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Oscar Bodansky &
A. L. Latner

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PREFACE

It is the hope of the Editors that this volume of the Advances continues to carry out the dual purpose that has motivated the series from its inception: the description of reliable diagnostic and prognostic procedures and the elucidation of fundamental biochemical abnormalities that underlie disease. Advances in technology and the increased pace of clinical investigation have resulted in lifting our dual purpose to new levels of effort and, we trust, achievement.

The Editors have striven to include reviews in this volume that reflect the growing role of the clinical biochemist in the modern hospital. Whitehead's review represents a critical evaluation of the information obtained from multiple analysis by current use of automated methods and data processing. Within recent years, the clinician's needs for additional laboratory aids have involved the clinical chemist in types of determinations formerly considered outside his province. Such fields are treated most comprehensively in the contributions of Stevenson and Loraine on Pituitary Gonadotropins and of Hobbs on Immunoglobulins. The rapidly burgeoning field of hereditary disease is represented by the reviews of Levin and of Saifer. Muscle diseases have been previously considered in this series, and Pennington's contribution scans this field again in terms of modern biochemical knowledge. Finally, in the paper by Halprin and Taylor on the Biochemistry of Skin Disease, the Editors have sought to include for review an area that has not hitherto been represented in the series but in which biochemical advances during the past decade warrant the active interest of the clinical chemist.

The present volume also represents a change in the editorship of the series. One of the coeditors (O.B.), while regretting deeply the retirement of Dr. C. P. Stewart, who was associated with the first thirteen volumes of this series, is delighted to have Professor A. L. Latner as his colleague.

As in the past, it is a great pleasure to thank our contributors and publisher for their excellent cooperation in making this volume possible.

OSCAR BODANSKY
A. L. LATNER

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PITUITARY GONADOTROPINS—CHEMISTRY, EXTRACTION, AND IMMUNOASSAY

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1. Introduction

1.1. The Gonadotropins as Biological Entities

In 1926 Smith (S14) demonstrated the existence of gonadotropic activity in the anterior pituitary gland of rats, and in 1931, Fevold, Hisaw, and Leonard (F4) showed that this activity was mediated by two components which they termed the follicle-stimulating hormone (FSH) and the luteinizing or interstitial-cell-stimulating hormone (LH or ICSH). Fevold *et al.* (F4) showed that FSH stimulated follicular growth in the ovary of hypophysectomized rats and caused spermatogenesis in male animals. LH transformed the follicles into corpora lutea in females, and in males stimulated the interstitial cells of the testis to produce androgens; these in their turn caused enlargement of the secondary sex organs, especially the prostate and seminal vesicles.

One of the major problems encountered by workers in the field of the gonadotropic hormones has been that of obtaining FSH preparations free from LH activity and LH preparations free from FSH. Accordingly, most of the work so far reported in the literature has been concerned with mixtures of these hormones. Even now after 40 years of active research, it remains a matter of considerable difficulty to provide a precise definition of the gonadotropins in terms of their biological activity.

1.2. The Gonadotropins as Chemical Entities

It is not yet certain that native FSH and LH have been prepared, although proteins containing predominantly one or the other activity have been isolated both from pituitary tissue and from urinary extracts. The two gonadotropic activities have been shown to be extraordinarily resistant to forms of treatment which generally destroy proteins.

Purified FSH and LH extracted from pituitary glands are both glycoproteins, containing approximately 26 and 19% of carbohydrate, respectively (B11, R15). In animal species which have so far been studied, both hormones have molecular weights of approximately 16,000 or multiples thereof (G8, P3, R13). LH, which is biologically active as a dimer, consists of two different glycopeptide chains (P3): it is not yet clear whether FSH is composed of one or more types of peptide. The biological activities of both hormones are stable for long periods of time at low pH (3 to 4) (S18) and withstand heating at 55°–60°C for 2 or 3 minutes (P5); LH retains some activity after precipitation with 5% trichloroacetic acid (R11).

Urinary glycoprotein with FSH activity is chemically different from FSH isolated from pituitary tissue (R15), and it is likely that urinary

preparations with gonadotropic activity consist of fragments of pituitary hormones bound to glycopeptides which originate in tissues other than the pituitary (D1). Such substances retain their gonadotropic activity even after being subjected to a very high pH (11–12) for several hours (J1).

FSH, derived both from pituitary tissue and from urine, is inactive if all the sialic acid has been removed from the molecule (G6). The sialic acid in LH is more stable than that in FSH, but treatment of the hormone with neuraminidase removes 75% of the LH activity (S13). It appears that tryptophan (P4) and probably a sulfhydryl group (A7) are necessary for FSH activity.

One of the aims of endocrinologists is to develop assay methods that will enable them to obtain quantitative estimates of FSH and LH in body fluids and glandular extracts. A major object of this review is to examine the basic principles underlying immunological assay methods for these hormones with a view to determining whether or not the results obtained by such procedures will be meaningful. Little or no mention is made of biological assays for these hormones because such techniques have been the subject of numerous reviews over the past two decades (A11, L5, L6).

Since the identities of the gonadotropic hormones remain uncertain, it is important before discussing assay procedures, to examine in some detail the various techniques for their preparation. Such methods may of themselves lead to differences in the composition or structure of the final products of extraction as well as to variations in the type of protein contaminants remaining in the purified hormone preparation. Since immunological procedures hinge on the availability of pure FSH and LH, it is likely that the results of such assays will vary depending on the methods by which these purified hormones have been obtained.

2. Properties of Glycoproteins with Special Reference to the Gonadotropins

The literature contains several recent reviews which discuss the structure and properties of glycoproteins (M4, R22). Much of the information in these articles is relevant to the assay of FSH and LH in that it emphasizes the conditions which should be avoided if native glycoproteins are to be prepared. True estimates of hormone levels in health and disease cannot be obtained if unknown and standard materials are markedly altered during the extraction procedure. This applies particularly to purified hormones used as the reference standards in radioimmunoassays.

2.1. The Importance of Sialic Acid

This substance is necessary for biological activity for several reasons: (a) its presence contributes to the maintenance of the tertiary structure of the glycoprotein (M4); (b) it protects the molecule from the action of proteolytic enzymes (M4, R26); (c) the ratio of sialic acid to non-polar and nonreducing end sugars (galactose and fucose) may determine the site of binding of the protein in its target tissue (D1). The *in vitro* work of Ryle *et al.* (R26) suggests that the main function of sialic acid in FSH is to protect it from proteolytic destruction.

Sialic acid is usually linked either to the 3-, 4-, or the 6-carbon atom of the adjacent glycosyl residue, and while the residues linked through the 3 and 4 positions are thought to be unstable to mild acid hydrolysis, those linked through the 6 position are more strongly bound (S10). Stability studies with the gonadotropic hormones suggest that the former linkage might be involved in binding sialic acid in FSH, and the more stable link is concerned in the case of LH. Two different types of neuraminidase (or sialidase) with different specificities are necessary to break the two kinds of linkage.

The importance of sialic acid in relation to the assay of the gonadotropins is discussed in subsequent sections of this review.

2.2. Bonding of Carbohydrate and Peptide Moieties in Glycoproteins

Carbohydrate chains may be joined to the peptide portion of the glycoprotein molecule by an N-glycosidic linkage with the β carbon of asparagine; Ward *et al.* (W2) have shown that this is probably the type of link formed in the case of LH. Ohgushi and Yamashina (O6) and Marshall and Neuberger (M4) have demonstrated that enzymes capable of degrading glycoproteins and breaking this bond occur in most tissues. A bond common in glycoproteins with similar compositions to FSH is the one that links the carbohydrate moieties to the peptide chain via the *N*-acetyl hexosamine and hydroxyl groups of serine or threonine (M4). This bond is hydrolyzed by cold alkali as well as by enzymes (G5). When the carbohydrate, in particular the sialic acid, has been removed from glycoproteins, the latter become susceptible to proteolytic digestion (M4, R26).

2.3. Polymorphism in Glycoproteins

There is among glycoproteins much heterogeneity due to small differences in the composition of the carbohydrate moieties attached to the

protein part of the molecule. This variation in carbohydrate composition is responsible for the immunological differences between blood cells in different human blood groups: variations have also been shown to exist in other glycoproteins extracted from tissues of different ethnic groups (S10). This phenomenon may be partly explained by the finding of Race and Watkins, who have shown that the enzyme α-D-galactosyltransferase is present in tissues of individuals with blood groups B and AB, but not with groups A and O (R1). It might be expected, therefore, that glycoproteins extracted from tissues collected from a cross section of the community and pooled will exhibit this inconsistency in composition. The human gonadotropins have not been examined for this type of heterogeneity, but it is reasonable to assume that it will be present. The loss of sialic acid residues during extraction also accounts for some of the polymorphism apparent in electrophoretic patterns (S11), and this, together with the heterogeneity of the other carbohydrate constituents of glycoproteins, makes it very difficult to prove that the protein portions of the molecule are homogeneous.

2.4. Depolymerization and Hybrid Formation

Another property of FSH and LH which must be taken into consideration when examining extraction procedures is that the hormones appear to be active as dimers with molecular weights of approximately 30,000. These dimers can readily be converted into monomers with molecular weights of approximately 16,000 by reagents which break salt linkages. Thus FSH is depolymerized by high salt concentrations (G8), whereas LH can be split either by subjecting it to extremes of pH or to high concentrations of urea or guanidine (P2, R13, S28). The two monomers of LH (ovine, bovine, and human) are different from each other both chemically and immunologically and are biologically inactive when separated (P2, P3, R13). There is some evidence, also, that human FSH consists of two different units; e.g., Ryan has shown that during electrophoresis in 8 *M* urea, FSH separated into two bands, as would be anticipated if it were composed of two nonidentical peptide units (R24). However, as Ryan himself has suggested, this might well be a function of the impurity of the preparation. Cahill and Li have isolated only one N-terminal and one C-terminal amino acid from ovine FSH, and this suggests that the monomer peptides are identical in this species (C1) However, their work is at variance with earlier results of Papkoff *et al.* and of Saxena and Rathman, who failed to demonstrate any free terminal amino acids in sheep and human FSH (P4, P5, S4).

It must be recognized that the forces which link the glycoprotein

monomers together are not covalent in nature and therefore are easily destroyed. They consist of interpeptide salt linkages and various non-ionic forces which act in areas of the subunits with complementary formation. As mentioned above, the strength of these bonds, which are relatively weak in the first instance, may be greatly decreased by increasing the ionic strength or changing the pH of the solution (G8, P2, R13, S28) so that the quaternary structure of the glycoprotein molecule is abolished. The subunits dissociate, but by returning the conditions to low ionic strength or to a neutral pH the quaternary structure is apparently regained (G8, P2). This process of dissociation and association permits the formation of hybrid molecules as well as the recovery of biological activity (S10). If LH and FSH are each composed of two subunits, then hybrid molecules containing one peptide unit of LH and one peptide unit of FSH might easily be formed during the initial stages of purification of these peptide hormones. Thyroid-stimulating hormone (TSH), a glycoprotein with similar chemical properties to LH, might also be involved in this interchange. It is therefore desirable at all times to work at low ionic strengths and at neutral pH's when handling the gonadotropic hormones.

3. Extraction and Purification of Gonadotropins from the Pituitary

3.1. Source of Pituitary Gonadotropins

FSH and LH are prepared from pituitaries which have been either stored frozen or stored in acetone until enough of the material has been collected to perform an extraction. Roos is of the opinion that frozen human pituitaries are a better source of native hormones than acetone-stored glands (R15). However, Stockell Hartree found no improvement in yields of gonadotropin from frozen pituitaries over those placed directly into acetone and stored (S25). Acetone-stored pituitaries are less likely to be affected by changes in temperature than are frozen glands: there is no evidence to suggest that enzymatic reactions proceed in cold acetone, but it is now established that chemical reactions applicable to proteins, particularly those involving imidazole groups, can occur in frozen solutions between 0° and —20°C (G7).

The term "fresh frozen" pituitaries can generally be applied only to animal glands since human pituitaries are usually removed some time after death. Accordingly, it might be anticipated that hormones obtained from fresh frozen animal glands would resemble more closely the native glycoproteins than those extracted from human pituitaries, whether frozen or stored in acetone.

3.2. Initial Extraction of Pituitaries

There are two basic methods for extracting gonadotropic hormones from pituitary glands: (1) extraction into organic salt solutions and (2) extraction with aqueous salt solutions.

3.2.1. *Extraction into Organic Salt Solutions*

In 1950 Koenig and King showed that 10% ammonium acetate in 40% ethanol at pH 5.1 was optimal for dissolving gonadotropins out of pituitary tissue (K5). This initial extraction has been used by various investigators including Butt and his colleagues, Steelman *et al.*, Stockell Hartree, and Ward *et al.* to prepare glycoproteins from acetone-dried pituitary material (A1, A7, B13, G8, S21, S25, W3). Duraiswami *et al.* (D5) extracted frozen pituitaries at pH 4.5 with ammonium acetate in 40% ethanol, while Saxena and Rathman (S4) employed 10% ammonium acetate in 35% ethanol at pH 6.1. By means of this initial extraction, FSH and LH were dissolved out of the pituitary material along with TSH and with other glycoproteins present in the pituitary, e.g., plasma proteins and materials derived from the structural and connective tissues. Proteins containing little or no carbohydrate such as growth hormone and ACTH are sparingly soluble in this solvent, although nucleic acids are extracted along with the glycoproteins.

3.2.2. *Extraction into Aqueous Salt Solutions*

Ellis showed that dilute salt solutions dissolved glycoproteins out of pituitary tissue and also extracted other substances including two proteinases, growth hormone, ACTH, albumin, and globulin (E2). Ellis's extraction method, with various modifications, has been employed by numerous investigators. Roos (R15) used dilute phosphate buffer at pH 5.7, and Papkoff *et al.* (P6) and Hashimoto *et al.* (H3) extracted with saline: dilute ammonium sulfate solutions of various concentrations at pH 4 have also been used by several workers (K3, P4, P6, P8, P9, R6, R12). Unfortunately the optimal pH for one of the proteolytic enzymes present is 4, and therefore the gonadotropins extracted at this hydrogen ion concentration could well be modified in their structure and might accordingly be different from hormones isolated by other methods. Ryan obtained a good yield of gonadotropins after extracting pituitary powder with an alkaline-salt solution (R24). Extraction of the gonadotropins into 1.0 *M* urea was favored by Cahill *et al.* (C2).

3.3. Initial Purification and Separation of LH from FSH

Problems in relation to the purification of the gonadotropins depend essentially on the properties of the contaminants, and, accordingly, the

problems will vary depending on the manner by which the hormones are first removed from the pituitary tissue. Stockell Hartree found that the gonadotropins obtained by extracting pituitaries with ethanol salt solution could be separated into FSH and LH fractions by ion-exchange chromatography (S25). However, workers who had used extraction into aqueous salt solutions as their first procedure found it necessary to purify the glycoprotein fractions further by various precipitation methods before subjecting the material to more elaborate techniques for the separation of the gonadotropins.

Ellis and Papkoff *et al.* removed growth hormone and some other contaminants from their pituitary extracts by successive isoelectric precipitations at pH 3 and 4 and again at 3; they then separated LH from FSH by ammonium sulfate fractionation at pH 7 (E2, P4). Ellis reported that LH and TSH were precipitated together in the course of this procedure (E2). On the other hand, Roos used ammonium sulfate fractionation to rid his preparations of growth hormone, and then precipitated the FSH and LH together (R15). At this early stage of purification there was already evidence that LH had been altered during the preliminary procedures. The human LH prepared by Roos was soluble in 50% saturated ammonium sulfate (i.e., 35% (w/v) at 0° and pH 7), while that of Papkoff *et al.* (P5) was precipitated with between 29 and 31% (w/v) ammonium sulfate at the same pH. This difference might have been caused by several factors including removal of neuraminic acid from the glycoprotein by hydrolysis, either chemically or enzymatically, or by hybridization of the molecules which may have occurred during treatment with high concentrations of ammonium sulfate or at low pH (see Section 2.4).

Reichert and Jiang collected bovine FSH which precipitated between 2 and 3 *M* ammonium sulfate (i.e., between 26 and 39%, w/v) at pH 4 (R4), while Reichert *et al.* and Wilhelmi precipitated all the human gonadotropic material together with 3 *M* ammonium sulfate at the same pH (R12, W10). Ryan used isoelectric precipitation to remove growth hormone from LH (R24), while Reichert and Wilhelmi, Saxena and Rathman, and Ryan all excluded other nonglycoprotein contaminants by subjecting their extracts to ethanol-salt and isoelectric fractionation (R11, R24, S4). Subsequently Reichert and Wilhelmi precipitated LH with 5% trichloroacetic acid (R11). Surprisingly, this LH still contained some biological activity after precipitation in concentrated acid, although later evidence suggested that the material had aggregated (R3).

3.4. Lytic Enzymes in Pituitary Extracts

Many workers take precautions during the final stages of a purification procedure to ensure that the hormones are not damaged by proteolytic

enzymes known to be present in pituitary extracts. It has recently been shown that one of these proteinases is identical with the serum protein plasmin (E4). Since this substance, as well as the other proteolytic enzymes concerned with blood clotting are glycoproteins, their presence can be expected in crude preparations of the pituitary gonadotropins.

Ellis investigated some of the properties of these proteolytic enzymes. He found that their optimal activities were at pH 4 and at pH 8–9, and that their minimal activity was at pH 6–7. The enzyme activity at pH 4 was inhibited by phosphate ions and could be separated from LH on the resin Amberlite IRC-50 (E2). Kathan *et al.* used the ion exchange technique to rid their LH preparation of proteolytic enzymes (K3), and Roos invariably employed phosphate buffers at hydrogen ion concentrations outside those at which the proteolytic activity was important (R15). Papkoff *et al.* inactivated the proteases by heating their gonadotropin extracts in acid solution at 50°–60° for 2 or 3 minutes (P4, P5). This procedure might be expected to cause some damage to the quaternary structure or carbohydrate content of the glycoproteins, and might contribute to the difference in solubility between the LH prepared by the above investigators, on the one hand, and by Roos (R15) on the other. However, Papkoff and co-workers claim that heating under these conditions does not reduce hormonal activity.

There has been no report of sialidase activity in pituitary gonadotropic extracts, but the results of Papkoff *et al.* suggest that such activity is present and is removed on heating along with the proteolytic activity. These workers report that when they omit the heating procedure their purified FSH contains considerably less sialic acid than does the FSH derived from heated extracts; in addition, only one-quarter of the biological activity remains (P5).

3.5. Further Purification of Pituitary Gonadotropic Activities

After the initial extraction of the protein fractions with gonadotropic activity from the pituitary, and preliminary separation from other components by ethanol, salt, and isoelectric precipitations, the materials are further resolved by ion exchange and adsorption chromatography, gel filtration, and electrophoresis.

3.5.1. *Ion Exchange in the Purification of Pituitary FSH and LH*

When properly employed, the resolving power of ion exchange columns is great, and very substantial purifications can be obtained by their use. However, in the work under consideration each investigator uses these exchange resins under different conditions of pH and buffer concentrations and so, although the specific activity of the gonadotropin itself is improved in most cases, the composition of the proteins contaminating

the different FSH and LH preparations must become more diverse with each step.

It is now known that the amino acid contents of FSH and LH are very similar (Table 4), but that the molecules differ in the amounts of charged carbohydrate moieties, such as *N*-acetylneuraminic acid, hexosamines, and *N*-acetylhexosamines. Therefore, it should be relatively easy to separate the two hormones on the basis of their different sizes and ionization behavior. However, there is a reversible reaction between reducing sugars and weak based amino exchange resins (M14), and so the glycoproteins may not necessarily behave as expected on the basis of their isoelectric points. The degree of separation of glycoproteins on ion exchange resins probably varies widely with very small changes in ionic strength and pH of the buffers used (D1). Unfortunately, precise details of the conditions employed in the purification of gonadotropins often are not reported in the literature.

3.5.1.1. *Anion Exchange of DEAE-Cellulose.* When employed at a pH above the isoelectric point of the gonadotropins and at low ionic strength, it has been shown that DEAE-cellulose, used either batchwise or in column chromatography, adsorbs most of the FSH from a pituitary extract, and leaves most of the LH unabsorbed.

Roos (R15) and Hashimoto, McShan, and Meyer (H3) resolved their DEAE-cellulose columns with dilute phosphate buffer at pH 7, eluting FSH by increasing the ionic strength of the phosphate buffer. Reichert *et al.* used a phosphate borate buffer at pH 8.0 to purify human FSH, and eluted the proteins from DEAE-cellulose by increasing the ionic strength of the buffer with sodium chloride (K3, R8). Despite the fact that the resolution was relatively poor when this buffer was used, the same system was employed by Reichert and Jiang to purify their bovine LH (R4), and by Reichert *et al.* and Ryan to improve the specific activity of their FSH (R12, R24). The elution patterns obtained by this system suggested that a difference existed between the FSH obtained by Ryan by means of his alkaline extraction methods (R24) and that of Reichert *et al.* (R12), who extracted their gonadotropins from the pituitary with acidified ammonium sulfate (see Section 3.2.2). The FSH of Ryan was eluted from the DEAE-cellulose column between 0.03 and 0.08 *M* NaCl, and that of Reichert *et al.* was eluted between 0.05 and 0.10 *M* NaCl. Stockell Hartree used DEAE-cellulose at pH 9.5 in a glycine-NaOH buffer to separate LH from TSH in a fraction already almost free of FSH. At this pH, most of the remaining FSH moved with the TSH fraction (S25).

3.5.1.2. *Cation Exchange on Amberlite-IRC-50.* Using a borate phosphate buffer at pH 8.0, Ellis showed that LH was retained by IRC-50,

while contaminating proteins including a proteinase were unabsorbed (E2). This method was adopted by Kathan *et al.* and by Reichert and Parlow for purifying human, bovine, and ovine LH (K3, R10). Reichert and Jiang found that relatively pure LH, prepared by chromatography on DEAE-cellulose, was adsorbed irreversibly onto IRC-50 in a phosphate borate buffer (R4). On the other hand, Stockell Hartree used phosphate buffer to elute LH from an IRC-50 column as the final step in her purification of that hormone without any great loss of material (S25).

3.5.1.3. *Cation Exchange of Carboxymethyl (CM) Cellulose.* Most workers employ this resin to absorb LH while the FSH activity passes straight through the column unimpeded. Stockell Hartree found that she obtained a better separation of LH from FSH in 0.004 *M* ammonium acetate buffer at pH 5.5 than when she used Butt's buffer, which was 0.010 *M* at pH 6 (B13, S25). Papkoff *et al.* purified ovine LH by absorbing it onto CM-cellulose, and discarding the unabsorbed fraction which contained FSH (P4), while Saxena and Rathman used the resin in the purification of human FSH, collecting the unabsorbed fraction and discarding the LH which was trapped in the column (S5).

3.5.2. *The Use of Gel Filtration in the Purification of LH and FSH*

Sephadex has been used repeatedly in the purification of FSH and LH either to change the medium in which the hormones are dissolved or to separate them from molecules with different molecular dimensions. Generally Sephadex is employed after much of the original material has been removed, and accordingly one would expect the contaminants to be proteins with much in common with the gonadotropins in respect to isoelectric points and carbohydrate content. Borate buffer might be expected to alter the properties of Sephadex gels since the latter react with carbohydrate moieties to form charged complexes (H10). However, at the time of writing, further information on this point was not readily available.

Roos, Papkoff *et al.*, Reichert *et al.*, Saxena and Rathman, and Butt and his colleagues all used Sephadex G-100 in the purification of FSH (A7, G8, P4, P5, R12, R15, S4). Roos claims to have increased the specific activity of his FSH almost 20 times by two successive filtrations on the gel (R15). Although both Roos and Ryan increased the specific activities of their LH preparations by filtration through Sephadex, using G-100 and G-200, respectively, Stockell Hartree did not find that any significant improvement in potency resulted from passage of her purified LH through Sephadex G-100 (R15, R24, S25). Up to the time of writing it is not known whether Sephadex gels will separate TSH from LH and FSH.

Ryan improved his FSH and LH preparations by partition on Biogel P150 (a porous polyacrylamide gel) in ammonium bicarbonate buffer, pH 8.0; however, the LH emerged still contaminated with TSH (R24). Gray used Biogel P30, Biogel P100, and Biogel P150 to demonstrate the existence of monomer, dimer, and tetrameric forms of human FSH in different salt concentrations; he noted that the dimer was split with 1 *M* NaCl (G8).

Roos increased the specific activity of FSH 2-fold by chromatography on hydroxylapatite (Biogel HT), and Amir *et al.* also used calcium phosphate gel to enhance their FSH activity. It should be emphasized that this last technique depended on adsorption chromatography, rather than gel filtration (A7, R15).

3.5.3. *The Use of Sephadex Linked to Ion Exchange Groups*

This procedure has the advantage of separating LH and FSH from impurities on the basis both of charge and of size of molecules. Papkoff *et al.* employed sulfoethyl Sephadex C-50 to separate both ovine and human FSH from contaminants, and then passed the human FSH through carboxymethyl Sephadex C-50 in the final step of their purification procedure (P4, P5). Papkoff *et al.* and Roos also used sulfoethyl Sephadex C-50 to purify human LH (P6, R15).

Amir *et al.* employed DEAE-Sephadex A-50 in a final purification step for human FSH; however, they lost 60% of the biological activity when they subjected their material to this procedure. They stated that the elution pattern of the proteins from DEAE-Sephadex was not reproducible because the LH, FSH, and albumin tended to form complexes which varied in their behavior on the column. This explanation should be accepted with reserve (A7).

3.5.4. *The Use of Preparative Electrophoresis*

3.5.4.1. *Electrophoresis in Cellulose.* Duraiswami *et al.* and Saxena and Rathman have used electrophoresis on cellulose columns in the purification of FSH (D5, S4), and Squire *et al.* have employed the same technique in the purification of LH (S18). These investigators found that considerable losses of gonadotropic activity occurred when cellulose was used to support the medium for electrophoresis; e.g., Saxena and Rathman found that 33% of their FSH was lost by this procedure (S4). The fact that LH is adsorbed onto cellulose has been used in the purification of that hormone after it has been labeled with radioactive iodine (S24). The LH is eluted off the cellulose with 50 mg of albumin/100 ml buffer at pH 8.6.

3.5.4.2. *Starch Gel Electrophoresis.* Ryan used starch gel electro-

phoresis for the purification of FSH and LH (R24), and Butt *et al.* employed the same technique in the purification of FSH (B14). Butt found that the major FSH peak, which he obtained at pH 8.6, resolved itself into two parts at pH 4, but noted that all the biological activity was lost at this low pH. This could well be an indication that human FSH consists of two nonidentical peptide units (see Section 2.4).

3.5.4.3. *Gel Electrophoresis.* Roos and Saxena and Rathman both used polyacrylamide gel in the final step of their purification of FSH (R15, S4). Roos lost 44% of his biological activity during this procedure with little improvement in purity (R15). Hunter and Midgley purified FSH on polyacrylamide gel at pH 8.5 after labeling it with radioactive iodine (H11, M8). Cahill and co-workers purified FSH after salt extraction by electrophoresis at the same pH on Sephadex G-25 (C2).

Most investigators carry out electrophoresis at the conventional pH of 8.6. Schmid, in his review of the isolation and characterization of glycoproteins (S10), showed that this pH is generally unsatisfactory for the separation of glycoproteins and that better results are likely to be obtained at an acid pH, which is nearer the isoelectric point of the substance. The resolution of glycoprotein materials by electrophoresis decreases rapidly on either side of their isoelectric points, and accordingly the optimal pH for electrophoresis of the gonadotropic hormones should be at about 5. Schmid pointed out that even partial resolution is often limited to a pH range of less than two units on either side of the isoelectric point, and consequently, when the conventional pH of 8.6 is used, homogeneity is often observed even when the material is, in fact, a mixture (S10). Claims with respect to purity of FSH and LH preparations based on data from electrophoresis carried out at this pH should be viewed with caution.

4. Extraction and Purification of Pituitary Gonadotropins from Urine and Plasma

4.1. Source of Urinary Gonadotropins

Gonadotropic hormones are extracted from urine for two main reasons: first, as a source of purified material that can be used for investigative work or for clinical administration; and second, as a means of concentrating the glycoproteins prior to the biological or immunological assay of FSH and LH in clinical conditions. For the former purpose the urine is generally derived from menopausal and postmenopausal women, in whom excretion values are high. Urine is an excellent bacteriological medium, and, since large quantities are often required, its collection and

storage frequently present problems. The urine is protected from bacteriological growth by a variety of methods, including (1) storage in the cold at 4°C or at temperatures below freezing point, (2) collection into glacial acetic acid (A6), and (3) addition of a bacteriostatic agent, such as hibitane (P1).

It is probable that differences in the storage of urine might be responsible for variations in gonadotropic activity between different preparations, because the chemical structure of the hormones has been altered. For example, storage at 4°C slows, but does not stop, bacteriological growth, and there therefore remains the possibility that enzymatic digestion of the glycoproteins may take place. Freezing may damage the urinary proteins by accelerating some chemical reactions (G7) or by concentrating the electrolytes, thus altering the three-dimensional structures of the molecules. In this connection recent experience in the Clinical Endocrinology Unit has indicated that, after freezing, LH in urine or in buffer solutions becomes progressively less active immunologically with the passage of time. Glacial acetic acid can remove sialic acid or carbohydrate chains from glycoproteins (see Section 2.3) and can cause denaturation of the gonadotropins; when urine is acidified with glacial acetic acid, yeast and molds continue to grow.

Present evidence suggests that none of the methods currently used for storing urine is completely satisfactory, and obviously the best solution to the problem is to extract the urine as soon as possible after collection.

4.2. Extraction of Gonadotropins from Urine

A great number of methods have been proposed for the extraction of human pituitary gonadotropins (HPG) from human urine, and an extensive literature already exists on this subject (L7). Comparisons of the yields found by the various techniques have been published by several groups of workers, and the original papers should be consulted for details of the results obtained (A4, B6, L9, W4).

4.2.1. *The Kaolin-Acetone and Tannic Acid Methods*

The extraction techniques in current use in most laboratories throughout the world are still based on the kaolin-acetone procedures of Albert (A2) and Loraine and Brown (L8) or the tannic acid method of Johnsen (J1). There is little information in the literature regarding the reliability criteria of these methods. Loraine and Brown tested the accuracy of their kaolin-acetone method in a series of recovery experiments in which a reference material prepared from urine, HMG-20A, was added to urine and recovered; the end point of the bioassay was the mouse uterus test for "total gonadotropic activity." The mean percentage recovery was 76,

a figure considered to be acceptable by the originators of the method. Loraine and Brown also studied the reproducibility of their technique by conducting replicate estimations on a pooled sample of postmenopausal urine; as with the recovery experiments they considered that reproducibility was satisfactory (L8).

McArthur *et al.* (M1) and others have demonstrated that extracts prepared by the tannic acid procedure are less toxic to experimental animals than those obtained by most other techniques including the variants of the kaolin-acetone method. Herbst *et al.* studied the accuracy of the tannic acid method by conducting recovery experiments involving the addition of the second International Reference Preparation for human menopausal gonadotropin (second IRP-HMG) to pools of normal male urine. They found that approximately 100% of the LH activity, but only 50% of the FSH activity, was recovered (H5).

Recovery experiments such as those performed by Loraine and Brown (L8) and by Herbst *et al.* (H5) for the kaolin-acetone and tannic acid methods, respectively, are open to the criticism that the material used had itself been extracted from urine. It is possible that more reliable information with respect to the accuracy of those techniques would have been obtained if material extracted from pituitary tissue had been used. However, at the time of writing, experiments of this latter type do not appear to have been conducted.

Both the tannic acid and kaolin-acetone extraction procedures employ extreme conditions which must certainly be damaging to glycoproteins such as FSH and LH. Thus, in the tannic acid procedure pH's of 4 and 11.6 are used in combination with high electrolyte concentrations, frothing under evacuation, and considerable temperature fluctuations. Extremes of pH and high electrolyte concentrations are also a feature of currently used kaolin-acetone methods. Gray, Justisz, Papkoff and Sammy, and Braikevitch and Stockell Hartree have demonstrated that the molecules of both FSH and LH from pituitary sources can be reduced to peptide chains of low molecular weight by high salt concentrations or extremes of pH (B8, G8, J2, P3). It is therefore virtually certain that such degradation will occur also when techniques such as the kaolin-acetone and tannic acid procedures are employed. Such extraction procedures are ideal, therefore, for the production of hybrid molecules (see Section 2.4).

4.2.2. *Other Currently Used Extraction Methods*

4.2.2.1. *Zinc Precipitation.* The initial stage in Courrier's purification of LH and FSH from the urine of postmenopausal women is the precipitation of all the gonadotropins from urine with 0.02 *M* zinc acetate

at pH 6. The zinc precipitate is washed with alcohol and dried, then extracted with ammonium acetate; the zinc ions are removed with Amberlite IRC-50 at pH 9.5, and the gonadotropins are further purified by alcohol fractionation (C7). The results of recovery experiments were not reported, but this procedure does appear to be mild in comparison with the methods in common use to extract HPG from urine.

4.2.2.2. *Acetone Precipitation.* Franchimont precipitated gonadotropins from urine at pH 5.8 with four volumes of pure acetone; the precipitate was collected after 24 hours at 4°C (F6). Franchimont used radioimmunoassays for FSH and LH as his end point and claimed recoveries of between 70 and 85% for the former hormone and 78 to 80% for the latter. Prior to radioimmunoassay the precipitate, which had been stored at low temperatures, was redissolved in phosphate buffer and dialyzed.

4.2.2.3. *Evaporation.* Such a technique has been used by Stevenson and Spalding to prepare urinary LH for radioimmunoassay. The urine was dialyzed against three changes of distilled water to remove all the substances of small molecular weight; the volume was then reduced 25 times by rotary evaporation at 30°C (S24). The overall yield of LH varied between 75 and 90%.

4.3. Extraction of Gonadotropins from Plasma

4.3.1. *Methods Employing Ethanol Precipitation*

The main steps in the procedure of Keller and Rosemberg are precipitation of the total plasma protein with 5 volumes of absolute ethanol followed by extraction of the glycoproteins from the precipitate with 10% ammonium acetate in 70% ethanol. The biologically active material is precipitated with absolute ethanol saturated with ammonium acetate (K4). The method was shown by its originators to achieve approximately a 10-fold concentration of gonadotropic activity using the mouse uterus test as end point. The technique is relatively sensitive and can estimate gonadotropins in the plasma of patients in whom concentrations are relatively low. The accuracy of the method, expressed in terms of the yield obtained, is approximately 87%. A technique similar to that of Keller and Rosemberg has been described by Mori for the extraction of FSH from serum (M12).

4.3.2. *Methods Employing Acetone Precipitation*

These have been described by Apostolakis (A10) and by Ekkel and Taft (E1). In the procedure of Apostolakis the gonadotropins are precipitated from plasma at pH 5 with 5 volumes of acetone. After cen-

trifugation, the supernatant fluid is discarded; the precipitate is washed with absolute ethanol and diethyl ether, and is dried over calcium chloride. The dried precipitate is ground to a fine powder and stored at 4°C prior to bioassay by the mouse uterus test. The recovery of added material by this procedure was approximately 100%.

In the method of Ekkel and Taft the acetone precipitate of plasma is washed with ethanol and then reprecipitated four times with an ammonium acetate–ethanol mixture (E1). Recovery experiments were conducted using the mouse uterus as end point, and the overall yield of gonadotropin varied from 89 to 100%.

4.3.3. *Method Employing Zinc Precipitation*

McArthur *et al.* prepared FSH and LH from postmenopausal plasma by fractionating the latter with alcohol as described by Cohn *et al.* (C6). The fractions containing gonadotropic activity were reprecipitated with zinc acetate at pH 6.5. The recovery of FSH, as judged by the rat ovarian augmentation test, was 78% and that for LH using the ventral prostatic weight (VPW) test in hypophysectomized rats was 64%. Zinc salts, which generally proved toxic to the experimental animals, were removed by dialysis prior to bioassay (M2).

4.4. Separation and Purification of Urinary FSH and LH

Much of the work on urinary gonadotropins has been concerned with the separation of LH from FSH rather than with purification of either of the hormones. Several systematic studies have been published describing the use of various types of chromatography and electrophoresis in the separation of the urinary gonadotropins, and there is now general agreement that it is somewhat easier to prepare LH relatively free from FSH than vice versa. Nevertheless, urinary FSH has now been obtained in a more highly purified form than has urinary LH.

4.4.1. *Purification of Urinary FSH*

The most successful study on the purification of urinary FSH was carried out by Roos (R15), who applied the same techniques of ion exchange and partition chromatography, polyacrylamide gel electrophoresis, and Sephadex filtration to his crude urinary gonadotropin preparation as to his pituitary material (see Section 3.5). The product of his extraction had a biological potency of 780 IU of FSH activity per milligram when assayed by the rat ovarian augmentation test of Steelman and Pohley (S20) and appeared homogeneous in both ultracentrifugal and free zone electrophoresis studies. It was shown by radio-

immunoassay that the purified FSH contained less than 0.1% by weight of LH.

The purification of urinary FSH has also been studied by Donini *et al.* (D3, D4), who tackled the problem by investigating the best order in which to use electrophoresis, ion-exchange chromatography, and filtration through Sephadex to obtain a good yield of the hormone. Although FSH of high specific activity was obtained when chromatography on DEAE-cellulose was followed by filtration on Sephadex, this procedure resulted in considerable losses of the hormone. Electrophoresis on cellulose powder followed by chromatography on DEAE-cellulose gave a good yield of FSH, but the material was contaminated with LH. They achieved a partial solution to this problem by treating their starting material with an antiserum to HCG. This reagent removed the LH activity from their extracts, and they proceeded to separate the LH antibody complex from FSH using DEAE-cellulose. Their final FSH preparation showed little LH activity as judged by bioassay but was contaminated with serum proteins.

Albert and Andersen and Blatt *et al.* have not been successful in separating LH from FSH in normal male and postmenopausal urine and in urine derived from eunuchs. The methods which they used were ion exchange chromatography on DEAE- and CM-cellulose, followed by filtration on Sephadex G-100 (A6, B5).

Andersen and Albert noted that the loss of FSH activity was very high if they omitted to presaturate their Sephadex gel with protein, and although the specific activities of both FSH and LH were improved by filtration through Sephadex, the procedure did not result in any further separation of the two hormones (A8).

Using his purified preparation of urinary FSH, Roos was able to demonstrate that his material differed from pituitary FSH in every physicochemical parameter studied (R15) (see Tables 3 and 4).

4.4.2. *Purification of Urinary LH*

Roos and Gemzell, Donini *et al.*, Reichert and Albert, and Andersen have all used DEAE-cellulose, either batchwise or in columns, to separate urinary LH from FSH (A6, D3, R2, R9, R15, R16). At a pH of 7 to 8 and at low ionic strength, LH is not adsorbed on the resin, while much unwanted protein together with FSH is removed. CM-cellulose has been used by Albert and Andersen to improve the specific activity of their LH fraction (A6), and Reichert found that residual FSH could be removed from the preparation by partition chromatography on CP_1-cellulose, which adsorbs the LH (R2). The results of various investigators show that Sephadex G-100 is unsatisfactory for the separation of urinary LH from FSH (A8, B5).

A pure sample of urinary LH has not yet been obtained. Nevertheless, there is evidence that urinary LH differs from pituitary LH in its behavior on polyacrylamide gel electrophoresis at pH 9.4 in that the former appears to be a more acidic substance than the latter (R2). However, Reichert and Jiang found that the v_e/v_0 ratios of human pituitary and urinary LH, when filtered through Sephadex G-100, were similar (R3).

At the time of writing, the purification of FSH and LH from plasma or serum has not been reported. This is obviously an important field for future studies.

4.5. Gonadotropin Inhibitors in Urine

Contributions to this field have been made by a number of investigators including Soffer and his colleagues (F5, F9, L1, S16, S17), Rosemberg *et al.* (R20), Saito (S2), Krishnamurti and Bell (K7), Ota *et al.* (O7, O8), Hipkin (H6, H7, H8), and Sairam (S1). The literature concerning gonadotropin inhibitors in urine is exceedingly confused, and in general refers to the interference of substances present in urine in biological assays. The most likely causes of such "inhibition" are, first, the presence in urine of unidentified materials which are toxic to the experimental animals; second, the occurrence of substances which may or may not be inactive derivatives of glycoprotein hormones but which are able to compete with gonadotropins at their sites of action; and finally, the presence of enzymes in urinary extracts which reduce or destroy the biological activity of FSH and LH before these hormones are injected into the experimental animals.

4.5.1. *Toxicity and Inhibition*

Rosemberg and her colleagues (R20) and Hahn and Albert (H1) failed to demonstrate the presence of a specific inhibitor of gonadotropic activity in human urine and concluded that the property of certain extracts could be explained on the basis of their toxicity to the experimental animals. Sairam (S1) has made a careful study of the gonadotropin inhibitors in both human and monkey urine. He has pointed out that toxicity of a given urinary extract can be assessed not only by the fact that it retards the growth of reproductive organs in the rat, e.g., ovaries and uterus, but also on the basis of other effects produced, e.g., an increase in the weight of the spleen and a decrease in adrenal weight.

4.5.2. *Inhibitors of Gonadotropic Activity*

Ota *et al.* (O7) demonstrated that effects such as ovulation induced by human chorionic gonadotropin (HCG) in the mouse and the decrease in ovarian ascorbic acid levels caused by LH (P7) could be inhibited

by the administration of a substance extracted by the method of Johnsen (J1) from normal male urine, the latter having been treated in a boiling water bath. Ota *et al.* precipitated this inhibitor by storing the preheated urine in the cold at pH 4 for 3 days. The inhibitory substance was heat stable and was not dialyzable. It had no effect on the activity of FSH when assayed by the method of Steelman and Pohley (S20). It appears possible that under these circumstances of heating and storage an analog of LH or an inactive polymer containing the hormone was formed.

Sairam confirmed that a high molecular weight glycoprotein which specifically inhibits the effect of LH on ovulation in mice and prevents ovarian ascorbic acid depletion in rats could be isolated from normal male urine (S1); such material was also slightly toxic to the experimental animals. The substance had a molecular weight of 68,000 as determined by gel filtration studies, was stable to heat, and had a high carbohydrate content. It might be anticipated that a sample of LH which had been denatured and aggregated would have such properties.

Ota *et al.* (O8) have also isolated a peptide from urine which inhibits the biological effects of LH. They claim that this peptide is a fragment of the LH molecule. It would be interesting to discover whether an inhibitor of this nature will compete reversibly with LH in its target tissue.

Various other substances have been shown to act as inhibitors of pituitary gonadotropic activity. These include synthetic arginine vasotocin (M13) and an extract prepared from the pineal gland of cattle (R14).

4.5.3. *Lytic Enzymes in Urine*

Sairam (S1) has succeeded in demonstrating the presence of an "inhibitor" to FSH in monkey urine. He was able to show that an extract of this type of urine inhibited biological activity if mixed with FSH before injection into rats; however, if the FSH and the inhibitor were injected at separate sites, such an inhibition was not observed. Sairam subsequently identified his inhibitor as sialidase, a glycoprotein enzyme which behaves similarly to LH during purification procedures. The sialidase was found to be capable of removing *N*-acetylneuraminic acid from a number of glycoproteins but was most active when the substrate was FSH (S1). Sialidase is not an inhibitor of FSH per se, but an enzyme which, when mixed with the hormone prior to injection into animals, destroys it by removing a part of the molecule necessary for biological activity.

A sialidase specific for FSH has not yet been demonstrated in human urine, although data derived from recovery experiments indicate that

such an enzyme may be present. Thus, as mentioned previously, Herbst *et al.* showed that most of the LH added to human urine was recovered during the extraction procedure while only 40% of the added FSH activity was retrieved (H5). Martin has also reported the loss of FSH activity added to human urine (M5). The reason for such losses has not been investigated, but presumably they could be due either to chemical inactivation of the hormone or to enzymatic hydrolysis.

Much further research in the area of gonadotropin inhibitors is obviously necessary. Such work must seek to establish whether such inhibitors occur naturally in human urine, whether they are also present in blood, and whether they subserve any physiological function in man and in other species.

4.5.4. *Effect of Inhibitors on Immunological Assays*

The presence of gonadotropin inhibitors in biological samples may or may not affect immunological assays. It is unlikely that substances which interfere with bioassays because they are toxic to experimental animals will influence immunological assays, or that substances such as arginine vasotocin, which are structurally unrelated to glycoproteins, will be of importance. On the other hand, the high molecular weight glycoprotein of Sairam (S1) and Ota *et al.* (O7), and, in particular, the LH fragment isolated by Ota *et al.* (O8) might well react with an antiserum to FSH or LH, thus giving falsely high results. It is unlikely that the sialidase characterized by Sairam (S1) would cause immunological inactivation of FSH since sialic acid does not appear to be necessary for the combination of a glycoprotein with its specific antibody (S3, S11).

It appears reasonable to suggest that if inhibitors have different effects on biological and immunological assays, satisfactory correlations cannot be expected when comparing the potency of gonadotropin extracts by the two different types of assay.

5. Comparison of Gonadotropin Preparations

A prerequisite for any immunological assay of the gonadotropins is the availability of "pure" hormone. However, the criteria for deciding the purity of a glycoprotein preparation are tenuous, depending on such investigations as sedimentation velocities and electrophoretic mobilities. The identity of the preparation can only be found by determining the biological action of the substance, and ideally the material used for immunological assays should be homogeneous by physicochemical standards, and have potent biological activity.

Several workers have observed that it is difficult, if not impossible, to compare the state of various preparations of FSH on the basis of their

specific biological activities (R15, R24), and this observation might well apply to LH also. There are at least four factors which render such comparisons unsatisfactory for judging the gonadotropin content of various preparations. These are: (1) instability of highly purified preparations, and of standards, (2) difficulties in estimating the amount of glycoprotein present in a solution; (3) the lack of a standard assay procedure; (4) the use of different standards for comparative assays. These factors will be considered in turn.

5.1. The Instability of FSH and LH Preparations

5.1.1. *FSH Lability*

It is relatively rare to encounter a pure protein which is stable when freeze-dried or frozen and thawed. FSH is no exception to this rule, and Reichert, Amir, Parlow, Papkoff, and their colleagues (A7, P5, P7, R12) have all shown that FSH loses its activity on lyophilization. This property of pure proteins has been recognized by enzymologists for decades, and the instability of purified enzymes is generally overcome by storing them as precipitates in ammonium sulfate solutions at +4°C. Furthermore, SH enzymes, such as pure lactic dehydrogenase, are often inactive after storage, but are routinely reactivated by mixing them with an SH agent, e.g., cysteine, glutathione, mercaptoethanol, or albumin; under such circumstances albumin is performing the function of a reducing agent. Current evidence suggests that FSH might fall into the category of compounds requiring a sulfhydryl group for activity, since Amir *et al.* showed that biological activity, which was lost after mild oxidation, could be restored by treatment with reducing agents (A7).

FSH is inactivated if sialic acid is removed from the molecule (G6, R24). Crooke and Gray (C8) pointed out that the activity of an FSH preparation varies with the sialic acid content of the glycoprotein—e.g., a preparation with 7% sialic acid had a biological activity of 14,000 IU of FSH per milligram, and a preparation with 5.2% sialic acid had a biological activity of 5000 IU of FSH per milligram; when the sialic acid content was lowered to 1.4% only 1250 IU of FSH per milligram was detected. Sialic acid, which constitutes up to 8% of the molecule by dry weight, might unwittingly be removed by hydrolysis either chemically, or enzymatically by sialidase, during the extraction of the hormone. Chemical hydrolysis occurs under mild acid conditions and could be caused by local acid concentrations during freezing, or freezing and thawing. Enzymatic hydrolysis is more likely to take place in solutions of impure extracts, such as biological standards in which sialidase,

extracted along with the gonadotropins, has not been removed. The results of Papkoff *et al.* (P5) suggest that sialidase is present in pituitary extracts (see Section 3.4), and it has been shown that such an enzyme is present in the urine of monkeys (S1). The urinary enzyme is a glycoprotein and is extracted with the gonadotropins (S1), but it has not as yet been directly demonstrated in human urine (see Section 4.5.3). Bacterial contaminants in solutions of gonadotropins will also remove sialic acid from glycoproteins. It has been shown that glycoproteins which contain sialic acid are in general resistant to attack by proteolytic enzymes, such as trypsin, chymotrypsin, pepsin, or plasmin (M4, R26). However, it has been demonstrated that glycoprotein becomes more susceptible to the attack of proteolytic enzymes as the sialic acid residues are removed (S3). Accordingly, a partly degraded FSH molecule is likely to have a much shorter half-life when injected into an animal, because it is liable to be destroyed by proteolytic enzymes present in plasma and tissue before it has reached its target organ. Therefore, a bioassay of an FSH preparation from which sialic acid has been removed will give no indication of its purity.

Ryan (R24) found that the activity of his FSH was lost when stored at −10°C for 2 months, but Amir *et al.* (A7) found no loss of activity when storing their FSH at −40°C for 20 hours or during dialysis at 4°C for 20 hours. This is in accordance with the experience of Grant (G7), who examined rates of some chemical reactions in ice and found more activity at −10°C than at +4°C or at temperatures below −20°C. Ryan found that FSH was apparently stable to reagents that break the weaker intramolecular bonds within a protein molecule, e.g., 8 *M* urea, guanidine sulfate, or sodium dodecyl sulfate (R24). However, Reichert and Midgley (R5) were able to demonstrate that the molecular dimensions of their FSH were reduced to half and the hormone lost its biological activity in the presence of 8 *M* urea. Butt *et al.* showed that the activity of FSH was lost when the N-terminal amino acid was acetylated (B15). However, if the N-terminal amino acid was necessary for activity, it might be anticipated that FSH would be inactivated by 6 *M* urea at 40°C; this has been shown not to be the case (E3). Although Butt *et al.* (B15) found that they lost their FSH activity when they acetylated the molecule, they did not show whether or not they had acetylated the carbohydrate moiety of the hormone; the loss of FSH activity may well have been caused by acetylation of one or all of the sugars attached to the peptide portion of the molecule, or by removal of the carbohydrate moieties during the acetylation procedure. Papkoff *et al.* (P4, P5) and Saxena and Rathman (S5) have all failed to demonstrate that there is a free N-terminal amino acid in FSH.

5.1.2. *The Instability of Highly Purified LH Preparations*

LH, which contains less sialic acid than FSH, is apparently a more stable glycoprotein. Reichert *et al.* (R12) claim that human pituitary LH has 1.4% dry weight of sialic acid. Butt (B11) found 3% sialic acid in his human LH, while Papkoff *et al.* (P4) could demonstrate no sialic acid in ovine LH. Donini *et al.* (D2) found 1.8% sialic acid in LH derived from postmenopausal urine. Since LH does not appear to be attacked by the same sialidase preparations as those that attack FSH, it seems likely that the bond which links the *N*-acetylneuraminic acid to the glycoprotein is different from that found in FSH. The authors who report that LH is not inactivated by sialidase (A1, E3) do not state the specificity of their enzyme. As pointed out in Section 2.1, sialic acid is usually linked either to the 3, 4, or 6 carbon atom of the adjacent glycosyl residue; different enzymes are required for hydrolysis of the primary and secondary linkages between the sugars. Results indicating the necessity of sialic acid for the biological activity of LH are therefore valid only if the activity is measured after treatment with both forms of sialidase.

Linked with the difference in sialic acid content is the finding that LH is inactivated more readily than FSH by proteolytic enzymes (A1). Reichert and Parlow showed that both FSH and LH preparations contained proteinase but could not demonstrate the relationship between the content of these proteolytic enzymes and the lability of the preparation (R7). Stockell Hartree found that one of her purified LH preparations, which had an activity five times that of NIH-LH-S1 when first prepared, lost half of its activity on storage at room temperature after drying in acetone and ether. However, other samples with this activity prepared by the same method were shown to be stable, and Stockell Hartree has suggested that the loss of activity in the one preparation was caused by failure to keep the substance properly desiccated (S27).

LH has a very high proline content (B11, R12) and consequently only a small part of the molecule is helical in structure. Accordingly, it is reasonably stable to heat. Adams-Mayne and Ward (A1) found that an absolutely dry preparation of LH was stable at 100° for 24 hours, but that as an aqueous solution it lost its activity after 6 minutes at 100°. LH is inactivated when frozen at −20° in a 0.01 normal acid solution (pH 2) (A1), and it is our experience that on repeated freezing and thawing in 0.07 *M* Veronal buffer pH 8.6 or in phosphate buffer pH 7.6, LH changes in such a way that it no longer reacts with an antiserum to the original hormone. Ellis (E3) found that LH was inactivated while standing at pH 4 for 24 hours, but Adams-Mayne and Ward showed that only slight inactivation occurred when pituitary LH was subjected to pH 12 for 15 minutes (A1).

Since human LH is composed of two dissimilar peptide chains (P2, R13), as is ovine LH (P3), one of the chief causes of lability during extraction and storage might well be the dissociation of the molecule. As mentioned previously, this is caused by extremes of pH or high salt concentrations (J2, P2, W1, W2); such conditions pertain in pockets of liquid during freezing as well as during the purification procedures. If conditions are such that denaturation can occur while the molecule is dissociated, then the peptide strand may not recombine to form active LH; however, Papkoff and Li (P2) have shown that in some instances active LH can be reconstituted. Furthermore, since the same conditions which lead to the dissociation of LH also lead to the dissociation of FSH, reconstituted molecules may consist of one peptide chain from FSH combined with one from LH. Reichert *et al.* (R13), have shown that, with their antiserum, one of the peptide strands from LH is almost as active immunologically as is the whole protein. Therefore it is unlikely that agreement will be found between biological and immunological assays of a preparation where this hybridization has occurred. It is obvious that in order to obtain reproducible results for the assay of gonadotropins, much attention must be given to the treatment and storage of assay samples and standards. If precise assays are required, adequate precautions must be taken in order to avoid extremes of pH, temperature, freezing, and frothing, as is the case when handling enzymes.

5.2. Difficulties in Estimating the Concentration of Glycoprotein in Solution

The definition of specific activity of a hormone is the number of units of activity per milligram of protein. Therefore, all estimates of potency must, by definition, depend not only on reliable assays of the hormone, but also on an accurate assessment of the weight of the protein or glycoprotein estimated.

There are three methods which have been used to obtain quantitative estimates of the weights of FSH and LH in a solution. These are: (1) drying and weighing a known amount of the material to be assayed; (2) a method depending on the UV absorption at 280 nm; (3) procedures depending on color reactions given by proteins or carbohydrates.

5.2.1. *Estimation of Glycoprotein by Weight*

The method of drying the protein followed by preparation of a solution containing a known weight of the substance per milliliter probably gives the best estimate of the amount of material being assayed. Many authors do not state clearly how the quantity of hormone used in their experiments has been estimated, but the technique of weighing the material

seems to be the one most often employed. However, this method is not ideal, since it has been shown repeatedly that FSH, and probably LH also, is labile to freeze-drying in all but crude preparations (B11). Accordingly, if the preparation is dried, then weighed and assayed, the result will not give a true estimate of the activity of the original material. Furthermore, it is known that there are different degrees of hydration of the material depending on the method of drying. Thus a protein dried by ethanol and acetone precipitation has had much more molecular water removed than one which has been dried by lyophilization (S27). Hence there is likely to be a discrepancy in the weight calculated even for the same protein preparation with two different methods of drying.

5.2.2. *The Method of Estimating Glycoprotein Concentration in Solution by UV Absorption*

Several workers have estimated the amount of protein used in their assays by measuring the UV absorption at 280 nm (B5, R15). Since absorption at this wavelength depends essentially on the amount of tryptophan and tyrosine in the molecule, this technique is obviously inaccurate for impure proteins, since the abundance of these amino acids varies from one substance to another. However, the method is reasonably reliable with pure glycoproteins, the molecular weights of which are known; the extinction coefficient of these may be accurately measured, or calculated from the amino acid composition (M3). Roos (R15) has considered these factors in estimating the protein concentration of his gonadotropic preparations, and he has found that a solution of pure FSH of concentration 1 mg/ml gives an absorption of 1 at 280 nm; this figure has been confirmed as accurate by Papkoff *et al.* (P5). Blatt *et al.* (B5) arrived at the extinction coefficient by the less satisfactory method of establishing the glycoprotein concentration using the biuret method and nitrogen determinations; neither of these is accurate when applied to glycoproteins.

5.2.3. *The Estimation of Glycoprotein Concentrations by Color Reactions*

The color reactions used for this purpose estimate the peptide, the protein, or the carbohydrate portion of the glycoprotein and depend, as do ultraviolet absorption methods, on information concerning the composition of the material being analyzed. The proportion of carbohydrate to amino acid in crude preparations of the gonadotropins from either pituitary or urinary sources probably varies with each step in the purification procedure, and therefore this method of determining glycoprotein can serve as only a rough guide to concentration. The same limitations apply to the use of nitrogen determinations for calculating

the amount of glycoprotein in a solution: a knowledge of the ratio of amino acids, sugars, and amino sugars in the preparation must be available before an accurate assessment can be made.

5.3. Bioassays Used to Compare the Potency of Different Gonadotropin Preparations

The main bioassay methods employed have been, for FSH, the ovarian augmentation test in rats, and for LH, the ovarian ascorbic acid depletion (OAAD) test and the VPW method. The precision of these techniques is often somewhat unsatisfactory, and Albert (A4) has stated that differences of potency of 25% or less between one preparation and another cannot be detected with certainty; a similar conclusion was reached by Adams-Mayne and Ward (A1). Other factors which render the results of bioassays difficult to interpret are, first, the fact that the results themselves may differ depending on the method used, and, second, the lack of a universally accepted standard preparation by means of which data from different centers can be compared.

A number of laboratories have published factors which they have used to convert the results obtained by one bioassay to those obtained by another, and Reichert and Parlow (R2, R6) have discussed this question in relation to LH estimations performed by the VPW and OAAD tests. In Reichert's experience, the factor necessary for conversion of results obtained by the former technique to those obtained by the latter was 25, whereas in the experience of Rosemberg *et al.* (R19) the comparable factor was 12. However, Rosemberg and Albert (R18) found in a further series of experiments that the conversion factor for the two LH assays in question varied considerably depending on the material being estimated. In their opinion such differences are a function of the half-life of the preparation after its administration to the experimental animal; the half-life will vary according to the number of sialic acid residues which have been removed from the molecule during extraction. The results of Rosemberg and Albert (R18) might also be interpreted as indicating that separate sites on the LH molecule are responsible, respectively, for prostatic weight increase and for depletion of ovarian ascorbic acid, i.e., that there exists a difference in the specificity of the binding sites for LH in the two organs.

Numerous investigators have emphasized the difficulties which arise in the field of the pituitary gonadotropins because of the use of a multiplicity of standard preparations (A5, B3, H4, R15, R17, S19). Bangham (B3) has especially criticized the practice of using material derived from sheep pituitary tissue (NIH-FSH and LH) to estimate the potency of human gonadotropins and has recommended that all assay results be expressed in terms of the second IRP-HMG. A similar view has been expressed by

Albert (A5). Bangham considers that a reference preparation from pituitary tissue should be used to assay pituitary LH fractions and that a urinary LH should be employed to estimate the potency of LH of urinary origin (B3). A similar statement might be made in the case of FSH, particularly since urinary and pituitary FSH have been shown to be chemically dissimilar substances (R15). Bangham regrets that, at present, serum and plasma standards for FSH and LH are not available and considers that standard preparations and test materials should be as similar as possible physically, chemically, biologically, and immunologically (B3). Some of the difficulties associated with the use of varying standard materials are illustrated in Table 1.

Comparisons of the potencies of gonadotropic preparations extracted in different laboratories do not provide meaningful information concerning the purity of the materials, particularly when factors (see Table 1) have to be used to convert the results obtained by one assay system to those obtained by another. An expression of the ratio of increase in specific activity of the purified to that of the starting material gives a better assessment of the value of an isolation procedure. If two preparations are compared on the basis of this ratio, problems associated with the use of different standards and different assay procedures are overcome. Unfortunately, few authors report their work in such a way that this figure can be calculated. The potencies of some of the highly purified gonadotropin preparations prepared by methods discussed in Sections 3 and 4 are shown in Table 2. These preparations are being used in the current work on the radioimmunological assay of the gonadotropins.

TABLE 1

CONVERSION FACTORS USED TO RELATE THE POTENCIES OF HUMAN GONADOTROPIC PREPARATIONS ASSAYED IN DIFFERENT LABORATORIES AGAINST DIFFERENT STANDARDS

Hormone	Comparative potencies of standards	Reference
Pituitary FSH	1 mg NIH-FSH-S1 = 3.33 mg Pergonal-23	(R6)
Pituitary FSH	1 mg NIH-FSH-S1 = 186 mg HMG-20A	(R6)
Pituitary FSH	1 mg NIH-FSH-S1 = 26.6 IU (2nd IRP HMG)	(R6)
Pituitary FSH	1 mg NIH-FSH-S2 = 22 IU (2nd IRP HMG)	(R15)
Urinary FSH	1 mg NIH-FSH-S1 = 9.2 "rat U" of FSH	(A3)
Urinary FSH	1 mg NIH-FSH-S2 = 22 IU (2nd IRP HMG)	(R15)
Pituitary LH	1 mg NIH-LH-S1 = 192.3 mg Pergonal-23	(R6)
Pituitary LH	1 mg NIH-LH-S1 = 1538 IU (2nd IRP HMG)	(R6)
Pituitary LH	1 IU HCG = 6.25 IU LH	(R15)
Urinary LH	1 mg NIH-LH-S1 = 8.33 mg Pergonal-23	(R18)
Urinary LH	1 mg NIH-LH-S1 = 66.6 IU (2nd IRP HMG)	(R18)
Urinary LH	1 mg NIH-LH-S1 = 24.3 "rat U" of LH	(A3)

TABLE 2

POTENCIES OF GONADOTROPIN PREPARATIONS PURIFIED BY METHODS DISCUSSED IN SECTIONS 3 AND 4

Human gonadotropin	Standard used for assay	Method of assay	Potency of purified hormone	Reference
Pituitary FSH	2nd IRP-HMG	Ovarian augmentation (rats)	14,000 IU/mg	(R15)
Urinary FSH	2nd IRP-HMG		780 IU/mg	(R15)
Pituitary FSH	NIH-FSH-S1	Ovarian augmentation (rats)	105 × NIH-FSH-S1	(R24)
Pituitary FSH	2nd IRP-HMG	Ovarian augmentation (rats)	3798 IU/mg	(R12)
Pituitary FSH	NIH-FSH-S1	Ovarian augmentation (rats)	40–60 × NIH-FSH-S1	(P5)
Pituitary FSH	NIH-FSH-S3	Ovarian augmentation (rats)	185 × NIH-FSH-S3	(S4)
Pituitary LH	NIH-LH-S1	OAAD test	5 × NIH-LH-S1	(S25)
Pituitary LH	NIH-LH-S1	OAAD test	3.5 × NIH-LH-S1	(R12)
Pituitary LH	HCG	Seminal vesicle test in immature rats	14,000 IU LH/mg	(R15)
Pituitary LH	HCG	Radioimmunoassay	17,000 U	(P3)
Pituitary LH	HCG	Radioimmunoassay	18,000 U	(R15)

5.4. Comparison of Gonadotropin Preparations by Physicochemical Methods

Another method of comparing FSH and LH purified by different techniques and of establishing their purity is to examine the physicochemical properties of the molecules. Table 3 summarizes some of the published data concerning molecular weight, sedimentation constants, and isoelectric points of FSH and LH prepared in different laboratories, and Table 4 shows some of the amino acid and carbohydrate analyses which have so far been published.

The tables indicate that there is still considerable divergence of opinion regarding both the properties and chemical composition of the gonadotropic hormones. Indeed, in some reports the amino acid analyses of two LH preparations have been found to be more dissimilar than those for FSH and LH. It should especially be noted that Butt (B11) and Stockell Hartree (S27) analyzed the same preparation of LH and arrived at very different results with respect to amino acid content; this finding sug-

TABLE 3
Some Physicochemical Properties of Highly Purified FSH and LH

Gonadotropin	$s_{20,w}$	Molecular weight	Isoelectric point	Reference
Human pituitary FSH	2.96	41,000, 43,301	—	(R15)
Human pituitary FSH	—	50,120 (by Sephadex)	—	(R12)
Human pituitary FSH	2.8	33,900, 51,500	—	(C4)
Human pituitary FSH	—	17,000, 34,000, 68,000 (by Biogel)	—	(G8)
Human pituitary FSH	2.04	31,000 ± 600	4.25	(S4, S5)
Human pituitary FSH	—	30,000 (by radiation inactivation)	—	(O5)
Human pituitary FSH	4.65, 3.45 (low pH)	—	5.6	(P5)
Human urinary FSH	1.9	28,000	—	(R15)
Ovine pituitary FSH	3.02	21,800	—	(P4)
Ovine pituitary FSH	3.5, 2.63 (low pH)	29,000-32,000	4.4	(C1)
Ovine pituitary FSH	2.63	—	—	(H3)
Human pituitary LH	—	40,740 (Sephadex)	—	(R12)
Human pituitary LH	—	30,000 (by radiation inactivation)	—	(O5)
Human pituitary LH	—	—	5.4	(S18)
Ovine pituitary LH	—	28,000-30,000 (at pH 7) 16,000 (at low pH)	—	(L2)

TABLE 4

EXAMPLES OF AMINO ACID AND CARBOHYDRATE ANALYSES OF HUMAN FSH AND LH[a]

Amino acid or Carbohydrate	Pituitary FSH	Pituitary FSH	Pituitary FSH	Urinary FSH	Pituitary LH	Pituitary LH	Pituitary LH
Lys	16	15	15.4	7	13.1	8	6
His	8	4.7	8	4	6.8	5.3	5
Arg	11	6.5	9.8	8	12.2	12.5	9
Asp	19	20.3	23.3	27	18	12.6	13
Thr	27	8.4	20.2	12	19	13.3	13
Ser	20	10.7	20.2	16	19.5	10.5	31
Glu	25	15.3	28.1	21	24.2	15.3	14
Pro	16	9.9	17.0	19	28.5	20.6	18
Gly	14	12.8	20.3	25	17.1	11.9	22
Ala	15	14.8	18.4	12	15.1	8.8	13
½-Cys	26	10.7 × 2	7 × 2	32	7.9	15.7	14
Val	18	11.4	18.4	11	20.8	16.4	20
Met	4	1.7	2.2	1	4.3	4.3	1
Ile	10	3.9	8	5	8	5.7	5
Leu	12	15.1	17.5	11	16.7	11.4	9
Tyr	13	6.7	4	4	5.3	4.8	4
Phe	9	8.2	10.3	7	8.5	5.0	5
Trp	4	2.2	—	2	—	—	
Cysteic acid	24.3	—	—	—	—	—	—
Met-S	4						
Hexose	39 (19.6%)	3.9%	11.6	14 (14.5%)	11%	—	13
Hexose NH_2	30	2.4%	9.1	7.7	3.1%	—	8
Fucose		0.4%					1.5
Sialic acid	8 (7%)	1.4%	5.2	2 (3.1%)	1.4%	—	3
Reference	(R15)	(P5)	(R12)	(R15)	(K3)	(S27)	(B11)

[a] Values: Residues per 27,000–30,000 MW as calculated from the published data.

gests that technical errors are probably responsible for some of the current conflict of opinion. However, some of the discrepancies might be explained by the finding of Holcomb *et al.* (H9), who showed that LH strongly binds some particular free amino acids: aspartic acid, threonine, glycine, and alanine. Since these amino acids can be removed under certain conditions (e.g., with guanidine), it is possible that the end products of different preparations contain different free amino acid complements.

6. Specific Antisera against FSH and LH

In order to assay gonadotropins by immunological methods, it is essential not only to have purified samples of FSH and LH, but also to possess antisera specific for these hormones. The preparation of purified FSH and LH has already been discussed. Accordingly it now remains to examine the question of antibody specificity in relation to the assay of these hormones.

Immunospecificity

As already stated, immunoassays depend on the fact that an antigen (in this case pure LH or FSH) reacts with a specific antibody. In the past the antibody–antigen reaction has been employed extensively, both qualitatively and quantitatively, for the identification of biological materials or for the elucidation of the chemical structure of complex molecules. However, in these studies use was made of specific immunological cross-reactions rather than the unique specificity necessary for immunological assays. It is well known that the antibody–antigen reaction is specific, but, it is often forgotten that specificity is directed against only a part of the antigenic molecule, and that any substance or substances which share this specific structure will also react with the same antibody.

6.1.1. *Immunodominance*

Gonadotropins show a high degree of antigenicity; i.e., they will readily stimulate the formation of antibodies in an experimental animal. However, one of the major difficulties in assays by immunological means is the finding of antisera which do not cross-react with other glycoproteins. It therefore seems appropriate to discuss the little that is known regarding the type of structure in an antigen or hapten which is responsible for the specific reaction with the antibody, i.e., the part which is immunodominant.

One of the original findings which pointed to the fact that antigenic specificity rested in only a small portion of a protein molecule was that of Obermeyer and Pick in 1904 (O1), who showed that antibody formed against a protein which had been treated with iodine would react with

many other unrelated proteins which had been similarly treated. Later Avery and Goebel (A13) showed that antibody prepared against egg albumin to which galactose had been attached combined well with horse serum globulin containing galactose, although it would not react with the original globulin, nor with the globulin to which glucose had been attached. In other words, the specificity of the reaction, or the immunodominant part of the antigen, was the galactose moiety. Subsequent work, which has been systematically carried out using synthetic macromolecules, has shown that immunodominance can lie in a wide variety of structures, and that different animals of the same species can produce antibodies with specificities for different regions in the one compound. This variation is thought to be under genetic control (B4, S12).

Using synthetic polypeptides and polysaccharides it has been demonstrated that immunodominance can reside in any of the following structures (i) terminal sugars (G4, K1, K2); (ii) the linkage between the sugars (K2, M11, T5); (iii) terminal amino acids (G4); (iv) amino acids positioned in accessible parts of a protein (G4, S12); (v) sections of a straight-chain polypeptide of up to 20 amino acids long (G3, S8); (vi) polysaccharide chains of up to six sugars (K1); (vii) portions of the protein molecule at which two peptide chains are cross linked (G3), and (viii) the linkage between the protein and carbohydrate moieties of a glycoprotein. On the other hand, sialic acid does not appear to play a role in antigenic specificity (S11, Y3). Natural glycoproteins are much larger and more complex than the synthetic macromolecules used for the above studies and probably have at least six or more immunodominant groupings per protein. It is much more difficult to study these larger molecules, but Sela (S12) is of the opinion that the conformation of a protein has a role in antigenic specificity, and Kabat and Leskowitz (K2), Avery and Goebel (A13), Rüde *et al.* (R23), and Clamp and Jones (C5) have all shown that the carbohydrate portion of a glyco- or mucoprotein contributes to the antigenic specificity of the substance.

6.1.2. *Heterogeneity in Antisera*

An antiserum is not homologous, but contains a number of populations of antibodies possessing combining sites with different binding affinities. For example, Kabat (K1), working with dextran, showed that in one antiserum some antibodies combined most satisfactorily with a chain five sugars long, some with six and others with seven. In the case of peptides, Benjamini *et al.* (B4) have shown that a part of an antibody population may be specific for an immunodominant section of an antigen which is 20 amino acids long, while another part of the population in the same antiserum is specific for a section only 10 amino acids in length.

6.1.3. *The Immunology of FSH and LH*

At the time of writing, nothing is known of the structure of the gonadotropic hormones in relation to their immunospecificity, and as mentioned in Section 5.4 even the amino acid composition of these glycoproteins remains uncertain. However, it is clear that FSH and LH are alike in their amino acid and carbohydrate composition, and immunological studies indicate that they have at least one immunodominant section in common with each other and with TSH (O3). Almost every worker in the field of the immunological assay of the glycoprotein hormones has had to face the problem of their similarity. A recent detailed review of this subject is that of Lunenfeld and Eshkol (L10).

Antisera raised against FSH and LH in either rabbits or guinea pigs are almost invariably nonspecific in that they cross-react with each other when tested in radioimmunoassay systems; in addition they frequently cross-react with TSH (O2, S15, S23). Occasionally in the one antiserum there is a population of antibodies specific for FSH, another for LH, and yet another for TSH (O2, S24), in which case the unwanted antibodies can be absorbed out by incubating with the appropriate glycoproteins, leaving intact the antibody population specific for one or both gonadotropins. However, more often the antibody population is directed against the immunodominant section common to both LH and FSH (H12, R25), and then the antiserum is unsuitable for use in an immunological assay.

HCG has been used successfully as the antigen for the production of antibody which will react with LH, but not with FSH (F6); however, as with antisera to the other gonadotropins, HCG antisera are frequently nonspecific, reacting with two or even three of the pituitary glycoprotein hormones (S24). It may be significant that Ryan using the same antiserum in all his experiments, found that different batches of his "immunologically pure" LH, all prepared by the same method, cross-reacted to varying degrees with HCG (R24). It is possible that the cross reactions which are observed between the glycoprotein hormones may be an artifact due to damage or hybrid formation of glycoprotein complexes during the purification procedure (see Section 2.4).

6.1.4. *Tests for Cross-Reactions*

It is clear that the degree of immunological cross-reaction between various antigens varies with the type of assay used. Nakai and Parlow raised an antiserum to highly purified LH and, using a microcomplement fixation test, were able to show that HCG did not cross-react with this antiserum. However, the same antiserum did react in both the hemagglutination-inhibition and radioimmunoassay systems (N1). Schuurs *et al.*

found that their antibody, if tested by hemagglutination-inhibition, could not differentiate between HCG which had been hydrolyzed by neuraminidase and by hydrochloric acid; however, when complement fixation was employed the antibody would react with neuraminidase-treated HCG, but not with the acid-treated hormone (S11). Stevenson showed that ovine LH did not cross-react with human LH in a radioimmunoassay system using an antiserum to human pituitary LH (S22, S23), but using the same antiserum Stockell Hartree found that human and ovine LH did cross-react in the hemagglutination-inhibition assay (S26). Maureer *et al.* discovered that with a single antiserum complement fixation and precipitin reactions gave different results (M6), while Gill and Matthews found that the importance of glutamic acid as an immunodominant factor in his synthetic polypeptide varied according to the assay method used (G2). All these discrepancies could be explained on the basis that one antiserum contained more than one population of antibodies with different characteristics.

6.1.5. *Change of Specificity in Antisera from the One Individual*

It has long been known in the preparation of antisera for analytical work based on immunological cross reactions, that the extent of such reactions is maximal when the antiserum is obtained after prolonged immunization (B10, S12). In the same context Benjamini *et al.* showed that with an antiserum containing two populations of antibodies, the ratio of these populations altered during successive inoculations with the antigen. Thus after 2½ weeks, antibody populations directed against the deca and eicosa portions of the protein were in the proportion of 1 to 100, and after 18 weeks the proportion was 40 to 100 (B4). Similarly Kabat found that early in immunization an antibody raised against an antigen containing 2,4-dinitrophenyllysine would react only to a very slight extent with the same antigen in which the 2,4-dinitrophenyllysine was replaced by 2,4-dinitrophenylalanine; however, this cross-reaction increased sharply as immunization continued (K1). Furthermore, Little and Eisen showed that the composition of the γ-globulin fragments involved in the binding of their antigen varied in amino acid composition between 5 and 10 weeks after the start of the course of injections (L3).

This variation in antibody specificity with successive inoculations of an animal has proved a matter of considerable difficulty in the field of the radioimmunoassay of the gonadotropic hormones. The work of Odell *et al.* (O3) demonstrates that, during the production of their antisera to human FSH, their experience was similar to that of Benjamini *et al.* (B4), namely, that early in immunization the population of antibodies which reacted with both FSH and LH was 100 times

smaller than the population which reacted with FSH alone. However, antisera from the same rabbit obtained after further immunization appeared from his results to contain approximately 50% of antibodies which would react with FSH alone and 50% which would react with both LH and FSH. A similar phenomenon has been noted by Stevenson (S23) with an antiserum to human LH where, early in the immunization an antiserum with one population specific for LH was produced, while later a large proportion of the antibodies cross-reacted with human LH, FSH, TSH, and HCG. Furthermore, repeated inoculations produced a detectable number of antibodies which would react with sheep LH by radioimmunoassay while the initial inoculations did not (S23).

6.1.6. *Raising Specific Antisera to the Gonadotropins*

Immunological techniques, especially radioimmunoassays, would appear to be ideal for work aimed at elucidating the chemical and immunological structures of some of the immunodominant sections of LH and FSH; in this connection it should be emphasized that the immunodominant sections of substances responsible for blood groups were identified with the aid of the immunological methods of Morgan and Watkins (M11) and Kabat and Leskowitz (K2). When the structures of FSH and LH are known, it should be possible systematically to raise antisera specific for either hormone by taking an immunodominant portion of one substance not shared by the other or by TSH, combining it with a foreign protein to render it antigenic, and using this as the agent to stimulate antibody formation. Alternatively, a hapten which corresponds to a portion of LH but not FSH (or vice versa) might be used as "the label" in a radioimmunoassay if such a substance could be iodinated or tagged in some way; if this were done, a nonspecific antiserum might be used for a specific assay.

The little that is known of the quaternary structure of the gonadotropins has not been investigated with a view to raising specific antisera. Both halves of the dimer which form active LH are antigenic (P2), and it remains possible that the antisera to one or other of these monomers will react with LH but not with FSH.

In the present state of our knowledge concerning the chemistry of gonadotropic hormones, the raising of antisera suitable for immunoassay is a matter of chance and has rarely been achieved. Until more is known regarding the immunodominant structures of FSH and LH, two points merit emphasis with a view to maintaining a supply of specific antisera. The first is that different individuals produce antisera with different specificities, and this specificity is under genetic control (see Section

6.1.1) (B4, S12); the second is that a monospecific antiserum is likely to be obtained early in a course of injections rather than later.

7. Immunological Assays of Gonadotropic Hormones

All immunoassays depend on the quantitative interaction of an antigen with its specific antibody; in the present instance, such assays are based on the interaction of the glycoproteins FSH and LH, with their respective antibodies. A relatively small part of the glycoprotein molecule is concerned with the specific reaction with its antibody. Provided that this part remains intact, the molecule can be modified in such a way that it still retains its immunological activity but is readily detected and easily differentiated from molecules which have been unchanged. Immunological methods such as those involving hemagglutination-inhibition and radioimmunoassay depend on competition at the antibody surface between the antigen (FSH, LH) and the detectable analog of the hormone.

7.1. Theory of Inhibition Reactions

It is possible to express antigen–antibody reactions and inhibition reactions used in immunoassays on a quantitative basis. Thus, ideally, a given number of molecules of antibody n will react or bind m molecules of antigen, whether labeled or unmodified, at a given concentration. If x molecules of unlabeled antigen are included in a reaction mixture which contains m molecules of labeled antigen and n molecules of antibody, then, assuming that the reaction goes to completion, the number of labeled (or detectable) molecules of antigen combined with antibody will be $m^2/(m + x)$. The amount of free labeled antigen in the medium will then equal the number of molecules of nonlabeled antigen bound to the antibody, i.e., $mx/(m + x)$. It is apparent that the amount of labeled antigen bound to antibody will depend on the quantity of unlabeled antigen present. Accordingly, by adding known amounts of unlabeled antigen to the mixture it is possible to construct a standard curve, which will be exponential if plotted arithmetically or sigmoidal in shape if plotted on a semilogarithmic scale. The slopes of the curves will depend on the equilibrium constants of the antibody–antigen reaction.

7.2. Hemagglutination-Inhibition Reaction

7.2.1. *General Considerations*

If a fixed amount of antigen is labeled by combining it with erythrocytes i.e., if the red blood cells are "coated with antigen" (B7), the

minimum amount of antibody necessary to cause the erythrocytes to agglutinate can be calculated. This predetermined amount of antibody is then mixed with unlabeled antigen, and the coated red blood cells are added. The unlabeled antigen then competes with the antigen bound to the red cells for the antibody binding sites and, as a result, varying degrees of inhibition of hemagglutination can be observed, depending on the concentration of the unlabeled antigen present. A standard curve is constructed by observing the amount of agglutination which occurs in each tube when dilutions of antigen are mixed with antibody and erythrocytes; the potency of the unknown sample is calculated by reference to this curve. A variant of the hemagglutination-inhibition technique is the use of latex particles coated with FSH or LH instead of erythrocytes; such a modification has been used by Tamada *et al.* (T1) and Taymor (T2).

Wide and his colleagues (W5, W7) were the first to apply the technique of hemagglutination-inhibition to the estimation of urinary LH. They found that some antisera raised against HCG were incapable of distinguishing between HCG and LH, and, accordingly, they were able to establish an assay system for LH using an antiserum raised to HCG and HCG-coated red blood cells. Taymor (T2) used a similar system to assay human urinary LH. He also employed an HCG antiserum and latex particles coated with this hormone; the system was specific in that a cross reaction with ovine LH was not observed; however, its specificity with respect to FSH was not reported. Taymor (T2) found it necessary to extract LH from urine prior to immunoassay. For this purpose he preferred precipitation with acetone to treatment with alcohol, since trace amounts of the latter interfered with the antigen–antibody reaction.

The results of Taymor and Wide and Gemzell, agreed that in normally menstruating women the pattern of LH excretion obtained by immunoassay was roughly similar to that previously found by biological methods (T2, W5), i.e., a peak of excretion occurred at midcycle while levels were low in the follicular and luteal phases of the cycle. However, when a comparison was made between the results obtained by bioassay and immunoassay using the index of discrimination (G1) the agreement was unsatisfactory, the results obtained by immunoassay being generally higher than those found by bioassay (L6).

Hamashige and Arquilla (H2) have strongly criticized the specificity of assays for LH based on an HCG–anti HCG system where crude HCG is used to coat the red cells. They suggest that such measurements may be inaccurate because of interference by urinary proteins, which are probably of placental origin and are present in relatively large quan-

tities in the commercial HCG preparations employed. Nevertheless, Stockell Hartree (S25, S27) reported successful results when the LH content of material obtained from human pituitary tissue was estimated using an HCG antiserum and red cells coated with either HCG or LH. She used this technique to measure LH in a series of pituitary fractions and found good agreement when parallel assays were performed by her method and the OAAD test of Parlow (P7). Taymor (T2), also working with pituitary extracts, reported a good correlation between an immunoassay based on the latex agglutination technique and a bioassay depending on the VPW test (S20).

Hemagglutination-inhibition and latex agglutination inhibition have also been employed to measure FSH in purified material derived from human pituitary tissue and human urine (B16, T1). Butt *et al.* using a highly purified pituitary FSH to coat the red blood cells, together with an FSH antiserum, were able to measure 0.3 μg of pituitary FSH per milliliter after the hormone had been purified by starch gel electrophoresis (B16). This system was unaffected by the addition of large quantities of HCG. Tamanda *et al.* (T1) coated latex particles with urinary FSH (Pergonal, Serono) and employed an FSH antiserum which had previously been absorbed with HCG in order to remove antibodies reacting with LH, and with the urine of children to minimize the reaction with glycoproteins of nonpituitary origin. By this means they were able to measure as little as 0.035 IU of FSH in urine, and they claimed that the presence of LH had little or no effect on their results.

7.2.2. *Reliability Criteria*

7.2.2.1. *Specificity.* Were it possible to coat erythrocytes with immunologically pure FSH or LH, and to use a monospecific antiserum to one or both of the hormones, then it is obvious that the hemagglutination-inhibition and latex inhibition tests would be specific for the substances in question. Unfortunately, it is necessary to coat the cells or the particles with milligram amounts of antigen, and since large amounts of the gonadotropic hormones in their purified form are not readily available, the substances generally used contain variable quantities of contaminants. In addition, specific antisera are also scarce. Because of these limitations the specificity of such methods must remain open to question.

7.2.2.2. *Precision.* Figures for the index of precision (λ) using such techniques do not yet appear to have been calculated. According to Stockell and Hartree (S25), the precision of hemagglutination-inhibition tests is reasonably satisfactory in skilled hands, the fiducial limits of

error of individual assays ($P = 0.95$) ranging from 66 to 152%. Methods depending on latex agglutination are probably more precise than hemagglutination-inhibition tests because their end points can be determined spectrophotometrically rather than by eye. The latex particles, suspended in solution, absorb light at 610 nm, and the percentage of light transmitted increases in the presence of agglutination; total agglutination is taken as 90% light transmission. In quantitative assays the dose-effect curve is constructed by plotting the light transmission against the dilution of the hormone under test on a semilogarithmic scale.

7.2.2.3. *Practicability.* Both hemagglutination-inhibition and latex inhibition tests are easy to perform and are inexpensive. In addition, the results of the tests can be read in a few hours. As mentioned previously, their main disadvantage is the need to use large quantities of purified hormones in order to coat the red cells. Stockell Hartree (S25) has shown that these assays are extremely useful in order to monitor hormone extraction procedures, but in the absence of a supply of purified hormones their sphere of applicability to clinical problems is limited. When crude hormones and nonspecific antisera are used the quantitative significance of assays based on hemagglutination-inhibition reactions is doubtful.

7.3. Radioimmunoassay

The basis of the radioimmunoassay is similar to that for hemagglutination inhibition, in that an antigen (or in radioimmunoassay it may be a hapten) is labeled, and used to compete, quantitatively, with unlabeled hormone (standard or test) at the antibody surface.

The substance used to label hormones for radioimmunoassays has always been radioactive iodine, although any nuclide or other compound which can be detected after combination with a protein, might be employed. However, the sensitivity of such an assay depends, in part, on the ability to trace very small amounts of the labeled hormone, and therefore it is necessary to combine it with a radioactive material which disintegrates rapidly and yet has a half-life long enough to complete the assay. Theoretically, ^{131}I with a half-life of 8 days fits the requirements, but in practice ^{125}I, with a half-life of 6 weeks, has been more useful because it can be obtained with a much higher specific activity. The isotopic abundance of ^{131}I is in the region of 25% while that of ^{125}I is nearer 90%; accordingly, if one iodine atom is introduced into each molecule of FSH or LH, every fourth glycoprotein molecule will be labeled if ^{131}I iodine has been used, but almost all will

be labeled if the isotope is ^{125}I. The advantages of using ^{125}I have been discussed by Freedlender (F8).

7.3.1. *Iodination*

Introduction of Iodine into a Protein. The method employed by the majority of investigators for the introduction of iodine into the gonadotropic hormones is that of Greenwood *et al.* (G9). In this procedure the commercial oxidizing agent chloramine T is used to convert iodine to its cationic form, when at an alkaline pH it combines with the negatively charged phenolic group of a tyrosine molecule. The ionization behavior of the tyrosine moiety is dependent on its position in the protein molecule (K6), and it has been shown that ease of iodination, as well as the importance of tyrosine as an immunodominant grouping, are both associated with the ease with which the phenolic group can be dissociated (S12).

Freedlender and Yalow and Berson, have discussed in detail the labeling of peptide hormones for radioimmunoassay (F8, Y1, Y2). Readers are referred to their papers for further information on this subject.

7.3.2. *Isolation of Labeled Hormone*

During the labeling procedure the protein is subjected in turn to an oxidizing agent, chloramine T, to cationic iodine which is itself a very potent oxidizing agent, and to a reducing agent, metabisulfite. Each of these reagents alone can damage or alter the structure of protein molecules (S22, Y1) and consequently, at the completion of the iodination reaction, the labeled hormone must be separated from the small molecular weight reactants as rapidly as possible.

Providing that the immunological activity of a hormone remains unchanged after labeling, it may not be important that the latter has been denatured, fragmented, or otherwise altered during the iodination. However, it is generally necessary to separate damaged hormone which is no longer immunologically active, from the labeled hormone which is to be used in the assay. This can be done separately, or at the same time as the free iodine and other small molecular weight reactants are removed. If an impure hormone has been iodinated, the unwanted protein contaminants are frequently removed at this stage also.

7.3.2.1. *Separation of Hormone from Free Iodine.* A number of methods have been used to separate free iodine from labeled FSH, LH, or HCG. Franchimont (F6) used Sephadex G-50 after the iodination of FSH, and Schalch *et al.* (S7) and Wilson and Hunter (W11) employed the same gel after the labeling of LH. Saxena and colleagues (S6)

removed excess ^{131}I from their FSH on an inorganic iodine resin, but did not state whether or not this substance removed the rest of the inorganic reactants from their solution; it was evident, however, that their hormone had been badly damaged. Midgley (M7) separated ^{131}I from labeled HCG with Biogel P60, and Schlaff *et al.* (S9) employed the ion exchange resin Dowex I to free their labeled FSH; Neill and co-workers (N2) separated LH from free iodine on Amberlite IRA-400.

7.3.2.2. *Separation of Labeled Gonadotropins from Iodine and Damaged Hormone.* Aono and Taymor removed both ^{125}I and damaged protein from their labeled FSH preparation using Sephadex G75 (A9), while Rosen *et al.* and Saxena *et al.* (R21, S6) employed Sephadex G-100 to remove damaged protein from FSH and HCG. Wilde *et al.* (B2, W8) and Franchimont (F6) separated damaged material from immunologically intact HCG on Sephadex G-200, while Stevenson and Spalding noted that the quick passage of labeled LH or HCG through cellulose columns completely separated the immunologically active from the inactive hormone (S24).

7.3.2.3. *Removal of Protein Contaminants from FSH after Labeling.* Several workers have removed contaminants which were present before iodination from their labeled FSH preparations. Franchimont (F6) carried out this purification in two stages, first by chromatography on DEAE-cellulose, and second by starch gel electrophoresis. Midgley (M8) and Hunter (H11) used polyacrylamide gel electrophoresis in one step to remove free iodine, damaged FSH, and contaminating proteins from iodinated FSH.

Some of the methods used for the purification of labeled FSH, LH, and HCG are summarized in Table 5.

Many workers consider that it is necessary to use freshly iodinated hormones for their radioimmunoassays. However, it has been shown that an iodinated hormone preparation can be kept for a period of several weeks provided that the damaged material is removed from the hormone each time before use (A9, B2).

7.3.3. *The Reaction between Antibody and Antigen*

As in hemagglutination-inhibition tests, a predetermined amount of antibody to the hormone in question is incubated with a series of dilutions of unknown or standard solutions of the gonadotropin. After a period of preincubation ranging from 0 to 3 days, labeled hormone is added to each tube, and the mixture is reincubated to equilibration.

At the completion of the reaction, which is carried out at dilutions at which the antibody–antigen complex is usually not precipitated, various techniques are employed to determine the amount of labeled

TABLE 5
ISOLATION OF LABELED HORMONE AFTER IODINATION

Hormone	Agent	Result	Reference
FSH	Sephadex G-50	Separated free I from hormone	(F6)
FSH	Inorganic iodine resin	Separated free I from hormone	(S6)
FSH	Dowex 1	Separated free I from hormone	(S9)
FSH	Sephadex G-25	Separated free I from hormone	(H11)
LH	Sephadex G-50	Separated free I from hormone	(S7)
LH	Sephadex G-50	Separated free I from hormone	(W11)
HCG	Biogel P-60	Separated free I from hormone	(M7)
LH	Amberlite IRA-400	Separated free I from hormone	(N2)
FSH	Sephadex G-75	Removed free I and damaged protein from hormone	(A9)
FSH	Sephadex G-100	Removed damaged protein from hormone	(S6)
FSH	Sephadex G-100	Removed damaged protein from hormone	(R21)
HCG	Sephadex G-100	Removed damaged protein from hormone	(S6)
HCG	Sephadex G-200	Removed damaged protein from hormone	(W8)
HCG	Sephadex G-200	Removed damaged protein from gonadotropin	(F6)
LH	Cellulose	Removed damaged protein from hormone	(S24)
FSH	Polyacrylamide gel electrophoresis	Separated free I, damaged protein, and contaminants from labeled hormone	(M8)
FSH	Polyacrylamide gel electrophoresis	Separated free I, damaged protein, and contaminants from labeled hormone	(H11)
FSH	DEAE cellulose + starch gel electrophoresis	Removed contaminants and damaged protein from labeled hormone	(F6)

hormone which has been bound to the antibody present. The quantitative expression of the antibody–antigen reaction cited in Section 7.1 applies to the radioimmunoassay system as well as to the hemagglutination-inhibition test; theoretically the shapes of the dose-response curves in the two types of method are the same.

7.3.4. *Separation of Antibody-Bound from Free Hormone*

Many different systems have been used successfully for the separation of "free" and antibody-bound hormones in radioimmunoassays. These depend on (1) differences in physicochemical properties of the glycoprotein hormones and the glycoprotein–γ-globulin complex; (2) immuno-

logical precipitation of the γ-globulin plus the antibody–hormone complex, leaving the free antigen in solution (F3); and (3) rendering insoluble one of the components of the reaction, usually the antibody, which is fixed to a solid matrix: at the end of the reaction it is an easy matter to wash away the unreacted hormone leaving the solid antibody–antigen complex to be counted.

The methods which have been employed to determine the amount of labeled hormone bound to the antibody are summarized in Table 6. Any of the systems is satisfactory provided that conditions have been adjusted so that complete separation of the free and bound hormone is obtained.

The double antibody method in which a second antiserum is used to precipitate the hormone bound to γ-globulin is the method most likely to produce incorrect results, unless it is carefully controlled. Errors may arise because the double antibody complex is soluble in the presence of excess antiserum or excess antigen. Accordingly, unless care is taken, falsely high values for the ratio of free to bound hormone may be obtained. Morgan (M10) has also shown that complement, which may be present in clinical samples as well as in the antisera, will inhibit the second antibody reaction. In addition, serum contains some very potent proteolytic enzymes which are active at 5°C and which are not inactivated by heating at 56° for 30 minutes (S8). Therefore, the addition of relatively large amounts of antiserum to the radioimmunoassay system in order to precipitate the bound hormone causes an increase in "incubation damage." This is invariably a problem in radio-

TABLE 6
METHODS FOR SEPARATING FREE AND ANTIBODY-BOUND HORMONES

Hormone	Method of separation	Reference
FSH	Starch gel electrophoresis	(F6)
FSH	Chromatoelectrophoresis	(S6)
FSH	Polyacrylamide gel electrophoresis	(H11)
FSH	Double antibody precipitation	(A9)
FSH	Double antibody precipitation	(R25)
FSH	Antiserum absorbed to bentonite	(C9)
LH	Starch gel electrophoresis	(F6)
LH or HCG	Chromatoelectrophoresis	(S6)
LH	Ethanol salt precipitation	(W11)
LH	Paper chromatography	(S22)
LH	LH adsorbed by dextran-coated charcoal	(N)
HCG	Dioxane precipitation	(T4)2
HCG	Double antibody precipitation	(B2)
LH and HCG	Antiserum absorbed to Protapol D1/1 disks	(C3)

immunoassays. In such techniques the hormones are likely to be damaged during a period of incubation which can last up to 1 week, and during which time disintegrating radioactive nucleides and serum or plasma enzymes capable of producing proteolysis are present in the reaction mixture.

Sources of error in the immunoprecipitation system of radioimmunoassay have recently been discussed by Quabbe (Q1), to whose article the reader is referred for further details.

7.3.5. *Reliability Criteria for Radioimmunoassays*

7.3.5.1. *Specificity.* The specificity of radioimmunoassays, like that of any other immunoassay, relies on the specific reaction between an antibody and an antigen. Since the identity of both is impossible to prove, the specificity must always be open to some doubt. The reaction of antibody with its antigen might be compared with that of an enzyme with its substrate thus:

$$\text{Enzyme} + \text{substrate} \rightleftharpoons \text{enzyme–substrate complex} \underset{\text{product}}{\Downarrow\Uparrow} \quad (1)$$

$$\text{Antibody} + \text{antigen} \rightleftharpoons \text{antibody–antigen complex} \quad (2)$$

Unlike the enzyme reaction (1), where the product provides some evidence that the right enzyme has combined with the correct substrate, there is no means of determining whether the right antibody has combined with the correct antigen. In immunological assays, evidence of specificity can only be indirect or negative. For example, it may be shown that the antibody does not bind with the other antigens studied or that the results are similar to those obtained using methods based on different criteria, e.g., bioassays.

The antibody is generally raised against preparations of gonadotropins which have been only partly purified, and the resultant antiserum probably contains antibodies to the contaminants as well as to the hormone itself. The hormone preparations used for iodination must be free of contaminants, and Sections 3 and 4 deal with methods for purifying FSH and LH for this purpose. However, any antigenic impurities which remain in the isolated hormone used as "the label" in the radioimmunoassay will be iodinated along with the gonadotropins, will become bound to the γ-globulin fraction, and will, therefore, influence the result of the test. If the contaminants are present in large amounts, and are sufficiently antigenic, the assay will be nonspecific in that the system will estimate the sum of the hormone plus contaminants. If the contaminant is present in small amounts it will

influence the quantitative significance of the result rather than the specificity.

Almost without exception, investigators who use radioimmunoassays for the estimation of gonadotropins in blood samples attempt to overcome this problem by "purifying" the antiserum. This is done by absorbing out foreign antibodies with serum and urinary concentrates prepared from children or from hypophysectomized subjects. In addition, other pituitary hormones are used to absorb out unwanted antibodies. Such procedures will increase the specificity of the assay if the interfering contaminants are of nonpituitary origin, or are due to contaminating hormones, but not if they are derived from some as yet uncharacterized pituitary glycoprotein.

The specificity of antisera has been discussed in Section 6, where it was pointed out that as yet antisera specific for either FSH or LH are rare and have been obtained by chance rather than by design. Many antisera appear to be specific for an immunodominant configuration shared by LH, FSH, HCG, and TSH; other glycoproteins do not so far appear to have been tested. However, several investigators have claimed that they have found antisera which can be satisfactorily used in radioimmunoassays. Thus Franchimont (F6) reported that he had an antiserum to FSH which was not influenced by 5000 IU of HCG, after it had been absorbed with HCG and serum proteins to remove antibodies to albumin and α_2-globulin. Aono and Taymor (A9) absorbed their FSH antisera with HCG and with sera from children under the age of 2 years, and Faiman and Ryan (F2) absorbed theirs with plasma from hypophysectomized patients. Both groups found that their assay results were affected by the presence of high concentrations of LH, although Aono and Taymor took the view that the ratios of LH to FSH in urine and plasma would not be high enough to affect their assays for FSH to a significant extent (A9). Neither group investigated the effect of TSH on their assay systems. Midgley and Reichert (M9) found that his FSH antiserum no longer reacted with LH after it had been absorbed with HCG, but Saxena and Rathman (S5) discovered that their antiserum was still nonspecific with respect to LH after absorption with the same hormone. Midgley and Reichert (M9), Saxena and Rathman (S5) and Faiman and Ryan (F1) all tested the specificity of their FSH antisera with respect to TSH by measuring the effect of serum from cretins on the labeled FSH antibody reaction; both concluded that TSH did not react with their FSH antibodies.

Franchimont (F6) stated that his HCG antiserum was suitable for use in an LH assay; however, the proof offered for this statement was not conclusive. He showed that FSH present in amounts 5 times as

great as HCG did not influence his inhibition curve, and that his HCG antiserum did not prevent the ovarian weight change induced by FSH in the augmentation test of Steelman and Pohley (S20); however, his results indicated that FSH probably did affect his assay since his estimates of LH were higher than expected when purified samples of pituitary FSH were being assayed. Franchimont did not test the reaction of TSH with his antiserum. Schalch *et al.* (S7) used an LH antiserum which did not cross-react with FSH, but did react with TSH. However, when they assayed LH in serum from patients with thyroid disease, they found that TSH probably did not interfere with the results.

Faiman and Ryan (F2) also showed that their LH antiserum reacted with FSH only in highly purified fractions, and that serum from cretinous patients, which was claimed to be high in TSH, did not influence their estimates of LH concentration. However, they found that with their LH antiserum there was no straightforward relationship between its reaction with LH and with HCG. Indeed at low concentrations HCG was bound to the antiserum more avidly than LH although at higher concentrations this was not the case. Stevenson and Spalding reported results obtained with an LH antiserum which contained discrete antibody populations for both TSH and FSH (S24); they noted that the presence of either of these hormones in a concentration 200 times greater than LH did not alter the standard curve of the latter. Bagshawe *et al.* do not claim absolute specificity for their HCG-LH radioimmunoassay system, but have stated that in clinical practice their assay is useful in the management of patients with trophoblastic tumors, both with respect to the natural history of the disease and to the effect of various types of medication (B2, O6a).

In conclusion, therefore, several groups of investigators have produced antisera which they find useful for the work which they propose to undertake. No systematic experiments have yet been carried out with these antisera to determine whether the specificity of radioimmunoassays for FSH and LH changes when hormones purified by different procedures are employed for the label. As shown in Section 2 there is a large variety of methods in current use for the preparation of the pituitary hormones, and there is evidence that the final products are not identical (see Tables 3 and 4). Accordingly, it is likely that different iodinated preparations will yield different results. In this connection Ryan (R24) found that several human pituitary LH preparations, all made by the same method, showed different cross-reactions with HCG in the presence of the one antiserum.

7.3.5.2. *The Influence of Electrolytes on Radioimmunoassays.* This topic is often discussed in the section dealing with specificity. How-

ever, it is more properly considered under a heading such as the effect of environment on the antibody–antigen reaction. The latter, like any other protein-protein interaction, depends on the formation of salt linkages, hydrogen bonds, etc., between the two kinds of molecule. These bonds cannot form in the presence of an adverse environment such as a high salt concentration, or an unfavorable pH, and care must therefore be taken in the assay of samples from patients in order to avoid such conditions. The concentrations of eletrolytes such as inorganic salts and urea in urine or a low urinary pH, can render the antibody incapable of combining with its antigen (F7), as can electrolytes, such as proteins and salts, in serum and plasma samples.

Electrolyte concentrations in urine are not always high enough to render the radioimmunoassay inaccurate, and many workers use untreated urine for their determinations. However, when electrolytes are present in high titer there will be less iodine labeled hormone bound to antibody than if these substances are in low titer, and this might mistakenly be interpreted as indicating a high gonadotropin concentration. This problem can be overcome only by separating the hormones from the small molecular weight substances in urine prior to assay, and methods for effecting such a separation have been discussed in Section 4. The effect of electrolytes on the results of radioimmunoassays in plasma is generally overcome by using the sample in a concentration no greater than 1 in 4 of the reaction mixture.

7.3.5.3. *Sensitivity of the Radioimmunoassay.* This depends on two factors. First, the hormone must be labeled with sufficient radioactive nuclide to allow the counting of free and antibody bound hormone fractions to be statistically significant at the end of the incubation period. Second, provided that the protein can be labeled to a high enough specific activity, the sensitivity of the test will depend on the equilibrium constant of the antibody–antigen system in use. The higher the constant in favor of the complex formation, the more sensitive will be the assay.

Table 7 summarizes some of the figures published for the specific activity of the labeled hormone, together with the sensitivities of the various assays.

7.3.5.4. *Precision.* The radioimmunoassay obeys the same laws of precision as any other chemical reaction, and therefore, under the same conditions, with the same standard and antibody preparation, and with the labeled hormone freshly purified, the results should be identical from day to day and from week to week. Variation is due to errors of technique and can be minimized. However, when a large number of tubes are being prepared with the addition of several reagents in micro

TABLE 7
SPECIFIC ACTIVITIES OF IODINATED GONADOTROPIC PREPARATIONS AND SENSITIVITY OF RADIOIMMUNOASSAYS

Hormone	Specific activity of labeled hormone (μCi/μg)	Material assayed	Sensitivity	Reference
FSH	100–160	Standard	—	(F2)
FSH	300–350	Plasma	5×10^{-5} IU	(S6)
FSH	194.5	Standard	4×10^{-9} IU/ml	(T3)
FSH	194.5	Standard	4×10^{-9} IU/ml	(A9)
FSH	100–350	—	—	(F6)
HCG	253	2nd IRP-HMG	2.7×10^{-9} IU	(M7)
LH	200–300	Standard	0.1 – 0.2 mg	(F2)
LH	300–350	2nd IRP-HMG	1×10^{-8} IU	(S6)
LH	25–30	Standard and plasma	0.2 ng/ml	(S7)
LH	—	—	2×10^{-9} IU/ml	(T2)
HCG	100–150	Urine, serum plasma	0.5 ng	(W9)
LH	5	Standard	0.5 ng	(S24)
LH	150–300	—	—	(F6)
LH	200–500	—	0.05 ng	(O4)
LH	200–300	—	—	(W6)

quantities, small errors are unavoidable. Results of radioimmunoassays will be more reliable if repeated on at least three different occasions, each time obtaining valid results, than by testing each sample in duplicate, or in triplicate.

Results of assays on patients may vary if the same sample is tested on different days, when there may be a progressive fall in potency with time; this is due to the lability of the gonadotropins. Stevenson found that the immunological activity of LH in urine and in standard preparations decreased progressively with repeated freezing and thawing; in addition Schechter *et al.* showed that plasma and serum contain potent proteolytic enzymes which may also influence the result (S8). Bagshawe *et al.* (B2) and Midgley (M7) have both performed statistical analyses of the results which they obtained in their radioimmunoassays. The former workers obtained figures for the index of precision (λ) ranging from 0.02 to 0.05; these must be regarded as highly satisfactory in comparison with the majority of bioassays (L6). Midgley (M7) reported λ values of 0.04 and 0.05 when he equilibrated his assays of the second IRP-HMG for 1 day; after equilibration for 3 days the corresponding figures were 0.02 and 0.03.

7.3.5.5. *Reproducibility.* Although the precision of assays in one laboratory may be high, it is by no means certain that the results will

be comparable with those from another center. The cause of this discrepancy is partly that each laboratory tends to use its own purified hormone for labeling, as well as its own standard. The World Health Organization has recently proposed that standards be made available for radioimmunoassay so that all investigators can express their results in the same way; this is obviously a step in the proper direction. In addition to the use of universal standards it is becoming increasingly clear that if results are to be truly interchangeable, it will be necessary to standardize the material which is being iodinated as well as the antisera being used for the radioimmunoassays (see Section 6).

7.3.5.6. *Practicability.* The radioimmunoassay is easy to perform, and unlike the hemagglutination-inhibition technique, requires only small amounts of purified gonadotropic hormones. The main disadvantage of radioimmunoassays is that they require costly equipment and laboratories which are specially constructed for the handling of relatively large quantities of isotopic iodine.

For the reasons discussed previously (Section 7.3.5.1.), radioimmunoassays are unlikely to be any more useful than hemagglutination-inhibition tests for work in which the identity of the hormone in question is important. There is no reason to expect that indices of discrimination between radioimmunoassays and bioassays will be any better than those between hemagglutination-inhibition tests and bioassays. However, radioimmunoassays for FSH and LH are extremely sensitive, and can probably provide a satisfactory indication of pituitary function in health and disease. For this reason, it is virtually certain that such procedures will be employed on an ever increasing scale in the management of patients.

7.4. Complement Fixation

Unlike the other immunological assays which have been used for the estimation of the gonadotropic hormones, complement fixation is not an inhibition reaction. Rather does it rely on the direct reaction between the hormone and its specific antibody.

7.4.1. *Theory of Complement Fixation*

The complement-fixation assay depends on the fact that during the reaction of an antibody with its antigen, added complement is also "fixed." The amount of complement fixed is directly proportional to the quantity of antigen which has reacted with the antibody, and therefore, in the presence of an excess of the latter, it is directly proportional to the quantity of antigen added. Another immunological system is

employed to detect the amount of complement remaining at the completion of the hormone-antibody reaction. The second system involves red blood cells which lyse when mixed with an appropriate amount of the antibody, hemolysin, in the presence of complement; the amount of lysis depends on the quantity of complement present.

Accordingly, when the system is working optimally the amount of hemolysis is quantitatively and inversely proportional to the amount of antigen added to the system. The hemolysis can be read spectrophotometrically (B9, M12), or the red blood cells can be tagged with ^{51}Cr, and the amount of lysis determined by counting the amount of ^{51}Cr which becomes soluble after lysis has occurred (B12). Not all antigen–antibody reactions will fix complement; e.g., horse antisera do not, nor do univalent antibody-antigen systems (A12).

7.4.2. *Complement Fixation as an Assay Method for Gonadotropins*

Complement fixation has not been widely used for the assay of hormones, although Brody has developed an immunological assay method for HCG depending on complement fixation and has discussed its reliability criteria (B9). Sensitivity was of a high order, it being possible to detect 0.4 IU of HCG per milliliter of serum. The precision of the test was very satisfactory, λ figures being as low as 0.01. When the complement fixation test was compared in the same serum sample with a bioassay for HCG depending on rat prostatic weight (L4), a reasonably good correlation was obtained as indicated by indices of discrimination which approximated to unity (B1).

The specificity of the complement-fixation system depends directly on the specificity of the antiserum, which should react exclusively with the gonadotropin being estimated. Butt and Lynch (B12) performed experiments designed to compare the specificity of a micro complement-fixation test with that of a radioimmunoassay for FSH. Their results demonstrated that their FSH antibody preparation cross-reacted with LH and HCG in the radioimmunoassay system, and could not be used because, when absorbed with these substances. it no longer reacted with FSH. However, with the same antiserum in the complement-fixation system, neither HCG nor LH fixed complement when present in concentrations ranging from 1 to 6000 ImU/ml, the maximum complement fixation occurring with 30 ImU FSH. It was concluded that their complement-fixation assay was specific for FSH. There are other examples in which complement fixation tests appear to be more specific than the radioimmunoassay systems (see Section 6.1.4.). This may arise from the fact that a system must be multivalent if complement is to be fixed, i.e., the antibody and antigen must have several complementary points

of attachment, while the radioimmunoassay can measure a substance which combines with only one site on the antibody.

In spite of its limitations, immunologists prefer to use a system such as the radioimmunoassay which involves only the reaction between an antibody and its antigen. Tests depending on complement fixation have found less favor largely because the reaction mechanism of this procedure is highly complex and is poorly understood.

8. Summary

One of the major aims of this review has been to examine the current status of immunological assays used to measure the pituitary gonadotropic hormones. Since all immunoassays basically depend on the interaction of an antigen of unique identity (in this case FSH or LH) and its specific antibody, it was necessary also to discuss the chemical nature of the various preparations of FSH and LH which are currently available and to consider the concept of antibody specificity as it applies in this field.

Glycoproteins with FSH and LH activities are both dimers with molecular weights of approximately 30,000. They can readily be reduced to monomers by treatment with high salt concentrations or extremes of pH, and this property permits the formation of hybrid molecules which may be present in some highly purified preparations. The implications of this property of the gonadotropin molecules have been discussed.

Both FSH and LH contain carbohydrate which is easily removed by mild hydrolysis. Sialic acid is necessary for the biological, but not for the immunological, activity of FSH. Consideration is given to the importance of carbohydrate moieties in the assay of both gonadotropic hormones.

FSH and LH can be extracted from pituitary tissue and from urine by a variety of methods; techniques for the purification of such extracts are also numerous. However, it must be emphasized that, at the time of writing, neither hormone has been fully characterized.

Physicochemical data with respect to FSH and LH remain scanty but suggest that materials of high biological activity prepared from the same source by different methods are not chemically identical. Evidence is now available that human pituitary and human urinary FSH are different chemical entities.

Antisera "specific" for FSH and LH are rare and have invariably been produced by chance. The specificity of such antisera varies depending on such factors as the purified material used as the antigen for testing and the types of assay method employed for the final determination. In general, it may be said that complement-fixation tests are more specific

than radioimmunoassays and techniques depending on hemagglutination-inhibition.

Despite some limitations with respect to specificity radioimmunoassays for gonadotropic hormones offer the advantages of high practicability, good precision and considerable sensitivity. It appears probable that in the future such techniques will be increasingly used in clinical studies.

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HEREDITARY METABOLIC DISORDERS OF THE UREA CYCLE

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1. Introduction

In man, as in other mammals, urea is the main end product of nitrogen metabolism. It is formed from the ammonia arising from the metabolism of the amino acids of protein by a sequence of five reactions, four of which comprise the urea cycle proper (Fig. 1). The end result is the conversion of ammonia into urea, with the re-formation of the individual reactants of the cycle.

Compared with other metabolic pathways, the urea cycle is short—possibly the shortest of all. The metabolic pathway of glucose, of amino acids, or of fatty acids are all very much longer, and even the citric acid cycle includes many more transformations. The number of metabolic disorders arising from enzyme defects involving the urea cycle is therefore limited. A consideration of the urea cycle must include the interrelationship between it, or any of its components, with other metabolic pathways, as well as other reactions of individual components. Thus ammonia can combine directly with α-ketoglutarate to form glutamic acid, which with a further molecule of ammonia forms glutamine (Fig. 2). This is an alternative pathway for ammonia utilization. As well as acting as a precursor for the synthesis of citrulline from ornithine, carbamyl phosphate also combines with aspartic acid in the initial step for the synthesis of pyrimidine and nucleic acids, and this pathway is to some extent in competition with the conversion of ammonia to urea (Fig. 3). There is a direct connection between the urea and citric acid

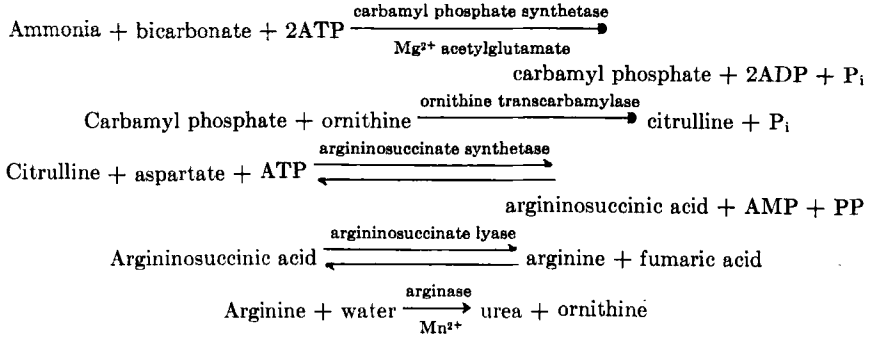

FIG. 1. Reactions of the urea cycle. P_i, inorganic phosphate; PP, pyrophosphate.

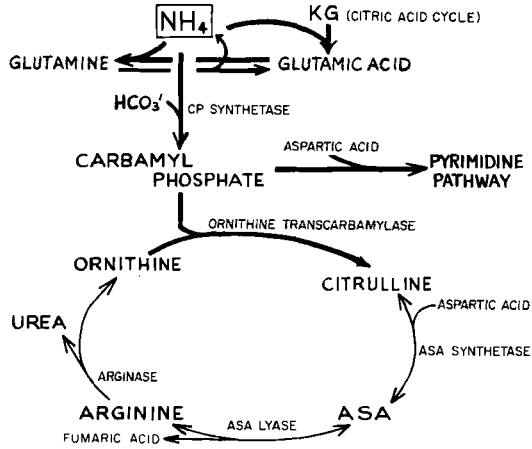

FIG. 2. Pathways of ammonia uptake, including urea cycle. CP, carbamyl phosphate; ASA, argininosuccinate; KG, ketoglutarate.

cycles since ornithine may be synthesized from α-ketoglutarate via glutamate and glutamate semialdehyde. These reactions are reversible (Fig. 4). A further link between these two cycles is in the conversion of fumaric acid, one of the products of hydrolysis of argininosuccinic acid,

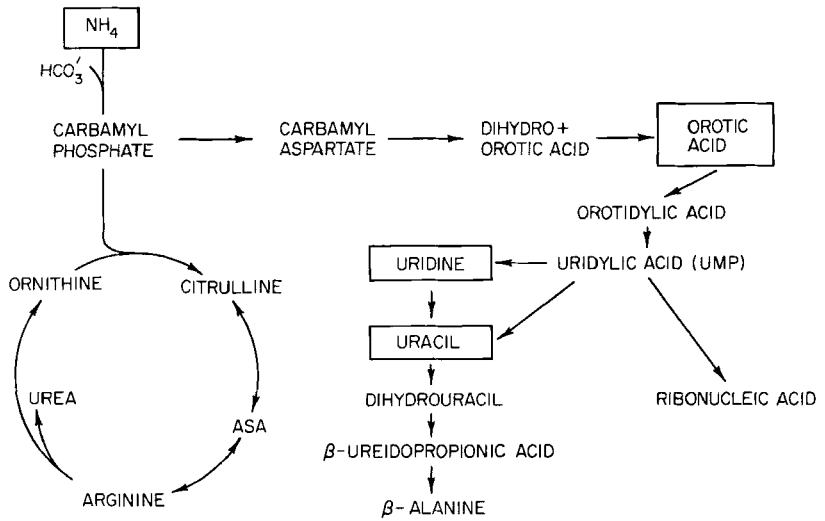

FIG. 3. Uptake of ammonia in pyrimidine synthesis and breakdown. Compounds found in excess in inherited disorders of urea synthesis are enclosed in rectangles. ASA, argininosuccinate.

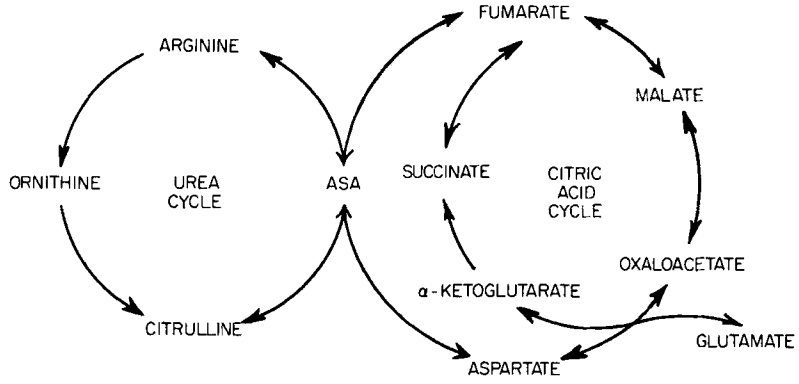

FIG. 4. Interrelationship of urea and citric acid cycles. ASA, argininosuccinate.

first to oxaloacetate in the citric acid cycle and then by transamination to aspartic acid for the Krebs–Henseleit cycle (Fig. 4). In addition to the direct hydrolysis of arginine to urea and ornithine, arginine can also reversibly react with glycine to form ornithine and guanidoacetate.

Ammonia arises in the body principally from the oxidative deamination of amino acids. In addition to its uptake in the reactions mentioned above, ammonia is also excreted in the urine as ammonium salts. This is not derived directly from the blood ammonia but is formed by the kidney from glutamine by the action of glutaminase. In metabolic acidosis, ammonia production and excretion by the kidney is greatly increased, and conversely it is decreased in metabolic alkalosis. This may be an important means of excreting excess ammonia. It must be remembered that ammonia formed by the action of intestinal bacteria on the protein hydrolyzates in the intestine can be also absorbed. The contribution of the ammonia formed in this way to the total ammonia in the body is unknown. Since this ammonia drains into the portal circulation, it is promptly removed by the liver.

Although the liver is the principal organ for the conversion of ammonia to urea, it has been demonstrated by Sporn *et al.* (S15) that this process can occur also in the brain, although the activity of the cycle is small. The urea cycle enzymes were later demonstrated in cerebral tissue by Tomlinson and Westall (T6), but the activities were very small, less than 1%, compared with the liver (R5). *In vivo* experiments in cats showed that ^{15}N-labeled ammonia as ammonium acetate injected in the brain was found largely in glutamine. That injected in the body was found mostly as urea and free NH3 in the liver (B3). This suggests that glutamine is more important for the removal of ammonia in the brain whereas in the liver the urea cycle is more important.

Enzyme Defects in Urea Cycle

In the years since the classic concept of Garrod (G1, G2) that certain disorders could arise from a deficiency of an enzyme mediating a single transformation in a metabolic pathway, such diseases have been recognized in ever growing numbers. The one gene–one enzyme hypothesis has been refined to that of one gene–one polypeptide, and the type of mutational errors has been extended from those involving the enzymes of metabolic pathways to those involving enzymes synthesizing structural proteins.

Defects of the enzymes mediating all four reactions of the urea cycle proper have now been established, and there is some evidence of the existence of a fifth enzyme defect, involving carbamyl phosphate synthetase, mediating the initial reaction of the pathway. As the first report of a metabolic disorder involving the urea cycle was only in 1958, it is not surprising that there have been very few reviews of this topic, that of Efron (E1) being the most complete to date.

Since one of the most important, if not the most important, result of a defect of the biosynthesis of urea is an increased level of blood ammonia, it is essential to consider other conditions that might affect indirectly the urea cycle or in some other way raise the blood ammonia. For example, it has been suggested that since lysine can act as a competitive inhibitor of the conversion of arginine to ornithine and urea, an increased level of plasma lysine may therefore inhibit the urea cycle (B12).

2. Biosynthesis of Urea and Enzymes of Urea Cycle

2.1. Enzymes of Urea Cycle and Review of Intermediary Metabolism

The first step in the formation of urea from ammonia is its combination with bicarbonate to form carbamyl phosphate (Fig. 1). This contributes only one nitrogen atom to urea, the other being donated by aspartic acid in the third step of the pathway. *N*-Acetylglutamate is required as cofactor, and the presence of Mg^{2+} is essential, ATP being converted to ADP in the process. The reaction is catalyzed by carbamyl phosphate synthetase (carbamate kinase EC 2.7.2.2). It has been shown that there are probably two forms of this enzyme, at least in rat liver. One is ammonia dependent, is primarily associated with mitochondria, and may be the enzyme responsible for the formation of carbamyl phosphate in the synthesis of urea. The other, which is glutamine dependent, is probably mainly extramitochondrial and may supply the carbamyl phosphate used

in the formation of carbamyl aspartate, the initial step in the synthesis of the pyrimidines (H1, K8, T1, T2). The existence of two enzymes might appear to complicate the question of carbamyl phosphate synthetase deficiency, since in the usual method, using liver homogenate, all the NH_3-dependent and over half the glutamine-dependent form are assayed. However, although the two enzyme activities are about equal early in fetal life in the rat, the NH_3-dependent activity is about 60 times that of the glutamine-dependent one in the 2-day-old animal. If these results are valid for the human, a marked deficiency in carbamyl phosphate synthetase as ordinarily measured must mean that it is the NH_3-dependent enzyme activity which is deficient. A deficiency in the glutamine-dependent carbamyl phosphate synthetase activity could not be detected by the usual assay since it forms such a small proportion of the total synthetase activity. In any case, a severe deficiency of glutamine-dependent synthetase activity is hardly possible, since this could be incompatible with life.

The first step in the ornithine cycle proper requires ornithine transcarbamylase (ornithine carbamoyltransferase, EC 2.1.3.3) which catalyzes the reaction of carbamyl phosphate with ornithine to form citrulline (Fig. 1). This is an irreversible reaction. The second involves the reversible condensation of citrulline with aspartate in the presence of ATP and magnesium ions to form argininosuccinic acid, and requires argininosuccinate synthetase (EC 6.3.4.5) (Fig. 1). This step was not formulated in the original pathway of Krebs and Henseleit (K12), but was demonstrated by Ratner and Pappas (R4, R6) and received further elegant confirmation by the original discovery of the metabolic error argininosuccinic aciduria. The energy required in this reaction is supplied by the breakdown of ATP to AMP and inorganic phosphate.

The third step, the cleavage of argininosuccinic acid to arginine and fumaric acid, is also a reversible reaction, which is mediated by argininosuccinate lyase (EC 4.3.2.1). The final one is the irreversible hydrolysis of arginine to urea and the re-formation of ornithine. It is mediated by arginase (EC 3.5.3.1) in the presence of manganese ions. It is probable that the ornithine cycle is the only source of endogenous arginine, and a deficiency of arginine may result if the urea cycle is blocked, especially in the case of a diet low or relatively low in arginine.

2.2. Organ Location of Enzymes of Urea Cycle

It has long been known that the liver is the main, if not the sole, organ of synthesis of urea. In the brain, only small amounts of urea can be formed. However, some of the enzymes of the cycle are widely distributed in the body tissues including plasma, although the liver is the only one

containing high enough amounts of these enzymes to account for the total urea synthesis. In addition to liver, other tissues have been used for the estimation of the urea cycle enzymes in cases of disturbed urea formation. Red blood cells and leukocytes have been used for the assay of argininosuccinate lyase, and, also although this is not so satisfactory, for ornithine transcarbamylase. Argininosuccinate lyase has been assayed in brain (C3) and kidney (C3, C8), both ornithine transcarbamylase and argininosuccinate lyase in kidney and jejunal mucosa (L8, V1), and argininosuccinate synthetase in skin (T3). Carbamyl phosphate synthetase has been assayed only in liver, although it is known to be present in the intestinal mucosa, spleen, thymus, and testis also (H2, T2). The difficulty in using tissues other than the liver is that the enzyme activities are normally low or very low so that low or absent activities may not be meaningful. Furthermore, because the enzyme is absent in one type of cell it does not follow that it is not present in the liver. The final criterion must always be a defective liver enzyme. The accessibility of such cells as leukocytes, which would be expected to contain a full complement of enzymes, often however makes it convenient to attempt the assay in these cells. Another alternative to the liver is to make use of tissue cultures. The growing cells of cultured skin explants have been shown to retain argininosuccinate synthetase, and such explants from a patient with citrullinemia had a markedly decreased argininosuccinate synthetase with altered K_m values (T3).

2.3. Intracellular Location of Enzymes of Urea Cycle

That form of carbamyl phosphate synthetase which may be responsible for the formation of carbamyl phosphate for urea synthesis is located mainly in the mitochondria, as is ornithine transcarbamylase (M10). On the other hand, argininosuccinate synthetase and lyase are cytoplasmic (M10), and arginase appears to be microsomal and also associated with the nucleus (D3). The synthesis of urea is thus neither wholly mitochondrial nor wholly in the cytoplasm. Citrulline formed in the mitochondria must diffuse out into the cytoplasma to form arginine, which in turn must diffuse into the microsomes to be hydrolyzed to urea and ornithine, which again must return to the mitochondria for the next stage, as portrayed diagrammatically in Fig. 5. This physical separation of the cycle of synthesis must involve an efficient system of transport across the membranes of the mitochondria and the microsomes which may be rate limiting. It can be envisaged that urea synthesis may be markedly impaired with a deficient membrane transport; thus a failure of ornithine to diffuse from microsome to mitochondria could result in both an accumulation of ammonia and an excess of ornithine (hyperornithinemia);

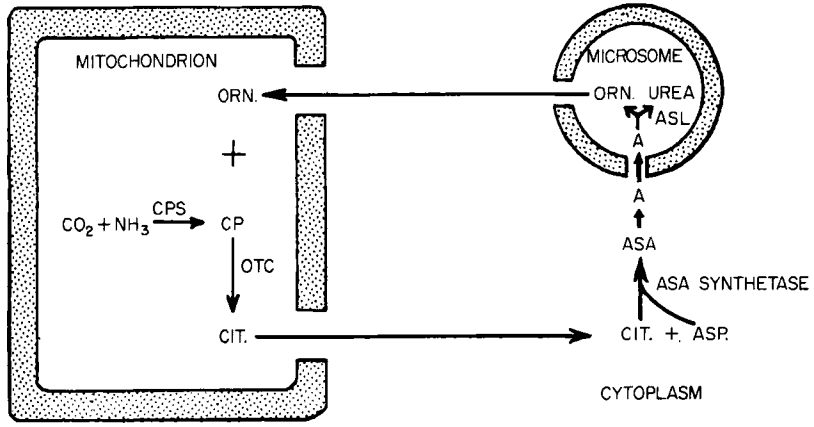

FIG. 5. Diagrammatic representation of intracellular localization of urea cycle pathway showing diffusion pattern. CPS, carbamyl phosphate synthetase; ASL, argininosuccinate lyase; ASA, argininosuccinate.

the failure of citrulline to diffuse out of the mitochondria could also result in deficient urea synthesis and citrullinemia.

3. Activities of the Urea Cycle Enzymes

The activities of the urea cycle enzymes in the liver have been reported by a number of workers, mostly using rat or mouse liver, but a few reports on the levels in the human liver have also appeared. The values reported vary in the different investigations even when the same animal or animal tissue is employed (B10, J2). Since both the method and the techniques employed affect the results, values for the normal should be established in each laboratory. Table 1 shows the normal levels of urea cycle enzymes obtained by various workers. For human liver Kennan and Cohen (K7) have reported on the urea cycle enzymes in two biopsy specimens obtained at operation. The author and his colleagues reported on the activities of all the urea cycle enzymes in the liver both from biopsy specimens and from those taken at necropsy (L1, L6, L8). Miller and McLean (M7) have also assayed these enzymes on a necropsy specimen of human liver. Kekomaki *et al.* (K5) have estimated the urea cycle enzymes in biopsy specimens of the liver of adults and children, not thought to have any abnormality of urea synthesis, as well as in liver biopsies from patients with familial protein intolerance. Their results are expressed as units per gram of nitrogen and not, as in other reports, as units per gram wet weight of liver. They are very different from the results we and others have obtained, although the ratios of enzyme activities are similar. However, all agree in showing a very wide

TABLE 1

ACTIVITY OF UREA CYCLE ENZYMES IN NORMAL HUMAN LIVER[a]

Age group	Source of tissue		CPS	OTC	ASS	ASL	A	Reference
Children	Biopsy	Mean:	320 (5)	5183[b] (13)	28 (3)	127 (3)	38200 (4)	(L3)
		Range:	180–615	3950–6550	21–41	100–177	24100–70200	
Children	Necropsy		448 (1)	6550 (1)	33 (1)	144 (1)	12000 (1)	(H5)
Adults	Necropsy	Mean:	156 (5)	4388 (5)	75 (5)	276	33600	(M7)
		SD	29	537	9	39	7535	
Children	Biopsy	Range:	7.8–28.8 (12)	105–131 (12)	—	21–50 (12)	2610–6134 (12)	(C14)
Adults	Biopsy		40.2 (2)	554 (1)	—	221 (1)	14640 (1)	(H4)
	Necropsy		36 (1)	327 (1)	—	—	—	
Adults	Biopsy		384 (6)	13340 (6)	120 (6)	600 (6)	134000 (6)	(K5)[c]
Children	Biopsy	Mean:	350 (5)	6640 (5)	49 (5)	218 (5)	68250	
		Range:	124–460	4130–8260	19–77	107–309	51600–87600	
Adults	Biopsy	Mean:	240 ± 17 (2)	8460 ± 550 (2)	51.6 ± 3.4 (2)	174 ± 6 (2)	7750	(B10)

[a] Values are expressed as micromoles per hour per gram wet weight. Numbers in parentheses indicate number of patients. CPS, carbamyl phosphate synthetase; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; A, arginase.

[b] Estimated at pH 7. Value at pH 8.3 = mean 5787; range 3900–9090 units.

[c] Author's results were expressed as μmoles/hr/mg liver protein. For comparison with the results from the other laboratories, the author's data have been converted to μmoles/hr/g wet weight of liver by multiplying by a factor of 200 as an approximation.

range of normal values, making the interpretation of a moderate reduction, indicating a heterozygote state, very difficult.

Factors Affecting the Activities of the Urea Cycle Enzymes

It must be noted that only few results have been obtained on fresh biopsy specimens; most have been from specimens which have been stored in the frozen state for some time or from specimens of liver which have been removed at necropsy at varying unstated periods after death and kept deep frozen at —15°C for various periods of time before analysis. There is some evidence from our results that at least two, carbamyl phosphate synthetase and ornithine transcarbamylase, of the urea cycle enzyme activities fall off on storage at —15°C for even 1 day, and this decrease continues over longer periods. Thus carbamyl phosphate synthetase activity in fresh mouse liver is in our experience appreciably higher than in liver kept frozen for some days or weeks. This is borne out by a comparison of the enzyme activities found in human liver obtained by biopsy, measured immediately, after storage at —15°C, and finally in liver obtained at necropsy (Fig. 6). Ornithine transcarbamylase activity in a human biopsy specimen of liver is greater when assayed immediately than when it is kept frozen even a short time or

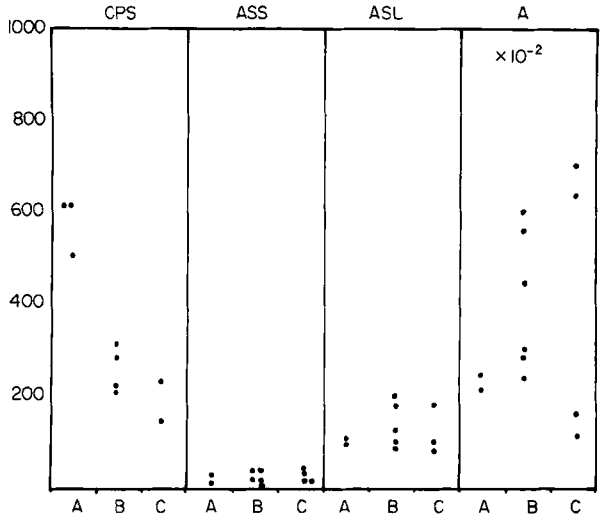

Fig. 6. Comparison of carbamyl phosphate synthetase (CPS), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), and arginase (A) activities in the human liver assayed immediately after biopsy (A), after storage at —15°C (B), and at necropsy (C).

when it is obtained at necropsy (Fig. 7). The levels obtained by us in the latter cases are similar to those found by Miller and McLean (M7) on necropsy liver. These authors, however, claim that no significant loss of activity in any of the urea cycle enzymes was detected in rat liver kept frozen for several days. Argininosuccinate synthetase and lyase appear to be more stable. Since the activity of arginase is extremely high, both absolutely as well as relative to the activities of the other enzymes of the urea cycle, the possibility of a falling off of its activity on storage is of less importance.

The loss of activity of ornithine transcarbamylase and carbamyl phosphate synthetase on storage is of some practical importance. When a very low activity is found, this must indicate a severe deficiency, but the significance of only a moderate reduction in activity may be difficult to assess, and in such circumstances, it is important to avoid the possible loss of activity occurring on storage. Sometimes, also, a severe primary deficiency of an enzyme may be associated with a reduction in the activity of another enzyme of the cycle, as has happened in two cases of hyperammonemia. It is important to determine whether this represents a true association of two enzyme deficiencies coexisting in one patient or whether it is due to a possible fall in activity of either enzyme on storage even for 1 day. Hers (H3) has argued that in glycogen storage disease such as association has never been proved and suggests that reports to the contrary are probably due to laboratory errors of assay.

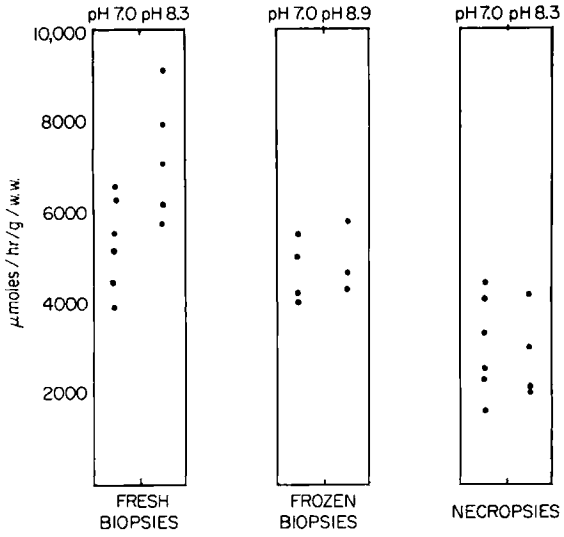

FIG. 7. Comparison of ornithine transcarbamylase activities in the human liver assayed immediately after biopsy, after storage at −15°C, and at necropsy.

However, not all the results in glycogen storage disease can be dismissed as resulting from imperfect laboratory technique. Enzyme deficiencies could occur if the mutation was in an operator gene controlling more than one adjacent gene (J1), and it is not impossible that such a situation exists in the hereditary metabolic errors of urea formation.

The activities of the urea cycle enzymes also vary according to protein intake, being increased in animals fed a high protein intake and diminished in animals on a low one (S4, S5). The increase in enzyme activities with increased protein intake applies equally to the enzymes with the highest activities, arginase and ornithine transcarbamylase, as well as to the rate-limiting enzyme, argininosuccinate synthetase. Similarly, hormonal factors which affect protein metabolism are also associated with altered enzyme capacities (M2). Urea cycle enzymes are also affected by agents such as carbon tetrachloride or azo dyes which damage the liver (M3, M4). Decreased activities of all enzymes result, normal levels being restored on withdrawal of the toxic substance.

4. Inhibition of Some Enzymes of the Urea Cycle

Arginine or citrulline in concentration in excess of the optimal can inhibit both argininosuccinate synthetase or argininosuccinate lyase in tissue cultures (S6). Whether this is applicable to the conditions of synthesis of the urea in liver is uncertain. α-Methylaspartic acid also specifically inhibits argininosuccinate synthetase (B7, C4, S10).

Lysine as well as ornithine and citrulline can inhibit arginase (C6, H5, S2). In the same way, citrulline can inhibit ornithine transcarbamylase (B13), which surprisingly is also inhibited by excess ornithine (S12).

5. Regulation of the Blood Level of Ammonia

Ammonia arises mainly from the oxidative deamination of the amino acid or by transamination of an amino acid with α-ketoglutarate to form glutamic acid, which is then deaminated by L-glutamic dehydrogenase to re-form α-ketoglutarate and to yield ammonia. The latter is also liberated in other reactions, for example in the hydrolysis of glutamine to glutamic acid and ammonia in the kidney, and in the synthesis of hemoglobin from *d*-aminolevulinic acid. Another source of ammonia is from the bowel contents. Urea and other nitrogen-containing substances are broken down to ammonia by the bacteria in the intestine, and this is absorbed into the portal circulation, from which it is removed by the liver and converted into urea. It is thought that appreciable amounts of ammonia are formed and absorbed in this way. Where the liver is

diseased, all the ammonia so absorbed is not completely converted into urea. In addition, in cirrhosis, where a collateral circulation between the portal and systemic veins has developed the absorbed ammonia may bypass the liver and gain direct access to the systemic circulation. This may also occur when the blood is diverted from the portal vein into the vena cava by an Eck fistula (M1) even in the presence of a normal liver, and in the absence of portal hypertension or collateral circulation.

The amount of ammonia in the blood is extremely small compared with the quantity of nitrogen-containing substances being metabolized every 24 hours. Despite an influx in an adult of 70 g more of protein per day, equivalent to 12 g of nitrogen, the level of ammonia nitrogen is maintained between 15 and 45 μg/100 ml blood. Ingestion by an adult of 30 g or more at one time does not raise the ammonia level beyond the normal, and the blood ammonia remains extremely small in relation to the amount of urea excreted.

It is clear that the blood level of ammonia is regulated between limits which although relatively wide are at low absolute levels. Although this is accomplished mainly by the conversion of ammonia into urea, which is rapidly excreted by the kidney, other mechanisms are available to regulate the blood ammonia levels. These are the reversible conversion of α-ketoglutarate to glutamic acid, and of glutamic acid to glutamine (Fig. 2), and the synthesis of pyrimidines for nucleic acid via carbamyl aspartate (Fig. 3). Both these mechanisms, unlike the open-ended one of urea synthesis, are necessarily limited. Ammonia cannot be stored as glutamine in indefinitely large amounts, although the latter is increased when an excess of ammonia arises. Since for glutamine formation, α-ketoglutarate is required, this substance is depleted when glutamine is formed in amounts greater than normal, thus reducing the activity of the citric acid cycle. A further consequence of the reduced amount of α-ketoglutarate is a failure of transamination with alanine, the level of which is therefore raised.

The utilization of ammonia resulting from the combination of carbamyl phosphate with aspartic acid, the initial reaction for the synthesis of the pyrimidine nucleotides, continues only as long as there is a requirement for them (Fig. 3). Regulation of this biosynthetic pathway is probably by way of feedback inhibition of aspartate transcarbamylase. The rat liver enzyme is inhibited by uridine, cytidine or thymidine or such derivatives as CMP, UTP, or TMP, all intermediates or products of this pathway (B8). This is not the only enzyme of the pathway which may be subject to feedback regulation. Dihydroorotase from rat liver is also inhibited by some pyrimidines and purines (B9).

Last, ammonia is excreted in the urine in the form of ammonium salts. Normally, however, this is relatively small, but it may be increased in metabolic acidosis, if kidney tubular function is normal. Ammonia is synthesized from glutamine by the kidney as required in order to conserve fixed base, e.g., sodium or potassium or to neutralize excessive amounts of acid excreted in the urine as, for example, in acidosis.

6. Regulation of Levels of the Intermediate Metabolites of the Urea Cycle in the Liver

The levels of the intermediates of the urea cycle in the liver also vary with protein intake, being appreciably higher on a high than on a low protein intake (K2). Presumably, this is because of an increased turnover of the urea cycle. Conversely the low amount of circulating amino acids resulting from a low protein intake will reduce urea cycle activity and therefore the level of the intermediates. Of the four amino acid intermediates of the cycle, neither argininosuccinic acid, which is a short-lived intermediate, nor citrulline participate in any other metabolic pathways, but ornithine and arginine do. The urea cycle is the only site of synthesis of arginine and citrulline. Arginine may be removed from the cycle by combination with glycine to form creatine and its level may need to be maintained by an exogenous source. Without such replenishment, the urea cycle may be slowed down, although the levels of the other intermediates of the cycle are maintained.

The synthesis of ornithine from α-ketoglutarate via glutamate may furnish this amino acid to the urea cycle. According to Katunuma *et al.* (K1), however, the reverse reaction, the conversion of ornithine to glutamate semialdehyde, is a major catabolic pathway for ornithine. Their results showed that ornithine-keto acid amino transferase activity is an important regulator of the level of ornithine in the liver and therefore of urea cycle activity. These authors have shown that ammonia inhibits the conversion of isocitrate to α-ketoglutarate, the decreased concentration of which means that ornithine is not depleted by combination with α-ketoglutarate to form glutamate. Thus a high level of ammonia results in a raised level of ornithine, and so an increased activity of the urea cycle.

In addition to these factors, any defect in absorption of any of the intermediate metabolites of the urea cycle, or an excessive urinary excretion of one of them, may result in a defective functioning of the cycle. Therefore the levels of these individual amino acids as well as of all the amino acids is important. Low levels of the plasma amino acids may result from a diet grossly deficient in protein, or from malabsorption in such conditions as celiac disease.

7. Laboratory Methods and Diagnosis

7.1. Ammonia

The determination of plasma ammonia is of great importance both for the diagnosis and for the treatment of hereditary metabolic disorders of the urea cycle. The level is always raised in these conditions since the other mechanisms for regulating blood ammonia mentioned above are not able by themselves to keep the ammonia level within normal limits.

Earlier methods of determination were based on the liberation of ammonia from whole blood by alkalis, the ammonia then being removed by distillation, aeration, or diffusion and trapped by acid, and the ammonia content estimated by titration with alkali or by a colorimetric reaction, Nessler's reagent, or phenol-hypochlorite solution (B4). The values obtained by these methods are subject to errors due to ammonia arising from the breakdown of such blood constituents as glutamine and other amino acids. Recent methods involve the immediate adsorption of ammonium ion on to a resin from which it is afterward eluted. In this laboratory, a micromodification of Fenton's method (F1) for capillary blood is used requiring only 0.2 ml plasma for duplicate estimations. Provided precautions such as careful cleaning of the skin before collection, keeping the blood cold during separation of plasma and using a resin column surrounded by ice during absorption and elution, only preformed plasma ammonia is estimated. This method gives a much lower and narrower range of values than the earlier ones and may be applied as a micromethod for the amounts of blood obtained by heel or finger prick.

The results of the determinations of blood or plasma ammonia levels are variously reported in the literature as micrograms of ammonia or μg of ammonia nitrogen per 100 ml. There is a difference of 21.4% calculated on ammonia. To avoid the necessity of recalculating all values reported so as to attain uniformity, all levels are given as ammonia, whether reported as ammonia nitrogen or not.

7.2. Urine and Plasma Amino Acids

Qualitative or semiquantitative screening by paper chromatography of the urine for amino acids is accomplished by the usual methods. In infants and children, as in adults, arginine and ornithine are normally present in the urine only in very small amounts, 1 mg or less being excreted in 24 hours (R9). Citrulline may be present in similar amounts, but it is usually absent in the urine. The other intermediate of the urea cycle, argininosuccinic acid, is also found in normal urine in amounts of up to 2 mg per day (P2) although it is not normally detectable in blood.

This is presumably because, although diffusion from the liver cell is only slight, such acid as does so is excreted into the urine, since the renal clearance is high.

Both in argininosuccinic aciduria and in citrullinemia, an excess of the amino acid concerned is easy to detect by paper chromatography. In the former, more than one large ninhydrin band may be present, corresponding to the acid and one or both of its anhydrides, especially if the urine has been standing for some time prior to chromatography (Fig. 13). These may be identified by their R_f values in solvents, butanol–acetic acid–water, and phenol–ammonia–water (L2). Argininosuccinic acid may also be isolated in quantity from the urine of affected children by a method similar to that used by Ratner *et al.* (R7) for isolating the acid after synthesis by an enzymatic method. Sparingly soluble barium salts are first separated by filtration after the addition of barium chloride solution (30 g/100 ml) (10 ml) followed by saturated barium hydrocide (20 ml) to each 100 ml of urine. To the clear filtrate is added 3 times its volume of absolute alcohol. The precipitated barium argininosuccinate is filtered and purified by dissolving in water and reprecipitating with alcohol 3 times (L1).

In hyperammonemia, however, there is no single large preponderant ninhydrin-positive band of amino acid visible after paper chromatography and the pattern of urinary amino acid found on chromatography may appear to be normal or nearly so. However, the glutamine band is usually more than normally prominent. Confirmation is by a quantitative ion exchange chromatography as below, which will also reveal the increased excretion of alanine.

The blood or serum may also be examined for amino acids by paper chromatography by the method of Efron *et al.* (E4), a simple method suitable for use as a mass screening procedure. Although this procedure will detect patients with citrullinemia or hyperargininemia, where high levels of the corresponding amino acids are found in the plasma, it is not so satisfactory for argininosuccinic aciduria, where the level of this acid is low, or hyperammonemia, where the rise in glutamine is not proportionately great enough to be discernible by a semiquantitative or qualitative method.

7.3 Quantitative Analysis of Amino Acids

The method of choice is ion exchange chromatography by automatic analysis (S14). Urine and cerebrospinal fluid can be applied directly to the column, but plasma must be first deproteinized. Since the accurate estimation of gultamine is of paramount importance in all inborn errors of the urea cycle, care must be taken to avoid the breakdown of glu-

tamine to glutamic acid or cyclization both during the preparation of the serum for chromatography and especially during analysis, and to effect adequate separation of glutamine from the other amino acids eluted about the same time. A suitable modification of the technique of Spackman *et al.* (S14) as adapted by Oreskes *et al.* (O1) has been devised by Palmer (P1).

7.4. Metabolites of Pyrimidine Synthesis and Breakdown

Certain metabolites of the pyrimidine pathway are excreted in excess of the normal in inherited metabolic disorders of the urea cycle. They include orotic acid, uridine, and uracil. Of these substances, only uracil is a normal constituent of urine, the other two either being absent or present in very small amount. They are readily detected qualitatively as dark bands at the appropriate R_f values when a paper chromatogram of the urine is examined under ultraviolet light. They may be estimated by an ion exchange method similar to that for urinary pseudouridine (R13).

8. Methods for the Assay of Enzymes of the Urea Cycle

8.1. General

The methods are essentially those described by Brown and Cohen (B10), with modifications to increase the sensitivity necessary for the small amounts of tissue obtained by biopsy. The simplest way of doing this was so to modify the reactions used for assaying the products of the enzyme reaction that smaller volumes and more dilute homogenates could be employed.

The substances assayed in the reaction mixture are citrulline, in determining carbamyl phosphate synthetase and ornithine transcarbamylase, and urea in determining argininosuccinate synthetase, argininosuccinate lyase, and arginase.

The color reaction for citrulline described by Brown and Cohen (B10) requires diacetyl monoxime in a mixture of commercial grade sulfuric and phosphoric acids, with the addition of catalytic amounts of phenylhydrazine. A more sensitive color reaction by Kulhanek and Vojtiskova (K13) in their method for estimating ornithine transcarbamylase used dimethylglyoxime in a somewhat more dilute mixture of sulfuric and phosphoric acids, containing phenazone. This was found to be satisfactory for measuring ornithine transcarbamylase (K13) using a buffer made with glycylglycine at pH 8.3 (K13) but was unsatisfactory when the incubation was carried out in Tris buffer at pH 7.0. Although a correct standard curve could be obtained using Tris buffer, the citrulline in

the reaction mixture was always lower than found by the method of Brown and Cohen (B10). Ornithine transcarbamylase is therefore measured by the unmodified Brown and Cohen's (B10) method when Tris buffer, pH 7.0, is used, and by that of Kulhanek and Vojtiskova (K13) with their more sensitive color reaction when glycylglycine buffer at pH 8.0 is used for the incubation.

Kulhanek's reagent (K13) is also used for the estimation of carbamyl phosphate synthetase. It is also considerably more sensitive for the estimation of urea than the α-isonitrosopropiophenone reagent recommended by Brown and Cohen (B10). It is, therefore, employed for the assay of argininosuccinate lyase and arginase, but not of argininosuccinate synthetase, because the reaction with the citrulline substrate masks the color production with urea. The α-isonitrosopropiophenone reagent is retained for the argininosuccinate synthetase.

8.2. Carbamyl Phosphate Synthetase

The fresh tissue is incubated in a CO_2/bicarbonate buffer, with ammonium bicarbonate, in the presence of ATP. The carbamyl phosphate formed reacts with added ornithine in the presence of ornithine transcarbamylase in the homogenate, to form citrulline, which is measured.

The liver is homogenized in a solution which contains ATP (final concentration 0.01 *M*) and $MgSO_4 \cdot 7H_2O$, (final concentration 0.01 *M*) adjusted to pH 6.7 at 37°C with solid $KHCO_3$. This homogenate is diluted 1:1 with cetyl ammonium bromide (CTAB); a final liver concentration of 1 in 10 is achieved, and the preparation is kept cold until used.

Two solutions are required to form the substrate mixture. Solution A contains 0.03 ATP and 0.03 *M* $MgSO_4 \cdot 7H_2O$, adjusted to pH 6.7 with solid $KHCO_3$. Solution B contains 0.04 *M* L-ornithine hydrochloride and 0.04 *M* *N*-acetylglutamic acid and is adjusted to pH 6.0 with approximately 1 *N* KOH solution, made up to 0.4 *M* with the calculated amount of solid ammonium bicarbonate and adjusted to pH 7.5 with solid $KHCO_3$. The substrate is prepared by mixing 2 parts of solution A with 1 part of solution B; pH about 7. It is gassed with CO_2 just before use until the pH is about 6.8 at 37°.

For the assay, 0.1 ml of homogenate is mixed with 0.3 ml of substrate solution in a small tube, and incubated at 38° for 20 minutes, taking 0.05-ml samples into 0.1 ml of 7% perchloric acid at 0, 5, 10, 15, and 20 minutes. After centrifugation, 0.05 ml of the supernatant is taken into a conical glass centrifuge tube, 0.05 ml of a 1% solution of dimethyl glyoxime in 96% ethanol added followed by 0.5 ml of an acid mixture made by dissolving 4 g of phenazone in a mixture of 76 ml of concentrated sulfuric acid, 11 ml of concentrated phosphoric acid and 163 ml

of water. After thorough mixing, the tubes are covered with vaccine caps pierced by syringe needles, and heated in a water bath at 100° for 20 minutes. A blank and standards of 0.2, 0.4, and 0.8 m*M* citrulline solutions are similarly treated. The tubes are cooled, and the optical density is read at 447 nm.

8.3. Ornithine Transcarbamylase

This enzyme catalyzes the transfer of the carbamyl group from carbamyl phosphate to ornithine, forming citrulline. Two methods are used, the first in Tris buffer at pH 7.0, and the second in glycylglycine buffer at pH 8.3.

8.3.1. *Assay at pH 7.0*

A 1:20 homogenate of liver is prepared in water. The reaction mixture consists of 20 μl of 1 *M* Tris buffer, pH 7.0, 60 μl of 0.1 *M* L-ornithine hydrochloride, and 400 μl of water. At zero time, 60 μl of a freshly made 0.1 *M* dilithium carbamyl phosphate solution is added. The solution is incubated at 37°C for 15 minutes, and 0.1-ml samples are taken into 0.2 ml of 7% perchloric acid at 0, 5, 10, and 15 minutes.

After centrifugation 0.1 ml of the supernatant of each sample is taken into a conical glass centrifuge tube and 0.3 ml of water, 0.2 ml of a solution prepared by mixing 12 ml of concentrated sulfuric acid (commercial grade), 12 ml of water and 36 ml of concentrated phosphoric acid, 40 μl of 2% diacetyl monoxime and 40 μl of 14 mg/100 ml phenylhydrazine hydrochloride are added. After thorough mixing, the tubes are covered with vaccine caps pierced by syringe needles, and heated in a water bath at 100° for 10 minutes. Standards containing 0.1 m*M*, 0.2 m*M*, and 0.4 m*M* citrulline are similarly treated. The tubes are cooled in the dark for 15 minutes and the optical density read at 490 nm.

8.3.2. *Assay at pH 8.3*

In this method, a blank containing an inhibitor is necessary since carbamyl phosphate will transfer its carbamyl group not only to ornithine, but also to the glycylglycine used for the buffer, and because there is a slow chemical combination of carbamyl phosphate and ornithine. The error is too small to be detectable by the color reaction of Brown and Cohen, but large enough to be apparent when the more sensitive reagent is used. The blank contains all the reactants, with the addition of phenyl mercuric borate (Famosept), which inhibits the enzyme-catalyzed formation of citrulline, but has no effect on its noncatalyzed chemical formation.

The incubation mixture contains, in each of 2 tubes, 100 μl of 0.06 *M*

glycylglycine; 0.06 *M* ornithine, pH 8.3; 50 μl of liver homogenate in water, and, in the blank only, 50 μl of saturated Famosept. At zero time, 50 μl of freshly made 0.1 *M* dilithium carbamyl phosphate is added to both tubes. The tubes are incubated at 37°C for 30 minutes taking aliquots of 40 μl from the test reaction mixture into deproteinizing solution B, and aliquots of 50 μl from the blank into deproteinizing solution A, at 10, 15, 20, and 30 minutes. The reaction becomes linear only after 10 minutes.

Deproteinization solution A contains a mixture of 40 μl of 7% perchloric acid and 60 μl of chloroform. Deproteinization solution B contains 10 μl of saturated (450 mg/100 ml) Famosept in addition.

After centrifugation the citrulline is measured in the supernatant by the method of Kulhanek and Vojtiskova (K13) as described for carbamyl phosphate synthetase.

8.4. Argininosuccinate Synthetase

In this assay the reaction is allowed to continue to the formation of urea, by the argininosuccinate lyase which is present in the liver homogenate, or which may be added if necessary in the form of normal liver homogenate.

To 0.5 ml of a 1:10 homogenate of liver in 0.1% CTAB is added 0.5 ml of substrate mixture. This contains 0.01 *M* L-citrulline, 0.01 *M* L-aspartic acid, 0.01 *M* $MgSO_4 \cdot 7H_2O$, 0.01 *M* ATP, 0.05 *M* KH_2PO_4, and 0.005 *M* K_2HPO_4, adjusted to pH 7.0 with KOH solution and 15 mg of arginase dissolved in 10 ml. The mixture is incubated at 37°C for 30 minutes, and samples of 0.2 ml taken into 0.3 ml of 7% perchloric acid at 0, 10, 20, and 30 minutes.

After centrifugation, 0.1 ml of each of the supernatants is taken into a conical glass centrifuge tube, and 0.3 ml of water is added. To each is added 0.3 ml of the acid mixture described for the estimation of ornithine transcarbamylase, followed by 20 μl of α-isonitrosopropiophenone (5 g/100 ml) in ethanol. After mixing, the solution is heated in a water bath at 100° in the dark for an hour and cooled in the dark for a further 15 minutes; optical density is then read at 540 nm.

Since the reaction mixture contains citrulline, which gives a color with the urea reagent, the urea standards must be prepared with added citrulline. To 1.5 ml of 7% perchloric acid is added 0.5 ml of water and 0.5 ml of substrate solution. A series of standards is obtained by adding to 0.1 ml of this mixture in each of 3 tubes, 0.3 ml of water or standard urea solution, such that the final concentration of urea in the 0.4 ml is 0.5, 1.0, or 2.0 mg/100 ml. The color is developed as described above.

It is important to note that argininosuccinate synthetase has a half-life of 172 minutes at 38°C, so that 25% of its activity is lost in 1 hour.

8.5. Argininosuccinate Lyase

Arginase is added to the reaction mixture to split the arginine formed by the lyase into ornithine and urea, which is determined.

To 0.05 ml of a 1:10 liver homogenate in 0.1% CTAB is added 0.25 ml of substrate mixture. This contains 0.03 *M* KH_2PO_4 and 0.03 *M* K_2HPO_4, adjusted to pH 7.3 with K_2HPO_4, with 0.0035 *M* argininosuccinic acid and finally 2 mg of arginase per milliliter. The reaction mixture is incubated for 30 minutes at 37°C, and 0.05-ml samples are removed into 0.1-ml of 7% perchloric acid at 0, 5, 10, 15, and 30 minutes.

After centrifugation, the urea is estimated in the supernatant by the method of Kulhanek and Vojtiskova (K13). A blank and standards containing 1, 2, and 4 mg/100 ml urea are treated in the same way.

8.6. Arginase

The urea formed from arginine by the action of this enzyme is directly measured.

Of a 1:2500 homogenate of liver in water, 0.8 ml is preincubated with 0.5 ml of 0.1 *M* glycine buffer, pH 9.5, and 0.05 ml of 0.1 *M* $MnCl_2 \cdot 4H_2O$ for 30 minutes at 37°C. This gives maximal activation of the enzyme by the manganese chloride. The reaction is then commenced by adding 0.65 ml of 0.85 *M* L-arginine monohydrochloride, adjusted to pH 9.5. The mixture is incubated for a further 20 minutes, 0.25-ml aliquots being taken into 0.5 ml of 7% perchloric acid, at 0, 5, 10, and 20 minutes. After centrifugation, the urea is measured as described for argininosuccinate lyase.

Provided the tubes of deproteinizing solution are prepared in advance of the biopsy, then the reaction mixtures, and last the homogenates, it is possible to measure all 5 enzymes, including ornithine transcarbamylase at two different pH's, in a tissue specimen in 1 hour. The homogenates are kept in ice until they are used, and the last incubation is begun about 30 minutes after the homogenates are made. At least 50 mg of liver is required.

8.7. Radioactive Methods

The enzymes requiring the largest amount of liver are carbamyl phosphate synthetase and, even more, argininosuccinate synthetase. It is therefore most desirable to develop more sensitive methods for their assay. Such methods using radioactive substrates can be devised for these enzymes.

8.7.1. *Carbamyl Phosphate Synthetase*

This method requires 10 μl of a 1:10 homogenate of liver in 0.25 *M* sucrose containing 0.01 *M* $MgCl_2$–ATP, brought to about pH 7 with 1 *M* Tris solution.

The reaction mixture consists of 0.2 ml of 150 m*M* potassium phosphate buffer, pH 7.5, 0.1 ml of 25 m*M* ATP, $MgCl_2$, 0.1 ml of 5 m*M* ornithine, 0.4 ml of ornithine transcarbamylase containing 7–15 units [prepared from ox liver by the method of Burnett and Cohen (B13)], 0.1 ml of 5 m*M* $NaH^{14}CO_3$ containing 10 μCi/μmole, 0.1 ml of 100 m*M* NH_4Cl with 50 m*M* acetyl glutamate, and 0.005 ml of homogenate. The mixture is incubated at 37°C for 30 minutes, aliquots of 0.2 ml being removed into 0.15 ml of 1 *M* $HClO_4$ at 0, 10, 20, and 30 minutes. As a blank, the same mixture, but with 0.1 ml of water substituted for the 0.1 ml of 5 m*M* ornithine is put through the same procedure at the same time.

To each, 0.05 ml of 0.02 *M* L-citrulline is added as a carrier. The tubes are placed in a vacuum desiccator over NaOH for at least 15 minutes to remove excess bicarbonate. The citrulline produced is estimated by a count of the radioactivity in 0.1-ml portions, using a TriCarb liquid scintillation counter.

8.7.2. *Argininosuccinate Synthetase*

To 0.25 ml of 1:50 homogenate in 0.1% CTAB is added 0.25 ml of the reaction mixture described in the original method, but with the inclusion of 1 μCi of radioactive citrulline, labeled with ^{14}C on the carbamyl group. The mixture is incubated for 30 minutes at 37°C, removing 0.1-ml aliquots into 0.15 ml of 1 *M* $HClO_4$ at 0, 10, 20, and 30 minutes.

The solution is chromatographed on paper to separate the radioactive citrulline from the urea formed from it. The portion of the paper containing the citrulline is cut, and the radioactivity is counted in a TriCarb liquid scintillation counter.

9. Clinical Aspects

9.1. Argininosuccinic Aciduria

Argininosuccinic aciduria results from a defective argininosuccinate lyase, mediating the cleavage of argininosuccinic acid to arginine and fumaric acid (Fig. 8).

Of all the inherited enzymatic errors of the urea cycle, this is the one so far most frequently reported, 19 cases in all (A1, A3, B2, C1, C3, C14, L2, L5, M7, M9, M13, S7, S13, W1). After the recognition of the

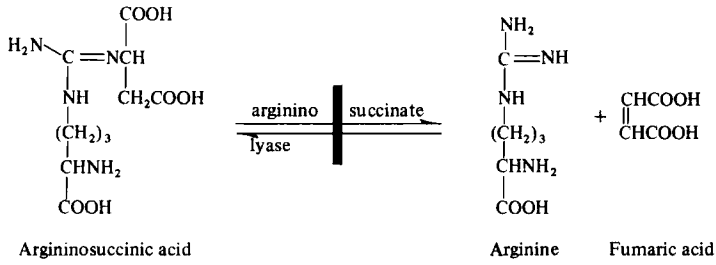

FIG. 8. Metabolic block in Argininosuccinic aciduria.

first 3 cases (A1, L2), many of the later ones were detected as a result of surveys of large groups of mentally retarded children (A3, C1, M13, S13). Because of this especial circumstance, the subjects were usually between the ages of about 3 and 18 years when first seen. In this group of cases, a history of an initial onset of illness in the first few months of life was sometimes elicited in retrospect, but often there was no such history, and the first complaint was that of physical and mental retardation or convulsions. Mental retardation is usually severe, but one subject was only mildly retarded, having an IQ of 92 (C1). The fits persist throughout, and the electroencephalogram (EEG) is usually abnormal. Ataxia is also a frequent feature. The liver is occasionally enlarged.

The first three children described with this condition had hair which was described as dry and brittle, so that it remained characteristically short (A1, L2). In this form of trichorrhexis nodosa, the breaks in the hair fluoresced red with acridine orange, as distinct from the more usual form of this condition in which the fluorescence is green (L2). In one of these children, the skin was rough in patches and there were numerous creases on the palms of the hand and soles of the feet. The disorder of the hair, originally thought to be a constant feature of argininosuccinic aciduria, is present in fewer than half, even of the older children.

Another group of cases includes infants presenting with severe illness suddenly occurring in the first week of life (B2, C3, L2, L5), and manifesting as lethargy, convulsions, and failure to thrive. Of four such neonates, only one survived, three dying within a few days despite vigorous intravenous therapy and exclusion of protein from the diet. In two instances (C3, L2), the patient was the first child; in the other two families, there was a history of the death of one or more sibs in the first week of life, following an illness similar to that of the proband.

Because of this characteristic onset and course in several infants, it has been suggested that there is, in addition to the less severe condition in the older child, a very severe and usually fatal variant of the disease

which occurs in the neonate. This has no biochemical justification as yet, although on general grounds, it is likely that more than one variant of this enzymatic defect can occur, resulting from different mutations of the same structural or control gene, as has been postulated to occur in other hereditary metabolic disorders, e.g., fructosemia (L13). The severe course could not have been due to a high protein intake in the form of artificial feeding since two of the infants were breast fed and in only one of the three who died was artificial feeding commenced from the third day of life.

9.2. Hyperammonemia

The specific syndrome arising from a severe deficiency of ornithine transcarbamylase has been termed hyperammonemia (L2) (Fig. 9). Next to argininosuccinic aciduria, this in the most frequently reported of the enzymatic disturbances of the urea cycle; eleven proved examples

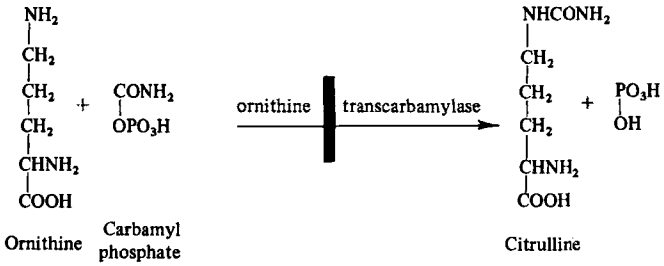

Fig. 9. Metabolic block in hyperammonemia.

have been described or are known from Great Britain, France, Switzerland, Yugoslavia, and Australia (C13, H5, L3, L6, L8, R14, S1). Unlike argininosuccinic aciduria, however, none of the reported cases have been reported as a result of screening tests applied to large groups of mentally retarded children. This is probably because argininosuccinic aciduria is readily recognized by the excretion of large amounts of argininosuccinic acid seen on paper chromatography, where no such heavily predominant amino acid is present in the urine in hyperammonemia.

The clinical manifestations of hyperammonemia are very similar to those of argininosuccinic aciduria or citrullinemia, but differ in that the neurological manifestations are in general more severe.

As in argininosuccinic aciduria, there are two main clinical categories, an infantile and an adult type. Adults with hyperammonemia may be symptom free, having only an aversion to protein foods. In the infant, symptoms are usually severe. Vomiting is frequently the first symptom. It may occur in the first few weeks of life, especially if artificial feeding

is commenced then, or may be delayed for several months, one affected child beginning to vomit only in the third year of life. The liver is usually enlarged. Later, there are episodes of hypotonia and lethargy developing into coma and this may be associated with convulsions. These episodes may occur shortly after the onset of the intermittent vomiting or may be delayed for a year or more. They are often associated with an increased protein intake, as when the infant is weaned to artificial feeding or mixed feeding. In one case, at least, the first such episode occurred as a result of the administration of ammonium chloride for the determination of the H^+ ion clearance index. Some affected infants may apparently thrive for the first few months of life or even longer, before physical and mental retardation become apparent. Later, ataxia may be a feature. In the severely affected infants there are profound EEG changes which indicate gross diminution of cerebral activity (S16).

Of the 11 known cases, 10 are female and 1 male. The prognosis is poor. Three have died, one in the second year of life (H5) and two in the seventh year of life (L8). On the other hand, one child, a girl aged 4 years, on a very low protein diet, is well and of normal intelligence (L10). Another child, a boy also on a low protein intake, has developed normally and has a normal IQ (L6). Other children, although still alive, have gross mental and physical retardation (C13, L3). The only adult with this condition so far diagnosed is quite normal apart from an elevated plasma ammonia (L3).

The abnormal hair or skin of argininosuccinic aciduria are not found in hyperammonemia.

Brain Damage in Hyperammonemia

There have been several reports on the nature of the brain damage occurring in hyperammonemia (B11, L3, S13), both from biopsy during life and from the brain at death. It seems probable that in all cases with neurological manifestations the brain has suffered damage, often severe; the damage is greater, the earlier the age at which the symptoms first appear. For this reason (L3) it has been suggested that the infant's brain is more susceptible to damage by high ammonia levels than that of the older child or adult. Bruton *et al.* (B11) have reported in their examination of the brains of two children who died from hyperammonemia that the common feature of both was the presence throughout the brain of large pale astrocytic nuclei, known as Alzheimer, Type II astrocytes. Similar cells were found in the brain of a child with argininosuccinic aciduria (S11). Other changes found, for example, shrinkage of nerve cells, were less constant. There is some evidence (B11) that all the changes, including the presence of Alzheimer Type II cells, are due

to the effect of high levels of blood ammonia, and that slight or moderate elevations of the blood ammonia are of little effect (B11, L6).

9.3. Citrullinemia

Citrullinemia results from a severe deficiency of argininosuccinate synthetase (Fig. 10). Only four cases of this condition have so far been reported (C2, M6, M12, V1), in one of whom (C2) no clinical details are recorded. Three of the children have so far survived, and one has died. The clinical features in the first two published cases were very similar. In both, early development was normal, until the 6th or 9th month of life, when vomiting was the first symptom. Both had convulsions from time to time; in both, the liver was enlarged. In one, growth fell off from the onset of vomiting and mental retardation was moderate; in the other, physical and mental retardation were noted apparently only after the first year of life, and mental retardation especially was severe, the IQ being only 20 at 2 years 4 months of age. This child also had more severe neurological manifestations, coarse Parkinsonian tremors of head and hands, as well as hypotonia. A pneumoencephalogram showed generalized cortical atrophy and the EEG was grossly abnormal. A surprising and probably unrelated feature was the finding of hypothyroidism, as indicated by a low ^{131}I uptake and a slightly low protein-bound iodine (PBI). He was treated with thyroid extract, and he became more alert, and vomiting decreased. It ceased altogether when his protein intake was moderately restricted at about 18 months of age. It is interesting to note that in two of the four cases, the parents were thought to be related. Unlike argininosuccinic aciduria, the hair and skin have been normal in all cases.

9.4. Hyperargininemia

Hyperargininemia, the most recently reported (T4, T5) condition, results from a block in the hydrolysis of arginine to ornithine and urea,

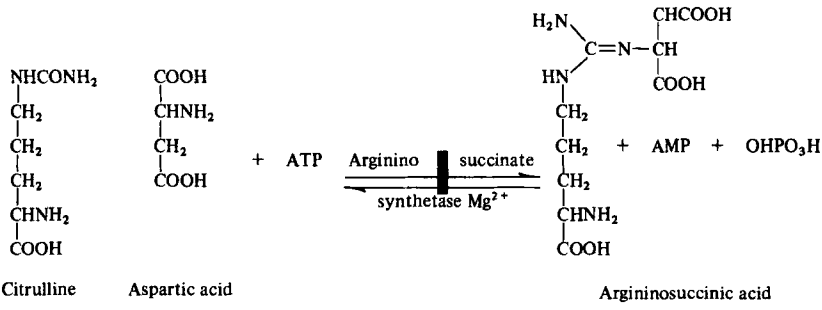

Fig. 10. Metabolic block in citrullinemia.

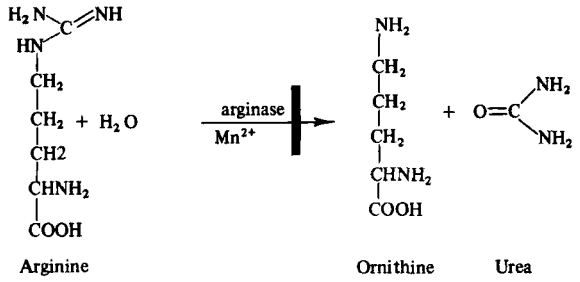

FIG. 11. Metabolic block in hyperargininemia.

mediated by arginase (Fig. 11). It has so far been recognized in only two children, sisters from a family of 5 children of healthy unrelated parents. The older of the two was apparently normal until 22 months of age, when she had a hyperpyrexia lasting over an hour leaving a left-sided paresis. A second convulsion occurred at 27 months of age. The waddling gait was first noticed at 2½ years of age, and later spasticity of legs and arms, the latter being only slightly affected.

In the second sib, vomiting was a prominent feature in early life. Pregnancy and delivery were normal, but by the end of the second month of life there was failure to thrive and vomiting began. This at first stopped on changing the milk feeds but recurred at 4½ months, and was now associated with psychomotor changes, increased irritability, and decreased interest in her surroundings. Convulsions first occurred in the third month of life, and at 7 months of age an episode of vomiting was associated with convulsions developing into coma. She was now inert and unable to hold up her head. There were other slight neurological manifestations, a persistent Moro reflex, and some hypertonicity of both arms and legs. Episodic vomiting has persisted, and further convulsions, associated with the onset of infection, have occurred. An air encephalogram showed dilated ventricles and an appearance of porencephaly, and the EEG showed dysrhythmia. The liver is enlarged. She, like her sister, is mentally retarded. The similarity in the clinical manifestations of the two sisters led to the analysis of the amino acids in the urine, serum, and cerebrospinal fluid, and to the discovery of the grossly raised arginine level.

Another possible example of the same disorder has been described by Peralta Serrano (P3) in a child born of consanguineous parents. Convulsions began on the 6th day of life. When the subject was first seen at 20 months of age, psychomoter development was retarded and there was muscular hypotonia. There was an associated hypertelorism and a peculiarity of the hair, which showed patchy differences of color. The routine biochemical tests, including the blood urea, appeared normal.

However, paper chromatography of the urine revealed a gross amino-aciduria, with a very high arginine excretion of 80 mg/100 ml. A high level of arginine was also found by chromatography of both the blood and the cerebrospinal fluid. A curious feature was the fact that the hyperargininuria was intermittent and apparent only during the periods of convulsions. Hyperargininuria was not found in either parent.

9.5. Summary of Clinical Features and Similarity of Clinical Features in Enzymatic Disorders of Urea Synthesis

The clinical features of argininosuccinic aciduria, hyperammonemia, citrullinemia, and hyperargininemia resemble each other closely. In all four diseases of the urea cycle the baby appears to thrive normally for the first few months of life, and sometimes, in very special circumstances, e.g., prolonged breast feeding, even as long as the second year. Vomiting for which there is no apparent cause is frequently the first sign, and in any case is almost always present at some time. In all four conditions, apart from mental retardation, there are other neurological symptoms, such as convulsions, spaticity of the limbs, and ataxia. These are perhaps more severe in hyperammonemia than in the others. The abnormal hair and skin found in many cases of argininosuccinic aciduria are not present in the other three disorders.

The simplest hypothesis is that in all four the neurological disorder is due to the raised blood ammonia (E1, L8). This elevation is more consistent in hyperammonemia, because the metabolic block directly affects the uptake of ammonia by the urea cycle whereas in the other three defects, ammonia may still be utilized to form citrulline, argininosuccinic acid, or arginine. On this basis the severity of the disease is thus probably directly related to the height of the blood and cerebrospinal fluid ammonia levels. This assumption would account not only for the differences in the severity in the four conditions, but also the largely symptom-free condition in the older child or adult. Blood ammonia level varies with protein intake. The protein intake of the artificially fed infant per kilogram body weight per day is very much greater than that of the older child or adult, so that an affected infant will have a much higher blood ammonia level than an affected older child or adult on a normal diet. On the other hand, breast fed babies get less than half the protein intake of those artificially fed on cow's milk. An affected breast fed infant will therefore have a lower blood ammonia level than an artificially fed affected baby and probably no higher than an affected adult. This is borne out by the fact that the most severely affected infants have been artificially fed almost from birth. Furthermore, in at

least two infants the most severe neurological episodes have been precipitated by giving ammonium chloride for tests of hydrogen ion excretion. The severe neurological manifestations of the infant may be therefore due to a high blood ammonia level resulting from the relatively high protein intake together with possibly a greater susceptibility of the infant's brain to damage by blood ammonia levels above the normal.

Brain damage although very frequent in these conditions does not invariably occur. Certainly one adult and one girl, now 14 years old, with hyperammonemia and two children aged 2 years and 4 years are normal, both mentally and physically, and one child of 4 years with argininosuccinic aciduria had a nearly normal IQ of 92 and showed no physical or neurological signs of the disease (C1). In the case of an adult with hyperammonia, the mother of a severely affected baby, it was plausible to suppose that this freedom from symptoms was due to an exceptionally prolonged period of breast feeding, and protein intake, even when mixed feeding was started, was low owing to wartime exigencies. Thereafter, an aversion to protein in the diet kept the protein intake low.

It is interesting to speculate on the reasons for the relative well-being in those patients with any hereditary metabolic disorder of the urea cycle in whom development appears normal for at least the first few months of life. Symptoms usually commence as soon as artificial feeding with cow's milk is introduced, because of the higher protein content of the feeds or when the infant is weaned from the breast with high protein foods. Presumably the fetus *in utero* is protected by the clearance of toxic substances, e.g., ammonia, by the maternal liver. There may also be another reason. According to Hager and Jones (H1), in fetal rat liver glutamine-dependent carbamyl phosphate synthetase is about equal in activity to the ammonia-dependent one, which is low. If this holds true for the human, the activity of the glutamine-dependent enzyme may be sufficiently great to restrain the fetal blood ammonia level to normal limits. This would also be consistent with the requirement of the fetus for rapid growth of tissue, which would necessitate the diversion of the available nitrogen for growth. It may be that, even in the postnatal period, a similar requirement for nitrogen for rapid growth is sufficient to maintain the blood ammonia to relatively normal levels for the first few weeks or even months of life, in children with disturbance of the mechanism of urea formation.

9.6. Carbamyl Phosphate Synthetase Deficiency

No proved example of a gross deficiency solely of carbamyl phosphate synthetase (Fig. 12) has as yet been recorded. Prior to the characterization and separation of the two carbamyl phosphate synthetases, it

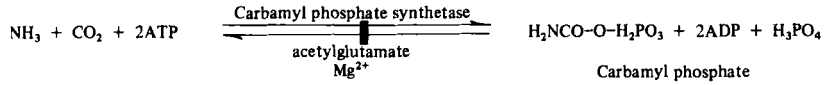

FIG. 12. Postulated block in carbamyl phosphate synthetase deficiency.

would have seemed highly unlikely that an infant with a gross deficiency of this enzyme could be viable since it would also have to follow that the first step in the synthesis or pyrimidine for nucleic acid would be blocked. Even a partial deficiency in this enzyme would have been unlikely. The discovery, however, of a distinct glutamine-dependent synthetase catalyzing the synthesis of carbamyl phosphate for the pyrimidine pathway, has made it possible to envisage the occurrence of a severe deficiency of the ammonia-dependent enzyme serving the urea cycle but with an intact or normal glutamine one. Such a defect would then be similar to the other four proven types of enzyme defect.

The first child with a deficiency of carbamyl phosphate synthetase to be briefly reported (F2) began to have periodic vomiting attacks from the second week of life. These recurred each time she was given proprietary milk feeds. There was lethargy, dehydration, and hypotonia. The symptoms subsided on intravenous therapy with protein free fluids. The blood and cerebrospinal fluid ammonia levels were both very high at 480 and 550 μg/100 ml, respectively. On a low protein diet of 1 g/kg body weight, she continued to thrive and appeared developmentally normal. After a laparotomy she developed severe acidosis and ketosis and died at 5 months of age. The hypothesis that there was a defect in urea synthesis was supported by the finding that there was little incorporation of ^{15}N into urea when glycine-^{15}N was given orally although citrulline-^{15}N was rapidly converted to urea. A specimen of liver obtained at operation proved to have a reduced carbamyl phosphate synthetase activity whereas the other enzymes of the urea cycle were normal but no quantitative results were recorded. In view of the lack of supporting data, there is no complete proof that this is an example of enzyme deficiency confined to carbamyl phosphate synthetase.

A second report (H4) concerned a female infant who was hospitalized at 20 days of age because of difficulty in feeding, lethargy, and convulsions. Two sibs had died with similar symptoms at 4 weeks of age, but two other sibs were normal. Blood ammonia levels on a relatively low protein intake (1.5 g/kg/day) ranged between 25 and 100 μg/100 ml, and blood urea between 2 and 14 mg/100 ml. Her general condition improved on the low protein diet, but later it deteriorated and she died at 7½ months of age, weighing little more than her birth weight of 3.25 kg. Liver function tests were normal; there was a slight metabolic alkalosis.

Plasma amino acids were incompletely analyzed, and the only significant feature was that the arginine was reported to be low, and this was also the case in the cerebrospinal fluid. High fasting blood glucose levels combined with low blood lactate and pyruvate levels suggested low capacity for glycolysis. This was ascribed to a defective insulin response to amino acid stimulation. The low levels of plasma and cerebrospinal fluid urea were thought to be due to defective urea synthesis. Of the five enzymes of the urea cycle estimated both in a biopsy of the liver before death and in liver taken at necropsy, only carbamyl phosphate synthetase was found to be low, less than half that of a control normal.

A third instance has been briefly reported by Kirkman and Kiesel (K10). They described a male infant who was admitted to hospital at 1 month of age because of vomiting, severe growth failure, and tremulousness. The blood ammonia was very high, 356 μg/100 ml. Neurological development was normal. Biochemical findings were an acidosis, organic aciduria, and lysinuria. There was also a moderate hyperglycinemia. When the protein intake was restricted to 1.5 g/kg/day, the blood ammonia fell to 80–260 μg/100 ml. The liver biopsy obtained by needle aspiration showed a normal ornithine transcarbamylase activity which also had normal K_m values. On the other hand, the carbamyl phosphate synthetase activity was only half that of specimens obtained at necropsy.

None of these cases can be considered as established examples of an isolated carbamyl phosphate synthetase deficiency. Although in the first the clinical history and the presence of severe hyperammonemia support the diagnosis of a defect of urea synthesis, the normal finding of levels of plasma amino acids, apart from glycine, is against it. No actual numerical data on the level of activity of the urea cycle enzymes are given.

In the second instance, blood ammonia levels were reported to be above normal on only two occasions and even then only slightly exceeded the normal.

An especial difficulty is that, while in one of these cases, the statement is made that carbamyl phosphate synthetase activity is reduced, and in the two others the more definite statement that it was reduced to about half the normal value, in only one out of the three was a numerical value actually recorded. In view of the rapid fall in the liver carbamyl phosphate synthetase activity which occurs on storage for even a short period of time, it is especially important that the enzyme be assayed in fresh tissue. No mention is made in these reports of the duration of time between the removal of tissue and its examination. Much of the reduction in activity that was found could be ascribed to delay. A final difficulty concerns the degree of reduction of carbamyl phosphate synthetase

activity which has been reported. In all the proved instances of a defect in the biosynthesis of urea, the reduction in activity of the enzyme concerned has been severe, 10% or less of the normal value. It would be surprising therefore if a reduction of only 50% in the activity of the carbamyl phosphate synthetase, which is not the rate-limiting enzyme, would be sufficient to cause the serious clinical effects which occur.

It may be concluded therefore that a metabolic disorder due solely to a deficiency of carbamyl phosphate synthetase has not yet been proved.

10. Biochemical Findings in Inborn Errors of the Urea Cycle

10.1. Argininosuccinic Aciduria

This was the first example of an enzyme defect of the urea cycle to be discovered (A1). A large amount of an unknown amino acid was detected in the urine by paper chromatography and was identified later by Westall (W3, W4) as argininosuccinic acid, an amino acid which had not hitherto been found in urine. All new cases since then with the exception of two not established with certainty have been diagnosed by the considerable amounts of argininosuccinic acid excreted in the urine.

In addition to argininosuccinic acid, the compound mainly present in freshly passed urine, two derivatives of this acid are also usually present (Fig. 13). These are both anhydrides of argininosuccinic acid, one being a six-membered ring form (anhydride B) and the other a five-membered ring form (anhydride C) (W4) (Fig. 14). The free acid is converted to the anhydrides if the urine is allowed to stand at room temperature for some time. Complete conversion can be effected by heating the urine with hydrochloric acid.

10.1.1. *Levels of Argininosuccinic Acid in Plasma, Cerebrospinal Fluid, and Urine*

The estimation of argininosuccinic acid in urine and plasma is complicated by the gradual conversion of most of the free argininosuccinic acid to the anhydride forms. In spite of this, the plasma levels of argininosuccinic acid with its anhydrides which have been determined by several groups of investigators, usually by column chromatography, are comparable, most reporting levels of 3–4 mg/100 ml (Table 2). However, in three recent reports of argininosuccinic aciduria in the neonate, all of whom have died, the blood levels of argininosuccinic acid have been extremely high. Baumgartner *et al.* (B2) reported a level of 11.5 mg/100 ml of total argininosuccinic acid and anhydrides, Carton *et al.* (C3) a level of 15.1 mg/100 ml of argininosuccinic acid and an-

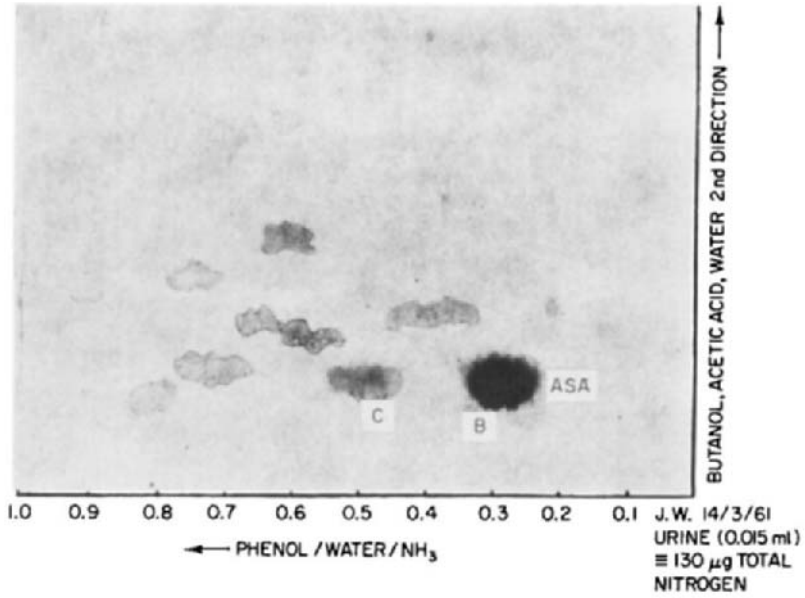

FIG. 13. Two-way chromatogram of urine from a case of argininosuccinic aciduria. The free acid and the two anhydrides are indicated (L2).

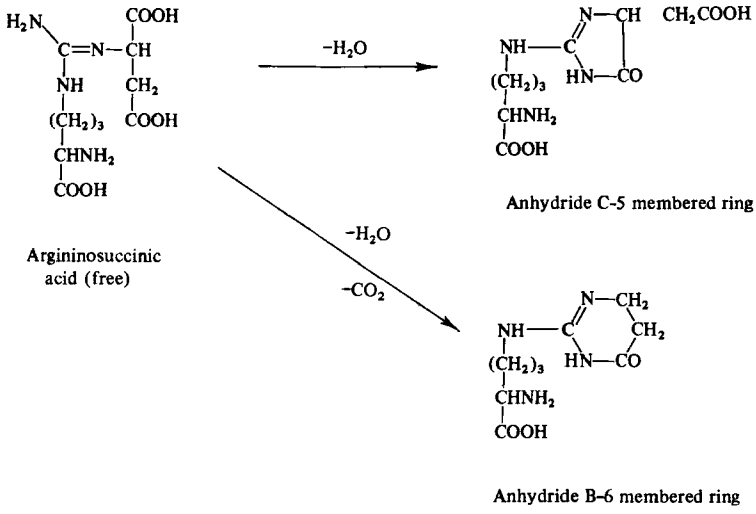

FIG. 14. Structural formulas of argininosuccinic acid and its two anhydrides.

TABLE 2

ARGININOSUCCINIC ACIDURIA: AMINO ACID LEVELS IN PLASMA, CEREBROSPINAL FLUID (CSF), AND URINE IN A 10-YEAR-OLD CHILD (NONFASTING) ON LOW PROTEIN DIET

	Plasma (mg/100 ml)		CSF[a] (mg/100 ml)		Urine[a] (mg/g N)	
Amino acid	Patient (L7, L10)	Normal 2M–11Y (P2)	Patient (L7, L10)	Normal 8M–11Y (P2)	Patient (L7, L10)	Normal 3M–12Y (P2)
Urea	17	26 ± 10	13	15 ± 4	630	1800
Taurine	0.44	0.83 ± 0.23	—	—	0.6	7.1 (0.56–22)
Hydroxyproline	0.18	0.21 ± 0.16	0	0	—	0–tr.
Aspartic acid	0	0–tr.	0	0	1.6	0–tr.
Threonine	1.3	1.35 ± 0.45	0.20	0.25 ± 0.08	1.0	1.4 (0.5–2.0)
Serine	1.5	1.50 ± 0.40	0.40	0.38 ± 0.12	6.7	2.8 (1.6–4.1)
Asparagine	—	0.31 ± 0.06	—	—	—	—
Glutamine	15.1	9.8 ± 1.3	21	8.3 ± 1.2	19	9 (5–16)
Glutamic acid	1.6	1.15 ± 0.45	0.21	0.02 (<0.04)	1.6	0.30 (0.07–0.50)
Proline	2.3	2.65 ± 1.00	0	0	0	0–tr.
Citrulline	2.6	0.32 ± 0.17	0.83	0.015 (<0.035)	16	0.23 (0–0.48)
Glycine	2.8	1.40 ± 0.40	0.037	0.045 ± 0.012	25	6.8 (1.5–11.2)

Alanine	3.6	2.55 ± 0.60	0.18	0.21 ± 0.06	4.5	2.6 (1.4–5.0)
α-Amino-*n*-butyric acid	0.15	0.28 ± 0.11	0.027	0.025 ± 0.005	—	0.18 (0.03–0.31)
Valine	1.6	3.00 ± 1.05	0.12	0.17 ± 0.045	4.5	0.53 (0.34–0.80)
Cystine	—	<1.0	—	—	1.2	0.52 (0.24–0.95)
Methionine	0.17	0.28 ± 0.11	0.14	0.040 ± 0.017	0.29	0.31 (0.08–0.65)
Isoleucine	0.48	1.05 ± 0.45	0.031	0.065 ± 0.030	—	—
Leucine	0.87	1.90 ± 0.65	0.12	0.17 ± 0.05	—	0.96 (0.39–2.4)
Tyrosine	0.58	1.35 ± 0.60	0.22	0.18 ± 0.07	3.7	1.9 (1.0–2.8)
Phenylalanine	0.61	1.10 ± 0.35	0.17	0.23 ± 0.12	1.6	1.2 (0.44–2.0)
Ornithine	0.37	0.79 ± 0.32	0.024	0.07 ± 0.05	0.50	0.15 (0.05–0.28)
Lysine	1.3	2.40 ± 0.80	0.19	0.25 ± 0.09	3.3	1.5 (0.75–2.5)
1-Methylhistidine	0.05	<0.19	0	0	0	6.7 (1.3–14)
Histidine	1.3	1.20 ± 0.30	0.46	0.18 ± 0.06	28	10.4 (5.7–17.5)
Arginine	0.43	1.20 ± 0.40	0.087	0.34 ± 0.10	0.35	0.25 (0.09–0.72)
Argininosuccinic acid, free	0.55	0	—	0	75	0
Argininosuccinic acid anhydride (B + C)	2.7	0	6.4	0	1500	0
Homocarnosine	0	0	0.19	0.14 ± 0.07	0	0
Carnosine	0	0	0	0	6.1	1.7 (0–3.3)

[a] Performed when the child was 6 years old. The normal levels are given as mean ± SD, and ranges are given in parentheses.

TABLE 3

ARGININOSUCCINIC ACIDURIA: AMINO ACID LEVELS IN PLASMA, CEREBROSPINAL FLUID (CSF), AND URINE IN A NEONATE WITH THE FULMINATING CONDITION

Amino acid	Plasma (mg/100 ml)		CSF (mg/100 ml)		Urine (mg/g N)	
	Patient (L5, L10)	Normal neonates (P2)	Patient (L5, L10)	Normal neonates (P2)	Patient (L5, L10)	Normal neonates (P2)
Urea	14	34 ± 21	5	40 ± 20	155	1800
Taurine	5.2	1.30 ± 0.80	—	—	61	13 (0–26)
Hydroxyproline	0.58	1.38 ± 0.22	0	0	16	11 (2.9–19)
Aspartic acid	0.99	0–tr.	0	0	0	<0.5
Threonine	2.0	1.75 ± 0.40	1.2	0.46 ± 0.20	4.5	2.8 (1.9–3.7)
Serine	2.6	2.10 ± 0.95	1.9	0.58 ± 0.18	16	6.3 (3.1–11)
Asparagine	1.5	0.42 ± 0.14	0.21	—	5.3	<1.7
Glutamine	48	10.5 ± 2.1	75	10.9 ± 2.1	61	14.5 (10.8–19)
Glutamic acid	5.8	1.95 ± 0.85	0.18	0.05 (<0.14)	1.8	0.80 (0.5–1.2)
Proline	7.7	4.15 ± 1.65	0	0	23	6.9 (1.8–15)
Citrulline	4.5	0.27 ± 0.22	3.0	0.04 (<0.06)	18	0.41 (0–0.79)
Glycine	6.0	1.80 ± 0.70	0.085	0.048 ± 0.014	64	24 (10–41)
Alanine	15.0	3.15 ± 0.70	1.1	0.35 ± 0.16	15	6.4 (1.8–17)
α-Amino-n-butyric acid	1.1	0.25 ± 0.12	0.50	0.05 ± 0.02	0.83	0.31 (0.045–0.65)

Valine	3.3	3.10 ± 0.95	0.70	0.34 ± 0.13	2.1	0.84 (0.50–1.6)
Cystine	1.5	<1.9	0.06	—	7.0	2.3 (0.55–4.4)
Methionine	1.1	0.39 ± 0.15	0.50	0.06 ± 0.025	2.3	0.90 (0.2–2.5)
Isoleucine	1.3	1.05 ± 0.35	0.17	0.12 ± 0.05	—	<0.26
Leucine	3.0	2.00 ± 0.55	0.53	0.24 ± 0.09	—	<3.7
Tyrosine	4.3	1.75 ± 0.55	3.5	0.36 ± 0.15	—	2.7 (2.0–3.5)
Phenylalanine	2.7	1.15 ± 0.30	1.0	0.31 ± 0.11	—	2.2 (1.4–4.3)
Ornithine	2.5	1.30 ± 0.55	0.10	0.09 ± 0.04	0.9	0.72 (0.17–1.4)
Lysine	7.6	3.00 ± 0.65	0.68	0.34 ± 0.07	7.7	6.4 (1.2–14)
1-Methylhistidine	0.1	<0.10	0	0	1.6	<0.52
Histidine	3.0	1.20 ± 0.30	1.5	0.26 ± 0.08	24	14.5 (6.6–23)
Arginine	0.11	0.79 ± 0.31	0.43	0.32 ± 0.09	1.0	0.44 (0.05–1.4)
Argininosuccinic acid, free	3.3	0	1.8	0	230	0
Argininosuccinic acid anhydride (B + C)	18.3	0	9.8	0	1550	0
Homocarnosine	0	0	0.40	0.15 ± 0.10	0	0
Carnosine	0	0.02 (0.14)	0	0	2.9	4.0 (0.80–12)
Ammonia N (μg/100 ml)	850	34 (18–50)	114	<20[a]	—	—

[a] Normal range for children and adults. The normal levels are given as mean ± SD, and ranges are given in parentheses.

hydrides, and Levin *et al.* reported (L5, L11) an even higher level of 21.6 mg/100 ml (Table 3). In all three cases the blood urea was within normal limits.

The marked difference in levels of argininosuccinic acid between the condition as seen in the older child and that found in the neonate tends to support the conclusion (C3) that the fulminating neonatal form of argininosuccinic aciduria represents a separate entity distinct from that found in the older child. Whether this is so or not, the reason for the strikingly high levels of argininosuccinic acid in the three reported cases of argininosuccinic aciduria resulting in death is not certain. One explanation may be that there was a diminished renal clearance of argininosuccinic acid. From the levels of argininosuccinic acid in plasma and urine in the earlier cases of argininosuccinic aciduria, the renal clearance has been calculated to be of the order of 100 ml per minute per 1.73 m^2. An impaired renal glomerular function would result in retention of argininosuccinic acid in a manner similar to urea. This may well partly explain the results obtained, since the plasma urea was perhaps higher than would be expected considering the defect in the synthesis of urea that is present, as well as higher in comparison with the levels of urea in older children with the same defect. However, no other evidence of glomerulotubular dysfunction of the kidney has been sought or found, and the suggestion, although interesting, remains unproved. An alternative explanation for the difference between these cases and those that survive to adolescence may be that the enzymatic defect is more severe in these neonates. There is no support for this hypothesis from the results of the levels of argininosuccinate lyase activity in two cases, one a boy still alive at 11 years of age (L7) and the other a boy who died in the first week of life (L5). In both cases there was no detectable argininosuccinate lyase activity in the liver. If the rapidly fatal course in these infants is due to a very high level of ammonia, this may be the result of a deficient uptake of ammonia by α-ketoglutarate and glutamate, or of poor renal excretion of ammonia compared with those children who survive. Finally, it is possible that an alternative method of converting ammonia to urea develops in those who survive the neonatal period, whereas it fails to develop in those who succumb.

Argininosuccinic acid also accumulates in the cerebrospinal fluid of children with argininosuccinic aciduria (Tables 2 and 3). In the surviving children in whom it has been measured, the levels have ranged from 8.2 to 10.0 mg/100 ml, levels much higher than those in the blood. In one of two neonates who died quickly, the level in the cerebrospinal fluid actually greatly exceeded even the high blood level of argininosuccinic acid (C2), whereas in the other it was lower (L5) (Table 3). The pres-

ence of argininosuccinic acid in cerebrospinal fluid could not have resulted from a simple diffusion from the blood since the level is usually higher in the cerebrospinal fluid than in blood. It can only be concluded that the brain is the source of the argininosuccinic acid and that the metabolic defect is present in the brain tissue, which therefore must normally be synthesizing urea. Such a capability has been shown to exist (S15). Argininosuccinate lyase activity has been detected in human brain tissue (K9, T6). To account for the normal levels of urea in the blood it has been suggested that the defect was present only in the brain and not in the liver (A1, D2, W3). This was disproved by the demonstration of the defective argininosuccinate lyase in the liver in argininosuccinic aciduria (L1).

The amount of argininosuccinic acid excreted in the urine in different patients varies greatly, from 1.5 to 9.3 g in 24 hours. The argininosuccinic acid nitrogen forms an appreciable proportion of the total urinary nitrogen content, in one case, amounting to 16%.

Argininosuccinic acid excretion also varied with protein intake. When Levin *et al.*'s patient was put on a protein free diet, the daily output of argininosuccinic acid fell to about 20% of the amount excreted when he was on a normal diet, i.e., from 3 g to 0.6 g a day. There was a somewhat larger drop in urea excretion, to about 6% (L7). Increasing the protein intake led to an increased excretion of argininosuccinic acid (L7). A similar relationship to protein intake was found also by Moser *et al.* (M13) in their patient.

10.1.2. *Levels of Ammonia in Blood and Cerebrospinal Fluid*

The effect on blood levels of ammonia of a defect in the biosynthesis of urea was not realized until Russell *et al.* (R14) reported the first instance of hyperammonemia, a deficiency of liver ornithine transcarbamylase. The earlier reports of argininosuccinic aciduria do not include levels of blood ammonia. Efron (E1) was the first to suggest that high levels of blood ammonia might be the underlying cause of the neurological symptoms and the brain damage in all those defects of the biosynthesis of urea which had then been characterized. Levin (L1) reported in three cases of argininosuccinic aciduria that although fasting blood levels of ammonia were within normal limits, they rose to levels of 200–300 μg/100 ml 2–4 hours after a meal containing 0.5 g to 1 g of protein per kilogram of body weight. These were much beyond the levels attained in normal children under the same conditions. The level of the ammonia in the cerebrospinal fluid in the fasting state in one child was 44 μg/100 ml, considerably in excess of the upper limit of normal of 20 μg/100 ml by the method used (L1). Similar results were obtained

by Moser *et al.* (M13), who also showed in one patient that while the fasting level of ammonia was within the normal range in the cerebrospinal fluid, it rose to a high level of 244 μg/100 ml 5 hours after a meal containing 1 g of protein per kilogram of body weight.

In one infant with the fulminating variant of the disease the level of ammonia in the cerebrospinal fluid was 114 μg/100 ml when the blood level was over 800 μg/100 ml (L5, L9) (Table 3). The only other reference to ammonia levels in cerebrospinal fluid or blood was by Carton *et al.* (C3), who observed in a neonate with argininosuccinic aciduria that the ammonia levels in both blood and cerebrospinal fluid as judged by column chromatography were high or very high.

Protein Intake and Urea Excretion. Levin *et al.* (L7) were the first to show that urea excretion and therefore presumably urea synthesis was increased with increased protein intake in argininosuccinic aciduria. They showed in their patient that an increase of 2.5 times in the protein intake resulted in a 4- or 5-fold increase in urea output. From the results of a feeding trial, in which the infant was given a casein hydrolyzate from which most of the arginine had been removed, they concluded from the small amount of arginine present and the relatively high amount of urea excreted daily, that most of the urea was derived from a urea cycle, presumably in the liver. Conversely, reduction of protein intake resulted in a marked decrease in the output of both urea and argininosuccinic acid.

10.1.3. *Levels of Glutamine and Glutamic Acid in Blood and Cerebrospinal Fluid*

The possibility of an increase in the level of glutamine in blood and cerebrospinal fluid was, like that of ammonia, overlooked in the first reported cases of argininosuccinic aciduria. This was partly due to the failure to note the rise in blood ammonia which would suggest a correlated rise in glutamine, and partly due to the ease with which glutamine decomposes to glutamic acid or undergoes cyclization, either on standing or even during analysis by column chromatography and which therefore makes an accurate assay difficult. Few reports therefore include the levels of glutamine and glutamic acid, and these are unreliable since no precautions were taken against the breakdown of glutamine during preparation of the serum sample for column chromatography. In one of their cases, Moser *et al.* (M13) reported fasting blood glutamine and glutamic acid levels which were above the upper limit of their normal range, and these both rose 1–2 hours after administration of protein to a higher level. In their second patient, however, no such abnormality was observed. The cerebrospinal fluid in their first patient also contained higher levels of these two amino acids than the normal. The patient of Baumgartner

et al. (B2) had a normal plasma glutamine, but the glutamic acid was grossly increased, almost certainly arising from decomposition of an originally high glutamine level. In the earlier case of Levin *et al.* (L7) the plasma glutamine was not estimated, but, in the cerebrospinal fluid, glutamine was found by a paper chromatographic method to be 8 mg/100 ml, a level which was taken to be within normal limits. A later analysis by column chromatography of a specimen of cerebrospinal fluid taken when the patient was 3 months old, but stored for several years at −15°C showed a raised level of glutamine. When the patient was 10 years old the plasma level of glutamine estimated when the patient was on a low protein diet was the high one of 15.1 mg/100 ml, although the glutamic acid level at 1.6 mg/100 ml was within the normal range (L7, L11) (Table 2). In Levin *et al.*'s second case, an infant who died in the first week of life, the plasma glutamine by column chromatography was very high, 48 mg/100 ml, with glutamic acid 5.8 mg/100 ml (L5, L11) (Table 3).

In summary, the reported results, while not entirely consistent, suggest that in argininosuccinic aciduria, the plasma and cerebrospinal fluid tend to have levels of glutamine and glutamic acid which are higher than normal. The inconsistencies may in part be due to the difficulty of accurate estimation and in part possibly to a variation of level with protein intake, since blood samples were not always correlated with times of meals or level of protein intake. Since the plasma ammonia levels in this condition are not as high as those attained in the other diseases of the urea cycle, e.g., hyperammonemia, the levels of glutamine would also not be expected to be as high.

10.1.4. *Levels of Other Amino Acids in Plasma and Cerebrospinal Fluid*

Particular interest attaches to these amino acids which are intermediary metabolites of the urea cycle, and which are normally present in the plasma, ornithine, citrulline, and arginine. Earlier reports do not mention the levels of these amino acids. Moser *et al.* (M13) found a normal plasma ornithine level in their two patients, but the citrulline was, as would be expected, several times the normal level, while the plasma arginine was at the lower limit of normal. In the cerebrospinal fluid also the citrulline level was greatly increased, while the ornithine and arginine levels were normal. Similar results were obtained also by Carton *et al.* (C3) in their neonate who died at 6 days old, and by Levin *et al.* (L5, L11), also in a neonate who died in the first week of life. In the last two cases, however, the levels of citrulline were considerably higher than in the older patient of Moser *et al.* (M13) at 2.2 mg and 3.0 mg/100 ml, compared with a level of only 0.77 mg/100 ml for Moser's

case (M13). These higher levels may be correlated with the higher levels of argininosuccinic acid found in the cerebrospinal fluid of these neonates again suggesting a more severe form of the disease in these infants.

10.1.5. *Intermediary Metabolites of Pyrimidine Synthesis and Metabolism*

The effect on pyrimidine synthesis and catabolism of a decreased uptake of ammonia resulting from a deficiency of the biosynthesis of urea has only recently been recognized (L8). The high level of blood ammonia accelerates pyrimidine synthesis, and this is shown by the increased urinary excretion of some intermediates of this pathway. Although this was first shown in hyperammonemia, in which very high blood levels of ammonia are found (L8), it was later shown to occur in argininosuccinic aciduria as well (L5, L9). The metabolites so far detected in the urine have been orotic acid, uridine, and uracil, occurring in amounts which vary with the level of blood ammonia. Orotic acid is greatly reduced in amount or disappears altogether when blood ammonia levels are reduced to normal, or even a little above normal. The presence of orotic acid in the urine forms a particularly useful confirmation of argininosuccinic aciduria as well as of the other enzymatic disorders of urea biosynthesis, since it is either absent or in extremely small amount in the normal urine. Its presence in high amount on screening the urine betokens a very high blood ammonia. For example, in the older children who are treated, these metabolites are only present in small amount in the urine, whereas in one neonate who died relatively large amounts of orotic acid were found (L9).

10.1.6. *General Biochemical Results and Liver Function Tests*

In general, most of the routine biochemical tests in these cases are within normal limits. However, some of the older children had slightly or moderately impaired liver function, as shown by the raised alkaline phosphatase and transaminases. Even in the neonates who were more severely affected, the results were similar. In one infant, hypocalcemia was also present (B2).

10.1.7. *Enzyme Levels in Liver, Kidney, and Red Blood Cells*

10.1.7.1. *Liver and Red Cell.* The identification by Westall (W3, W4) of the amino acid excreted in large amounts as an intermediate in the urea cycle led him to postulate a deficiency of argininosuccinate lyase in this condition. He confirmed this in the red cells of two affected children. In all other cases of argininosuccinic aciduria in which this assay has been performed, no enzyme activity has been detected in the red cells

(Table 4). However, since urea is mainly synthesized in the liver, an enzyme deficiency must be proved in this organ before such an assumption is to be conclusively confirmed. Assay of urea cycle enzymes on a liver biopsy from an affected child (L1) showed no detectable argininosuccinate lyase although the other urea cycle enzyme activities were present in normal levels (Table 5). An identical result has been obtained on a necropsy specimen of liver from a neonate with the fulminating variant of the disease (L5). Miller and McLean (M7) also found very low activities of the argininosuccinate lyase, less than 5% of the normal, together with normal values for the other enzymes of the cycle in a necropsy specimen of liver from a 14-year-old boy with argininosuccinic

TABLE 4

ARGININOSUCCINATE LYASE ACTIVITY IN RED BLOOD CELLS OF PATIENTS WITH ARGININOSUCCINIC ACIDURIA AND THEIR FAMILIES

No.	Reference	Case	Sex	Age	Units	Normal range
1	(L4, 7)	Patient	Male	2 yr	0	3.72–9.3
2		Mother			2.7	
3		Father			0.77	
4		Grandfather (maternal)			7.7	
5		Grandmother (maternal)			3.1	
1	(L4, 5)	Patient	Male	5 days	0	
2		Mother			2.9	
3		Father			4.7	
1	(T7)	Patient	Male		0	1.9–6.4, mean = 3.4 ± 1.2
2		Patient	Female		0	
3		Father			1.6	
4		Mother			1.8	
5		Sib			1.1	
6		Sib			1.6	
1	(M13)	Patient	Male		0	2.6–6.9, mean = 3.8 ± 1.1
2		Patient	Male		0	
3		Father			2.1	
4		Mother			2.1	
5		Sib			2.6	
1	(G3)	Patient	Female		0	
2		Father			1.4	
3		Mother			1.3	
4		Sib			1.6	

1 unit = 1 μmole of urea formed per gram of hemoglobin per hour.

TABLE 5
ARGININOSUCCINIC ACIDURIA: UREA CYCLE ENZYME LEVELS IN LIVER[a]

Case	Source	OTC pH 7.0	OTC pH 8.3	CPS	ASS	ASL	A	References
1	Biopsy[b]	—	3900	222	122	Nil	24,600	(L1)
2	Necropsy	3295	—	195	87	13	20,600	(M5)
3	Necropsy	—	3250	182	?	Nil	64,200	(L4)

[a] Levels are given in micromoles of product formed per hour per gram wet weight of tissue. OTC, ornithine transcarbamylase; CPS, carbamyl phosphate synthetase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; A, arginase.

[b] Estimation performed on liver kept at −15°C for 3 years.

aciduria (Table 5). These authors also attempted a partial purification of the enzyme from the liver of their patient and obtained a preparation with only 3% of the enzyme activity per milligram of protein of a preparation similarly obtained from a normal liver.

10.1.7.2. *Kidney.* In two cases of the neonatal fulminating types of the disease, urea cycle enzyme activities have been measured in kidney tissue obtained at necropsy. In one, Colombo and Baumgartner (C8) found activities of argininosuccinate lyase in the kidney which were as high as those found in control necropsy specimens from children not suffering from the condition. The levels were 2 or 3 times lower than those of the liver, however, and the K_m value of the kidney enzyme was different from that of the liver enzyme, the latter having an affinity for argininosuccinic acid which was 4 or 5 times greater than the kidney argininosuccinate lyase. Colombo and Baumgartner suggested that the two may be isoenzymes, and they speculated furthermore that the kidney enzyme was sufficiently active to explain largely the findings of normal concentrations of urea in plasma and urine. On the other hand, in another case Carton *et al.* (C3) could detect no argininosuccinate lyase activity in the kidney, although the arginase activity in the same specimen was normal. They detected also no lyase activity in the red cells from their patient, and less than 3% of the normal activity in the gray matter of the brain. They do not appear to have measured the lyase activity in the liver, but on the assumption that here again no activity was present, it seems that, in contrast with Colombo and Baumgartner's (C8) patient, the defect of argininosuccinate lyase activity was a generalized one. The explanation offered by the latter authors for the normal urea formation could not therefore hold for the case of Carton *et al.* (C3).

10.1.8. *Inheritance of Argininosuccinic Aciduria and Detection of the Heterozygote State*

The approximately equal number of males and females affected suggests that argininosuccinic aciduria is an autosomal disease. That it is recessive in character is shown by the fact that no parent of such a child has been affected. No proof of the heterozygote state has as yet been afforded by determinations of argininosuccinate lyase activity in the liver from parents of children with argininosuccinic aciduria. However, there have been a number of such assays on red blood cells from parents and siblings of affected children. In all cases except one, the parents' red cells have levels of enzyme activity lower than the lower limit of the normal, the exception possessing a level of activity just within the range of normals (Table 4). In three families, the sibs had levels similar to those found in the parents' red cells.

A further indication of the heterozygote state is indicated by the excretion of argininosuccinic acid by parents of affected children. Fifty members of three generations of the family of the patient of Coryell *et al.* (C14) were examined, and in ten of them, including the affected child's mother, argininosuccinic acid was found in the urine in amounts ranging from 4 mg to 29 mg per gram of creatinine, none being detected in normal subjects. Baumgartner *et al.* (B2) found an excretion of 6 and 14.3 mg of acid per day in the parents of their patient, and similar amounts were found in the urine from the parents of an affected child by Palmer *et al.* (P2). On the other hand, no argininosuccinic acid was detected by Moser *et al.* (M13) in the parents of the two affected sibs. These results taken together suggest that argininosuccinic aciduria is an inherited disorder, the heterozygote state being indicated by a low lyase activity in the red blood cells and an excretion in the urine of small amounts of the acid. However the latter distinction is not so clear cut as has been thought. Palmer *et al.* (P2) have demonstrated that, contrary to what has been stated (C14, E1, W2), normal adults excrete up to 1.8 mg of argininosuccinic acid per day in the urine, although only a small series of normal adults has been evaluated. These results suggest that the heterozygote state is present only when argininosuccinic acid excretion exceeds 2 mg per day.

10.2. Hyperammonemia

10.2.1. *Levels of Ammonia in Blood and Cerebrospinal Fluid*

In this condition, unlike the other types, the expected accumulation of ornithine does not occur, presumably because this amino acid participates

in other metabolic pathways, especially in its conversion first to glutamate semialdehyde, then to glutamate and α-ketoglutarate (K4), and also possibly because the block is situated at the point of entry of ammonia into the urea cycle so that ornithine is not re-formed. The level of ornithine is not therefore a satisfactory indication of deficient ornithine transcarbamylase, although it tends to remain at the upper limit of normal. Since carbamyl phosphate probably exists only as an unstable and short-lived intermediate, the main effect of the block then is a marked elevation of blood ammonia.

Since different methods of measuring ammonia levels have been employed with resulting variation in the range of normal values, a strict comparison between the results in the different reports is not possible, especially when some have been given as ammonia and others as ammonia nitrogen. However the blood ammonia levels have usually been high when first estimated and when the patients were on normal protein intake. They have ranged up to 1200 μg/100 ml, or more, with the cerebrospinal fluid levels correspondingly increased up to 540 μg/100 ml. In those children who died, the blood ammonia rose considerably just before death, even when the levels had previously been successfully controlled.

10.2.2. *Relation of Ammonia Levels to Protein Intake*

Most of the data regarding the relationship of ammonia levels to protein intake in inborn errors of urea synthesis have been obtained from the results in hyperammonemia, because of the relatively higher blood levels in this condition. Levin *et al.* (L3, L6, L8, L12) have shown that the ingestion of a single meal containing protein to affected children causes the blood ammonia to rise to a maximum at 3 or 4 hours after ingestion, when the level is several times the fasting level, whereas in normal children, the blood ammonia does not rise beyond the normal range even at its maximum. The blood ammonia levels measured at intervals during the day when an affected child is stabilized on varying levels of protein intake show a consistent fall the lower the intake (L3, L6). Complete exclusion of protein results in a fall from very high levels to normal ones (L8). It was on the basis of such results as these and similar results in patients with argininosuccinic aciduria (L1, M13) that the dietary treatment with restricted protein intake, spread over frequent small meals throughout the day, was recommended (E1, L1, L12). The cerebrospinal fluid ammonia level also falls on a low protein diet (L8).

10.2.3. *Urinary Excretion of Ammonia*

The first observation that there was a high urinary ammonia excretion in hyperammonemia was made by Russell *et al.* (R14). They observed in their first case that hydrogen ion excretion was mainly in the form of the ammonium salt which constituted over 95% of the total hydrogen ion excretion. A search for a cause of this unusual result led to the finding of a high blood ammonia. Presumably the high urinary ammonia excretion is directly related to the high blood glutamine level, which is itself related to the raised blood ammonia (see below). This would imply that whenever the blood ammonia, and therefore the blood glutamine, are raised, ammonia excretion would increase. This has been well shown by Levin *et al.* (L3), who found in one infant with hyperammonemia that the rate of excretion of ammonia went up to nearly four times the fasting rate after a protein meal, the maximum excretion rate being achieved by the fourth hour after the meal.

10.2.4. *Levels of Glutamine and Glutamic Acid in Blood, Cerebrospinal Fluid, and Urine*

Despite the importance of an increase in the level of glutamine in the blood in the diagnosis of hyperammonemia, few reports of glutamine levels in this condition have appeared. This is probably in part due to the practical difficulty of the estimation of this amino acid by the usual quantitative ion exchange chromatography. Nearly all the data have been provided by Levin *et al.* (L3, L6, L8). In all their cases of hyperammonemia the plasma glutamine has been raised, from a mean level of 9.8 mg/100 ml in the normal nonfasting child to 25 mg/100 ml or over in the affected child (Table 6). In those cases in which care has been taken to avoid breakdown of glutamine to glutamic acid, the latter has also been found to be raised although only slightly, from a mean normal of 1.15 mg/100 ml in the nonfasting to about 1.7 mg/100 ml. This rise is not always observed, as the glutamic acid level is sometimes within normal limits, despite a coincident very high glutamine level. The very high level of glutamic acid in the plasma reported in one case of hyperammonemia coupled with a relatively low level of glutamine was almost certainly due to decomposition of glutamine before analysis.

The rise in glutamine level in hyperammonemia is highly significant. The formation of glutamic acid from α-ketoglutarate, and ammonia and its further conversion to glutamine by its reaction with ammonia, serves to regulate the level of ammonia in the blood, albeit to only a limited extent. Thus the initial effect of a defect of urea synthesis in the body

TABLE 6

HYPERAMMONEMIA: AMINO ACID LEVELS IN PLASMA, CEREBROSPINAL FLUID (CSF), AND URINE[a]

Amino acid	Plasma (mg/100 ml)		CSF (mg/100 ml)		Urine (mg/g N)	
	Patient (L10)	Normal 2M–11Y (P2)	Patient (L10)	Normal 8M–11Y (P2)	Patient (L10)	Normal 3M–12Y (P2)
Urea	10	26 ± 10	9.5	15 ± 4	1750	1800
Taurine	1.7	0.83 ± 0.23	—	—	12	7.1 (0.56–22)
Hydroxyproline	0.16	0.21 ± 0.16	0	0	0	0–tr.
Aspartic acid	0.12	0–tr.	0	0	0	0–tr.
Threonine	0.77	1.35 ± 0.45	0.22	0.25 ± 0.08	8.0	1.4 (0.5–2.0)
Serine	1.95	1.50 ± 0.40	0.52	0.38 ± 0.12	9.4	2.8 (1.6–4.1)
Asparagine	0.25	0.31 ± 0.06	tr.	—	0.54	—
Glutamine	21.3	9.8 ± 1.3	13.5	8.3 ± 1.2	22	9 (5 – 16)
Glutamic acid	1.45	1.15 ± 0.45	0.003	0.02 (<0.04)	2.2	0.30 (0.07–0.50
Proline	3.5	2.65 ± 1.00	0	0	0	0–tr.
Citrulline	0.17	0.32 ± 0.17	0	0.015 (<0.035)	0	0.23 (0–0.48)
Glycine	3.9	1.40 ± 0.40	0.042	0.045 ± 0.012	19.5	6.8 (1.5–11.2)
Alanine	10.3	2.55 ± 0.60	0.52	0.21 ± 0.06	5.7	2.6 (1.4–5.0)
α-Amino-*n*-butyric acid	0.15	0.28 ± 0.11	0.003	0.025 ± 0.005	0.14	0.18 (0.03–0.31)

Valine	1.4	3.00 ± 1.05	0.14	0.17 ± 0.045	0.72	0.53 (0.34–0.80)
Cystine	—	<1.0	—	—	0.90	0.52 (0.24–0.95)
Methionine	0.28	0.28 ± 0.11	0.08	0.040 ± 0.017	0.90	0.31 (0.08–0.65)
Isoleucine	0.43	1.05 ± 0.45	0.06	0.065 ± 0.030	0.70	—
Leucine	0.54	1.90 ± 0.65	0.075	0.17 ± 0.05	1.4	0.96 (0.39–2.4)
Tyrosine	0.53	1.35 ± 0.60	0.16	0.18 ± 0.07	3.2	1.9 (1.0–2.8)
Phenylalanine	0.60	1.10 ± 0.35	0.15	0.23 ± 0.12	1.5	1.2 (0.44–2.0)
Ornithine	0.64	0.79 ± 0.32	0.075	0.07 ± 0.05	0.07	0.15 (0.05–0.28)
Lysine	0.61	2.40 ± 0.80	0.18	0.25 ± 0.09	1.9	1.5 (0.75–2.5)
1-Methylhistidine	0.02	<0.19	0	0	1.8	6.7 (1.3–14)
Histidine	1.2	1.20 ± 0.30	0.29	0.18 ± 0.06	36	10.4 (5.7–17.5)
Arginine	0.63	1.20 ± 0.40	0.42	0.34 ± 0.10	0.26	0.25 (0.09–0.72)
Homocarnosine	0	0	0.22	0.14 ± 0.07	0	0
Carnosine	0.005	0	0	0	4.9	1.7 (0–3.3)
Alloisoleucine	0.02	0.025 (<0.050)	0	0	0	0
Homocitrulline	0	0–tr.	0	0	0.90	0.70 (0.22–15)
β-Amino-*i*-butyric acid	0	0–tr.	0	0	3.0	2.2 (0–9.0)
Ethanolamine	0.015	0–tr.	0.005	0.03 (<0.18)	2.5	1.7 (0.72–3.1)

[a] The normal levels are given as mean ± SD, and ranges are given in parentheses.

will be a high glutamine rather than a high blood ammonia level and only later will the blood ammonia rise. Such a defect could be reflected therefore in both high glutamine and ammonia blood levels, or in high glutamine alone, but should not appear as a high ammonia level alone. It is remarkable that glutamic acid levels are only slightly, if at all, increased. This would be expected if the equilibrium of the reaction of ammonia with glutamic acid lies toward the formation of glutamine. That this is so is apparent from the normal ratio of glutamine to glutamic acid concentration in the blood of about 10 to 1. In the cerebrospinal fluid too, the glutamine levels are raised in hyperammonemia, even higher than in the blood. In one child, the cerebrospinal fluid glutamine was as great as 92 mg/100 ml (L11). Again the glutamic acid levels are either normal or a little higher than normal.

The effect of protein intake on the levels of glutamine in the blood and cerebrospinal fluid is very interesting and confirms the close association between it and ammonia level. In one child, the blood glutamine fell from 25.5 mg/100 ml when she was on a normal diet to 17.0 mg/100 ml when on a low protein intake and 13.8 mg/100 ml when protein was excluded altogether (L3). These were coincident with a fall in the blood ammonia to relatively slightly increased levels (L3). The glutamine level in the cerebrospinal fluid fell from 45.5 mg to 9.0 mg/100 ml. In two other cases, one adult and one child, the plasma glutamine rose 2–3 hours after a protein meal, again coincident with a rise in blood ammonia (L3, L6).

As would be expected, the urinary excretion of glutamine is greatly increased, frequently in greater amount than any other amino acid in the urine (Table 6). When dietary protein is augmented, glutamine excretion rises (L6).

10.2.5. *Levels of Other Amino Acids in Blood and Cerebrospinal Fluid*

There are small but significant alterations in the plasma levels of two of the amino acids involved in the urea cycle. Both citrulline and arginine levels are low, about one-third to one-half the normal levels (L11) sometimes even lower (Table 6). These changes would be expected since these acids are in the metabolic pathway beyond the block. What appears surprising is that the level of ornithine, the amino acid whose further metabolism is blocked, which would be expected to rise, is actually usually within normal limits. There are two possible explanations for this. The biosynthesis of urea forms a cycle in which each intermediate is re-formed in the process. The block in the synthesis of citrulline from ornithine results in a decreased formation of arginine which in turn will

result in less ornithine. Alternatively, as Kekomäki (K3) pointed out, ornithine-ketoacid aminotransferase activity is high in human liver. Ornithine can be converted to Δ'-pyrroline-5-carboxylic acid and subsequently to glutamic acid by this enzyme. There is therefore a natural degradation pathway for any excess ornithine resulting from a metabolic block in the conversion of ornithine to citrulline. That this is a major pathway is supported by the report of Bickel *et al.* (B5a) of two siblings with hyperornithinemia due to a defect of ornithine-ketoacid aminotransferase.

The blood alanine level is also always increased, sometimes markedly to about 2–4 times the normal value (L3, L6). This is presumably because the normal transamination of alanine to pyruvate, which requires α-ketoglutarate, is inhibited both by the excess of glutamine in the blood and by the drain on α-ketoglutarate. One other amino acid, carnosine, has been found to be present in the plasma or in raised amounts in the urine, in those cases of hyperammonemia where it has been sought (L10). There are no consistent changes in any of the other amino acids, including lysine, in the blood.

The changes in levels of amino acids other than glutamine in the cerebrospinal fluid in hyperammonemia are variable. In two reported instances (L3, L6), the arginine level was low, 30–50% of the normal. This could be a reflection of the decreased plasma arginine level or possibly of the block in the urea cycle in the brain itself. On the other hand, in another instance (L10), the arginine level was normal. The changes in the other amino acids are also not consistent. In any case, the cerebrospinal fluid levels of amino acids are so low that such changes as were found are difficult to interpret with certainty (Table 6).

10.2.6. *Urea Excretion and Protein Intake*

In one of the first cases to be reported, Levin *et al.* (L8) showed that urea excretion increased with a rise in protein intake over a 3-day period. In another patient (L3), this was strikingly shown to occur immediately after a single protein load, the rate of urea excretion rising 3-fold by the 4th hour after ingestion. The authors took these results to indicate that even the diminished capacity to synthesize urea was not fully utilized on the usual protein intake.

10.2.7. *Intermediary Metabolites of the Pyrimidine Pathway*

The first evidence of the effect of elevation of blood ammonia on the synthesis and breakdown of pyrimidine was found in cases of hyperammonemia (L8), presumably because it is in this condition that the highest levels of blood ammonia are likely to be found. Orotic acid was first detected in the urine of these patients, because on one occasion so

great an amount was excreted as to form a copious crystalline deposit. Its discovery prompted a search for other metabolites of this pathway, and uridine and uracil were also detected (L8). Uridine has not so far as is known been previously detected in urine. The concentration of orotic acid excreted by affected children on a normal protein intake may be as high as 240 mg/100 ml, almost as much as in hereditary orotic aciduria, while the excretion of uridine and uracil may be as high as 140 mg and 40 mg/100 ml, respectively. Orotic excretion does not normally exceed 1.5 mg per day in an adult, or about 0.1 mg/100 ml. Corbeel *et al.* (C13) also found that orotic acid excretion in hyperammonemia varied with protein intake.

On a low protein diet, when the blood ammonia is considerably decreased, the urinary excretion of these compounds is also reduced. Even so some orotic acid and uracil is always found, although uridine may be completely absent from the urine (L6).

10.2.8. *General Biochemical Results and Liver Function Tests*

Most of the general biochemical results are within normal limits. The blood urea is also normal, except in subjects on a low protein diet, when it is decreased. The blood pH is frequently near the upper limit of normal, and sometimes even above it. Although some liver function tests are normal, the serum GOT, GPT, and the LDH are raised, sometimes markedly, especially when a normal protein diet is taken. The alkaline phosphatase is also usually raised.

10.2.9. *Ornithine Transcarbamylase Levels in Liver and Other Tissues*

Although ornithine transcarbamylase is fairly widely distributed in the tissues, only the enzyme levels in the liver in hyperammonemia have been adequately reported (Table 7), except that in one affected infant and the mother of two others, the small intestinal mucosa has also been examined (L8). In a series of 8 children, 7 females and 1 male, the liver ornithine transcarbamylase activity was 10% or less of the mean normal value, with the exception of the male, in whom the residual enzyme activity was as much as 25% of the mean normal. In 6 cases in whom the activity was also determined at pH 8.3, the reduction was much more variable, the activity varying from 11% to 40% of the mean normal; an exception is the case mentioned previously, in whom the activity was actually within the normal range at that pH although slightly lower than the mean normal. These results together with the patient reported by Hopkins *et al.* (H5) proved conclusively that hyperammonemia is due to a gross deficiency of ornithine transcarbamylase activity. One other case, reported by Corbeel *et al.* (C12, C13), showed a somewhat

TABLE 7
HYPERAMMONEMIA: UREA CYCLE ENZYME LEVELS IN LIVER[a]

Case	Source	OTC pH 7.0	OTC pH 8.3	CPS	ASS	ASL	A	References
1	Biopsy[b]	250	—	112	21	1000	9,000	(L6)
2	Necropsy[b]	370	—	199	44	178	16,480	(L6)
3	Biopsy	1288	4332	250	13.5	115	24,830	(L5)
4	Biopsy	432	1051	152	37	122	45,100	(L4)
5	Biopsy	415	2288	153	41	190	56,300	(L4)
6	Biopsy	249	—	159	18	87	15,350	(H6)
7	Biopsy	38.7	—	41.4	—	34.3	9,060	(C14)
8	Biopsy	528	2090	31	23	97	31,600	(L4)
9	Biopsy	308	950	108	—	—	—	(L4)
10	Biopsy	528	622	111	12	176	—	(L4)

[a] Levels are given as micromoles of product formed per hour per gram wet weight of tissue. OTC, ornithine transcarbamylase; CPS, carbamyl phosphate synthetase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; A, arginase.

[b] Estimation was performed on liver kept at −15°C for 3 years.

less convincing reduction in ornithine transcarbamylase activity, to only about 35% of the normal levels obtained by them. However, the comparison between the latter and the results of Levin *et al.* (L3, L4, L6, L8, R14) and Hopkins *et al.* (H5) is rendered difficult by the very large differences in the levels in the normal liver reported by them, those of the latter two groups agreeing closely in their normal levels, whereas those of Corbeel *et al.* (C13) were very much lower.

It is significant that the ornithine transcarbamylase activity in the intestinal mucosa of one patient was only 20% of the mean normal compared to a reduction to less than 5% of the mean normal in the liver of the same patient (L8) (Table 7). This manifestation of an enzyme deficiency in more than one organ in the body has a parallel in argininosuccinic aciduria, where lyase deficiency has been shown to occur both in the liver and in the red cells and, in one case, in red cells, brain, and kidney.

10.2.10. *Association of Carbamyl Phosphate Synthetase with Ornithine Transcarbamylase Deficiency*

In one of the first two reported cases of hyperammonemia, it was noted that in addition to the gross reduction in ornithine transcarbamylase activity the liver carbamyl phosphate synthetase activity was reduced to 30% of the mean normal level (L8, R14). Levin *et al.* (L3) also reported in two further cases a reduction, although not severe, of car-

bamyl phosphate synthetase activity in association with the marked reduction in ornithine transcarbamylase activity. A similar reduction of carbamyl phosphate synthetase activity associated with a very low ornithine transcarbamylase level was reported in another case (H4). An even more remarkable instance (S1), investigated in the author's laboratory, shows an even greater reduction, to only 10% of the mean normal level of carbamyl phosphate synthetase activity, associated with an equal reduction in ornithine transcarbamylase activity, with resulting hyperammonemia. Finally, Corbeel *et al.* (C12, C13) reported a child with hyperammonemia in whom the liver enzymes showed again a reduction of carbamyl phosphate synthetase activity albeit only to 50% of the lowest normal value, and a somewhat greater reduction, to about 35% of the lowest normal level, in ornithine transcarbamylase activity.

It may be concluded that in many cases of an ornithine transcarbamylase deficiency, there is an associated carbamyl phosphate deficiency, usually of moderate degree. It is because of this that it has even been suggested (E2) that hyperammonemia might not be due solely to ornithine transcarbamylase deficiency. This view cannot be correct in at least two cases where the carbamyl phosphate synthetase was within the normal, albeit low normal, range.

The significance of this association is still not clear. It may be that the observed reductions in carbamyl phosphate synthetase activity are due to delay in assaying the enzyme. However, this cannot explain those cases in which this estimation was carried out on fresh liver immediately after removal at operation. It is also noteworthy that in the only necropsy liver specimen examined in the author's laboratory, the carbamyl phosphate synthetase activity was within the normal range, so that at least in this case the delay in carrying out the estimation did not result in severe reduction.

It is possible that diminution in carbamyl phosphate synthetase activity may be an adaptive change secondary to the slowing down of the urea cycle because of the ornithine transcarbamylase deficiency. Such an explanation was adduced by Hers (H3) to account for the low glucose 6-phosphate activity in glycogen storage disease due to a defective debrancher enzyme. A further possibility is that these cases are examples of simultaneously occurring or multiple enzyme defects. Although this is improbable, it cannot be regarded as impossible. There are, for example, cases of two types of glycogen storage disease occurring in different individuals belonging to the same kinship. The simultaneous occurrence of two enzyme defects in one individual has been fully discussed by Steinitz (S17), who subjected recorded cases to critical review. He concluded, with Hers (H3), that such an association in the same

patient or the same family has not been proved. This, however, is a purely negative conclusion, against which the further evidence of the coexistence of ornithine transcarbamylase and carbamyl phosphate synthetase deficiencies in one and the same patient may be cited. It is possible that all the enzymes of the urea cycle may be linked in such a way that a reduction in one enzyme leads to a reduction in all. This is unlikely (see below), but there may be a special link between ornithine transcarbamylase and carbamyl phosphate synthetase. The patient with hyperammonemia where both enzymes were equally and severely reduced in activity could be an extreme example of such a link. This suggestion receives some support from the evidence that in *Neurospora crassa* the pyrimidine synthesis-directed carbamyl phosphate synthetase enzyme and aspartate transcarbamylase are associated as a single bifunctional protein (R1, R2, W5). By analogy, it is not unlikely that the urea-directed carbamyl phosphate synthetase and ornithine transcarbamylase in mammalian liver also form a single bifunctional protein.

10.2.11. *Changes in Levels of Other Urea Cycle Enzymes in the Liver*

In addition to ornithine transcarbamylase, the other enzymes of the urea cycle have also been estimated in the liver in hyperammonemia (H5, L4, L6, L8) (Table 7). It has proved difficult to assess the differences from the normal which are found in some of the enzyme activities. There is little doubt that the level of arginase is always normal. Of the remaining three enzymes of the urea cycle, the argininosuccinate lyase activity is also probably within normal limits, although both for this and for the synthetase too few assays have been performed for the normal range in the liver to be accurately determined. It is remarkable, however, that of 7 determinations of argininosuccinate synthetase in patients with hyperammonemia, two had levels one-third that of a control normal liver. This suggests that, in some patients at any rate, there is a coincident reduction of argininosuccinate synthetase activity. Hopkins *et al.* (H5) found a similar though less marked reduction in synthetase activity in their case of hyperammonemia compared with a normal control.

10.2.12. *Inheritance and Sex-Linkage*

Of a total of 11 known cases, 10 are females and 1 is a male, who had a differing type of ornithine transcarbamylase deficiency (L6). Such a preponderance of females suggests that the condition is sex-limited, finding expression only in the female. Of the ten female cases, two sibs have been affected in one family as well as, in all probability, the mother. In another family, both mother and her female infant were certainly affected. The latter in particular suggests that the defect is inherited as

a dominant character, since it is unlikely that mother and infant were both homozygotes, a situation which would require that the homozygote parent had married a heterozygote father. It is possible that the defect is limited to females, because its expression was lethal in males. Although in most families there is no living male sib, in one reported instance (L3) a brother of the affected adult female is alive and apparently well. Preliminary investigations suggest that this relative may also be affected, but unfortunately full investigations have not been possible.

10.2.13. *Determination of Possible Heterozygote State*

Several tests have been suggested to determine the existence of an heterozygote state, e.g., the response of blood ammonia and glutamine levels to a meal containing protein or to the oral ingestion of a standard dose of ammonium chloride. These tests have been attempted in too few cases of relatives of hyperammonemia for their usefulness to be adequately assessed. In some cases, the results have been interpreted to indicate that the full expression of the defect existed in one parent of an affected child (L8), since the mother was shown to have abnormal rises in blood following ingestion of ammonium chloride, whereas the father had not. This was merely presumptive since no enzyme assays of the liver were attempted in either parent. However, in another family where this was done, liver enzyme assays confirmed the abnormal findings in one parent, and the normal results in the father (L6).

10.2.14. *Enzyme Variant in Hyperammonemia*

Levin *et al.* has shown that whereas the ornithine transcarbamylase deficiency is severe and critical in most cases of hyperammonemia, in one child, a boy, the enzyme activity was reduced to a lesser extent (L6). Further investigations showed that other differences between the enzyme in this child and in others affected, existed. Thus the affinities of the enzyme for both carbamyl phosphate and ornithine were markedly different in this child from the others. On the basis of these findings they suggested that this constituted a distinct genetic variant, which could explain its occurrence in a male infant whereas all other reported cases of hyperammonemia were in females.

10.3. Citrullinemia

10.3.1. *Levels of Citrulline in Plasma, Cerebrospinal Fluid, and Urine*

In citrullinemia, plasma levels of citrulline, the amino acid whose further metabolism is blocked, are greatly increased, 30 mg/100 ml or more compared to a normal level of about 0.4 mg/100 ml (Table 8). McMurray *et al.* (M6) showed that their patient was able to absorb

citrulline normally when a loading dose was given by mouth. The plasma citrulline rose nearly 50% at the maximum, and about 40% of the load was excreted during the period of 24 hours after the ingestion. The administration of other amino acids, ornithine, arginine, glutamic acid, and proline failed to alter plasma citrulline levels or citrulline excretion (M6).

In two cases, the level of citrulline in the cerebrospinal fluid was between 2.5 and 6.0 mg/100 ml, much lower than that in the blood. This is the reverse of that found in argininosuccinic aciduria. The lower level of

TABLE 8
CITRULLINEMIA: AMINO ACID LEVELS IN PLASMA AND URINE[a]

	Plasma (mg/100 ml)		Urine (mg/g N)	
Amino acid	Patient (V1)	Normal 2M–11Y (P2)	Patient (V1)	Normal 3M–12Y (P2)
Taurine	3.2	0.83 ± 0.23	12.0	7.1 (0.56–22)
Hydroxyproline	0.70	0.21 ± 0.16	0.7	0–tr.
Threonine	1.1	1.35 ± 0.45	7.4	1.4 (0.5–2.0)
Serine	1.25	1.50 ± 0.40	7.5	2.8 (1.6–4.1)
Glutamine	41.0	9.8 ± 1.3	63.0	9 (5–16)
Glutamic acid	5.8	1.15 ± 0.45	10.8	0.30 (0.07–0.50)
Proline	9.0	2.65 ± 1.00	6.1	0–tr.
Citrulline	38.0	0.32 ± 0.17	415.0	0.23 (0–0.48)
Glycine	3.1	1.40 ± 0.40	17.0	6.8 (1.5–11.2)
Alanine	42.0	2.55 ± 0.60	38.0	2.6 (1.4–5.0)
α-Amino-*n*-butyric acid	1.7	0.28 ± 0.11	5.1	0.18 (0.03–0.31)
Valine	3.0	3.00 ± 1.05	9.2	0.53 (0.34–0.80)
Cystine	—	<1.0	0.68	0.52 (0.24–0.95)
Homocitrulline	0.72	0–tr.	18.0	0.70 (0.22–15)
Methionine	0.40	0.28 ± 0.11	2.5	0.31 (0.08–0.65)
Alloisoleucine	0.03	0.025 (<0.050)	—	—
Isoleucine	1.2	1.05 ± 0.45	2.8	—
Leucine	2.7	1.90 ± 0.65	6.5	0.96 (0.39–2.4)
Tyrosine	0.93	1.35 ± 0.60	8.8	1.9 (1.0–2.8)
Phenylalanine	2.2	1.10 ± 0.35	9.4	1.2 (0.44–2.0)
β-Amino-*i*-butyric acid	0.12	0–tr.	2.8	2.2 (0–9.0)
Ethanolamine	0.80	0–tr.	4.3	1.7 (0.72–3.1)
Ornithine	1.65	0.79 ± 0.32	0.34	0.15 (0.05–0.28)
Lysine	5.9	2.40 ± 0.80	12.5	1.5 (0.75–2.5)
Tryptophan	—	—	5.2	1.0 (0–1.9)
Histidine	2.15	1.20 ± 0.30	15.0	10.4 (5.7–17.5)
Carnosine	0.16	0	3.4	1.7 (0–3.3)
Arginine	0.02	1.20 ± 0.40	1.2	0.25 (0.09–0.72)
Homoarginine	0.01	0	0.09	0

[a] The normal levels are given as mean ± SD, and ranges are given in parentheses.

citrulline can be entirely accounted for by diffusion from the plasma, without postulating its accumulation in the cerebrospinal fluid by a block in urea synthesis in the brain. However, from the findings in argininosuccinic aciduria, it is certain that urea synthesis in the brain must account for the presence of some citrulline in the cerebrospinal fluid. It is possible that the relatively low level may be due to a more active utilization of citrulline by brain tissue by other pathways than urea synthesis, whereas argininosuccinic acid cannot be so utilized. It is much more likely that whereas the renal clearance of argininosuccinic acid is complete, there is a high renal tubular reabsorption of citrulline, resulting in a high plasma level, which will therefore be higher than in the cerebrospineal fluid.

As in the other disorders of urea synthesis, the plasma level of the blocked amino acid, in this case citrulline, varied with protein intake, the level dropping to half with restriction of protein (M6, M8, M12). Excretion of citrulline in the urine was high, in one case ranging from 0.5 g to 2.5 g per day, and in the other from 0.15 g to 1.39 g per day, depending on dietary intake of protein. This compares with a normal excretion of less than 1 mg per day.

10.3.2. *Levels of Ammonia in Blood and Cerebrospinal Fluid*

Effect of Protein Intake. In McMurray *et al.*'s case (M8), the fasting blood ammonia levels were usually a little above the normal, but rose to 800 μg/100 ml, about 5 times the fasting level, 2 hours after protein ingestion, and remained high even at 4 hours. These levels are similar to those found in argininosuccinic aciduria. However, the method of estimation used by McMurray *et al.* (M8), the diffusion method of Seligson, gives much higher levels of ammonia for the normal than the ion exchange method, so that comparison is difficult. Giving arginine, ornithine, aspartic acid or glutamic acid did not increase the blood ammonia level, but glutamine administration resulted in a considerable increase in blood ammonia to about 4 times the initial level, 2 hours after ingestion. This rise can readily be explained by a mass action effect suppressing the uptake of ammonia by glutamic acid. The effect of a protein load and glutamic acid ingestion on the ammonia level in the cerebrospinal fluid was similar to that on the blood. With both, the ammonia rose from a fasting level of 60 μg/100 ml to over 300 μg/100 ml 2 hours after administration.

In Morrow's case the blood ammonia, also measured by Seligson's method, was within their normal limits, 67 μg/100 ml, when she was on a low protein intake of 1.5 g per kilogram of body weight per day. When the protein intake was increased to double that amount, the blood ammonia rose to 269 μg/100 ml, falling again to 78 μg/100 ml when the

protein intake was again reduced (M12). The level of ammonia in the cerebrospinal fluid was 37 and 31 μg/100 ml on two occasions, lower than in the plasma. In the patient of Vidailhet *et al.* (V1), the blood ammonia which was measured only when the child was severely ill and comatose, was 1300 μg/100 ml. This was reduced to 643 μg/100 ml by peritoneal dialysis.

10.3.3. *Levels of Glutamine and Glutamic Acid in Blood, Cerebrospinal Fluid, and Urine*

As in other reported examples of urea cycle defect, levels of glutamine and glutamic acid in the plasma have not been satisfactorily measured. In McMurray's case (M6), only the glutamic acid level was reported, and this was within normal limits. The patient of Morrow *et al.* (M12) was said to have a plasma glutamine level of 4.65 mg/100 ml, actually lower than that of normal adults, while the glutamic acid level, 2.86 mg/100 ml, was higher. These levels are clearly erroneous for the reasons already given. In Vidailhet *et al.*'s case, however, where glutamine and glutamic acid levels were accurately determined, both were high, especially glutamine levels—41.0 mg and 5.8 mg/100 ml respectively—at a time when the plasma ammonia was 1300 μg/100 ml (V1) (Table 8). Thus it may be concluded that in citrullinemia, as in other types of defect in the biosynthesis of urea, glutamine levels in the plasma are always raised, forming a sensitive indicator of failure of ammonia uptake in the urea cycle. The levels of these two amino acids in the cerebrospinal fluid have not been recorded in any of the three cases. In two, however, where the levels of glutamine and glutamic acid were measured in the urine, they were shown to be high or extremely high, reflecting the raised levels in the plasma (M12, V1).

10.3.4. *Levels of Other Amino Acids in Plasma, Cerebrospinal Fluid, and Urine*

The other two amino acids of the urea cycle, arginine and ornithine, were found usually to be low in plasma (Table 8), but in one instance, whereas arginine was very low, almost absent in fact, ornithine was actually higher than the normal. Of the other amino acids to show changes from the normal, alanine is the most significant. In all three cases, it was elevated, in one case considerably so, and this was reflected in the urinary excretion which was also high. This finding is similar to that reported for hyperammonemia, and the reasons for the elevation are the same. Homocitrulline and lysine were increased in two of the three cases. The urinary amino acids show similar changes, the aminoaciduria being mainly of renal overflow type.

10.3.5. *General Biochemical Results and Liver Function Tests*

Again most of the general biochemical tests yielded normal results. However, of the liver function tests the serum transaminases were usually raised, especially during the severe phase when the child was comatose. On the other hand, the alkaline phosphatase has been reported normal. Renal function is normal. Blood urea was normal in two of the children, but in one case, it was usually low or very low.

10.3.6. *Intermediary Metabolites of the Pyrimidine Pathway*

In the first case of citrullinemia reported (M6), intermediary metabolites of the pyrimidine pathway were not sought in the urine. In the second, orotic acid was tested for, but not detected (M12). In the third child, orotic acid, uridine, and uracil were found in relatively large amounts in the urine when the plasma glutamine was at the very high level of 41.0 mg/100 ml, showing that in this genetic disorder, as in the other disorders of urea synthesis, these metabolites are always excreted in excess when the glutamine is raised (V1).

10.3.7. *Urea Excretion and Protein Intake*

The effect of protein intake on urea formation and excretion has been extensively investigated. McMurray *et al.* (M6) showed that with an 8-fold variation in protein intake, from 12.5 g to 100 g of protein per day, each for a period of 1 week, the urea excretion varied 6-fold, rising and falling with increase or decrease in intake. Single protein loads also induced increased urea excretion, although not so markedly as with increased ingestion of protein over a longer period. Morrow *et al.* (M11, M12) obtained a similar result, but they concluded that the persistently low blood urea in their child and the less than expected rise in urinary excretion with the increase of protein intake, was evidence of a block in urea formation, contrary to previously reported cases where normal amounts of urea were produced. This distinction is doubtful, being presumably based on the relatively normal levels of blood urea reported in previous cases. It cannot be assumed, simply because of one normal level, that normal amounts of urea are produced. It seems highly probable that in all diseases of the urea synthesis resulting from enzymatic errors there is a defective formation of urea, at least under stress of a normal protein intake.

10.3.8. *Urea Formation from Citrulline and Arginine*

Studies with ureido citrulline-^{14}C showed that the child could convert about 30% of the injected labeled citrulline to urea in a 24-hour period.

Plasma citrulline did not rise significantly after the oral ingestion of a test load of arginine and ornithine. Urea excretion was increased after loading with arginine or glutamic acid. It was interesting that the addition of arginine to the diet resulted in an increase of urea output equivalent to that of the added arginine, showing that there was no block in the conversion of arginine to ornithine.

10.3.9. *Urea Cycle Enzymes in Liver and Other Tissues*

The liver argininosuccinate synthetase in the patient of McMurray *et al.* (M5) was found to have an activity of only 5% that of normal liver. Although no estimations of urea cycle enzymes of the liver were performed in the patient of Morrow *et al.* (M12), argininosuccinate synthetase was assayed by Tedesco and Mellman (T3) in cultured fibroblasts from the skin of the patient. They showed that although the enzyme was present in the cells, the K_m value for the mutant enzyme was at least 25 times greater than normal, suggesting an aberrant enzyme.

Vidailhet *et al.* (V1) assayed all five enzymes involved in the urea cycle, and found no detectable activity of argininosuccinate synthetase, whereas the other enzymes were present in normal activity (Table 9). It is of interest that an appreciable synthetase activity was detectable in the kidney tissue in their patient, at a level of about 20% of that found in normal liver, despite its absence in the patient's liver (L4). This observation is similar to that of Colombo and Baumgartner (C8), who found argininosuccinate lyase in the kidney of their child with argininosuccinic aciduria, in spite of its absence from the liver. The question is again raised whether it is possible thus to account for the production of urea in these cases. However, since arginase is not present in the kidney, the arginine formed would have to be transported to the liver

TABLE 9
CITRULLINEMIA: UREA CYCLE ENZYME LEVELS IN LIVER AND KIDNEY[a]

			OTC						
Case	Source	Tissue	pH 7.0	pH 8.3	CPS	ASS	ASL	A	References
1	Biopsy	Liver	5500	—	82	1.4	290	59,000	(M4)
2	Necropsy	Liver	4920	5020	229	Nil	210	51,000	(V1)
2	Necropsy	Kidney	49	689	Nil	7.3	1.8	Nil	(V1)

[a] Levels are given in micromoles of product formed per hour per gram wet weight of tissue. OTC, ornithine transcarbamylase; CPS, carbamyl phosphate synthetase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; A, arginase.

for conversion to urea. Such a system, although possible, would be inefficient (see later discussion).

10.3.10. *Other Examples of Citrullinemia*

Visakorpi (V2) has described a severely retarded adolescent who at 15 years of age was found to be excreting a large quantity of citrulline, approximately 500 mg/24 hours, an amount much more than that found in the other cases of citrullinemia. In addition, there was a gross aminoaciduria, especially cystine, lysine, arginine, ornithine—a pattern suggestive of cystinuria. No further investigations were carried out. While the clinical picture especially the periods of unconsciousness could accord with a diagnosis of citrullinemia, this must be considered doubtful.

10.3.11. *Inheritance*

There are too few cases of citrullinemia on which to assess with certainty the character of the inheritance. It is not confined to one sex, since of three cases, two were males and one was a female. No tests have been performed on the parents of any affected patient to determine the heterozygote state. It seems likely, however, since no parent of such a child was found to be affected, that the disorder is inherited as an autosomal recessive.

10.4. Hyperargininemia

10.4.1. *Levels of Arginine in Blood, Cerebrospinal Fluid, and Urine*

The serum level of arginine was, as expected, very high, attaining levels of 17.3 mg and 11.5 mg/100 ml, respectively, in the two cases so far reported, levels 7–10 times higher than the normal (T4, T5). Similarly, the arginine level in the cerebrospinal fluid was also raised, 1.65 mg and 0.93 mg/100 ml, compared with their own mean normal level of 0.25 mg/100 ml. The lower level in cerebrospinal fluid than in the plasma is similar to that found in citrullinemia and contrasts with the higher levels of argininosuccinic acid found in the cerebrospinal fluid than in plasma in patients with argininosuccinic aciduria. The reason is probably the same as that given for citrullinemia. There was also gross argininuria, up to 2.4 g per gram of creatinine compared with their own normal of about 5 mg per gram of creatinine.

10.4.2. *Levels of Other Amino Acids in Blood, Cerebrospinal Fluid, and Urine*

It is surprising that despite the high blood and cerebrospinal fluid ammonia levels, glutamine and glutamic acid were not raised in either

of the two patients, although in one the blood glutamine was a little above the normal (T5). In view of the close correlation between the blood and cerebrospinal fluid levels of ammonia and glutamine found in hyperammonemia it is likely that a similar relationship exists in hyperargininemia. Again it is possible that the two amino acids were not accurately determined. This is supported by the finding that large amounts of glutamine, greatly exceeding the normal, were excreted in the urine (T5). Of the other two amino acid intermediates of the urea cycle, the ornithine levels in both children were low, but citrulline was normal.

The pattern of excess excretion of cystine, lysine, and ornithine in addition to arginine found in the urine suggested a striking analogy with cystinuria. This similarity was further strengthened by the detection of the disulfide of cysteine and homocysteine, also found in cystinuria. That the patients had this condition in its usual form was excluded by the more nearly normal levels of the renal tubular readsorption capacity found for these four amino acids in the two patients compared with the low values found in cystinuric patients. The similarity of the pattern of renal excretion of these four amino acids to that seen in familial cystinuria was most likely due to the blocking of the common transport mechanism of the kidney by the necessity of maximal reabsorption of the excessive amount of arginine (T5). This supposition was supported by the observation that in one child where the arginine excretion was much less than in the other, the excretion of the other three amino acids was also considerably smaller.

10.4.3. *Levels of Ammonia in Blood and Cerebrospinal Fluid*

The levels of ammonia in both blood and cerebrospinal fluid were very high, up to 671 μg/100 ml and 514 μg/100 ml, respectively, measured on one patient when she was on a normal protein intake of 2.7–3 g per kilogram body weight per day. When this was reduced to 1.6 g per kilogram body weight per day, the blood and cerebrospinal fluid ammonia levels were reduced to 134 μg/100 ml and 44 μg/100 ml respectively, a little above the upper limit of normal levels for the method used. The blood urea levels were always within normal limits, the highest level being 26 mg/100 ml.

10.4.4. *General Biochemical and Liver Function Tests*

Again, most of the routine biochemical tests in these two children were within normal limits. However, there was a moderate impairment of liver function, as judged by the serum alkaline phosphatase and both transaminases, GOT and GPT, all of which were slightly or moderately raised.

10.4.5. *Enzyme Levels in Red Blood Cells*

The levels of arginase and argininosuccinate lyase were measured only in red blood cells in these cases. In one child, no detectable activity of arginase was found; in the other only 12% of the mean normal level was found (Table 10). Lyase activity was normal in both children. The two enzyme activities were also measured in the parents, and both were found to be below the lowest level found in a series of normal controls, suggesting the heterozygote state. In both the lyase activity was normal.

TABLE 10
HYPERARGININEMIA: LEVELS OF ARGINASE AND ARGININOSUCCINATE LYASE IN RED BLOOD CELLS[a,b]

Case	Sex	Arginase	Argininosuccinate lyase
Patient	F	127	41
Patient	F	Nil	26
Mother	F	573	30
Father	M	743	34
Normal control (10)	—	793–1830	18–40

[a] Data from Tomlinson and Westall (T7).
[b] Levels given as micromoles of product formed per hour per gram wet weight of tissue.

11. Production of Urea in Enzymatic Defects of Urea Cycle

The fact that relatively large amounts of urea can be produced by subjects in whom an enzyme defect of the urea cycle has been proved has perplexed many investigators. Despite the fact that the activity of the rate-limiting enzyme has been reduced even to zero as measured by a sophisticated method, urea production still continues. It must therefore be concluded that, in all these cases, urea production is only impaired, not abolished. In all normal circumstances, all but a small fraction of the nitrogen in excess of tissue protein requirements is still excreted as urea and/or as the amino acid whose further metabolism is blocked. The impairment shows itself in the elevated levels of blood ammonia and consequently of glutamine, which vary according to the stress placed upon the urea cycle by varying the rates of protein intake.

Several hypotheses have been propounded to explain the continued formation of urea. One of the first was the suggestion that to the synthesis of urea there was a pathway alternative to the Krebs-Henseleit one (L7). Evidence for such a pathway had earlier been adduced by Bach (B1), who concluded that, in the liver, glutamine formed from glutamic acid and ammonia could combine further with ammonia and

carbon dioxide to re-form glutamic acid and yield urea. However, Archibald (A2), who at first found evidence to support this, discovered later that no urea was produced by liver slices from glutamine, if the pure substance free from arginine was employed.

Cedrangolo *et al.* (C5) and De Lorenzo (D1) also postulated an alternative pathway on the basis of their experimental results on rats. The animals were injected with α-methyl aspartate, a specific inhibitor for argininosuccinate synthetase. No effect on urea excretion was observed, but there was complete inhibition of urea synthesis from citrulline in liver homogenates prepared from injected animals, as well as increased susceptibility to ammonia intoxication. However, this inhibition was not confirmed by Crokaert and Baroen (C15, C16, C17), although they did confirm the lack of any effect on urea excretion. The experimental basis for this suggestion is therefore in doubt.

A possible alternative pathway for urea synthesis has also been put forward by Cohen *et al.* (C7). They detected a new nitrogeneous product, guanidinosuccinic acid, in the urine of azotemic patients (B6, N1) and suggested that this might be an intermediate in an alternative route of urea synthesis via a series of 6 reactions:

$$\text{Ammonia} + CO_2 + 2\text{ATP} \xrightarrow[\text{phosphate synthetase}]{\text{carbamyl}} \text{carbamyl phosphate} \quad (1)$$

$$\text{Carbamyl phosphate} + \text{ornithine} \xrightarrow[\text{transcarbamylase}]{\text{ornithine}} \text{citrulline} \quad (2)$$

$$\text{Citrulline} + \text{aspartate} \xrightarrow[\text{synthetase}]{\text{argininosuccinate}} \text{ASA} \quad (3)$$

$$\text{ASA} + NH_3 \rightarrow \text{ornithine} + \text{carbamyl aspartate} \quad (4)$$

$$\text{Carbamyl aspartate} + NH_3 \rightarrow \text{guanidinosuccinic acid} \quad (5)$$

$$\text{Guanidinosuccinic acid} \rightarrow \text{urea} + \text{aspartate} \quad (6)$$

There are difficulties in accepting this alternative pathway. Reactions (4), (5), and (6) are speculative, and the postulated enzymes mediating them have not been identified. Furthermore, although the hypothetical cycle would explain the normal urea formation in argininosuccinic aciduria, it could hardly be satisfactory, for example, in citrullinemia, in which the necessary intermediate, argininosuccinic acid, is not formed at all.

The explanation most favored at the present time to account for the continued urea formation despite a defective enzyme of the cycle is not that there exists an alternative pathway, but that the defect is only partial, not complete, and that the residual activity of the defective

enzyme *in vivo* is sufficient to account for the amount of urea synthesized. This is not likely to be the case at any rate for argininosuccinic aciduria. One child with this condition excreted as much as 5 g of urea in 24 hours when less than 6 months old. The activity of the liver argininosuccinate lyase in his case was zero, which according to the sensitivity of the method used must have been less than 2% of the normal level (L1), an activity well below that of the rate-limiting enzyme of the cycle, argininosuccinate synthetase. With this activity, and assuming a liver weight up to 350 g, the maximal amount of urea which would be formed would be only 1.0–1.5 g/day.

In hyperammonemia, however, the residual ornithine transcarbamylase activity is still in excess of that of argininosuccinate synthetase, so that on this basis, urea synthesis could proceed even at the normal rate. This however may not be so. It is known, for example, that when the overall rate of gluconeogenesis alters as a result of physiological or pathological circumstances, the changes of enzyme activity are not limited to the pacemaker stages (K11). Other enzymes in this pathway also increase in parallel, although their capacity may be much greater than the rate-limiting enzyme. A similar observation has been made for the enzymes of the urea cycle. Both arginase and ornithine transcarbamylase activities increase in parallel with the other enzymes as a result of increase in dietary protein intake, in spite of the fact that their normal activities are far in excess of argininosuccinate lyase and synthetase (S3). These findings form the basis of the so-called principle of the "constant proportions" groups of enzymes (K11). It may well be, therefore, that a severe decrease in ornithine transcarbamylase or arginase activity will greatly reduce the rate of synthesis of urea even though the residual activity is still above that of the rate-limiting enzyme. Thus the suggestion that the residual activity of a defective enzyme of urea synthesis is sufficient to permit normal amounts of urea to be formed is still debatable.

Still another possibility has been discussed by Scriver (S8, S9). Two assumptions are necessary. The first is that patients who survive have mutant but not absent enzymes, and the second that the abnormal enzyme has a much higher K_m, i.e., a lower affinity, for the substrate of that enzyme, the maximum rate of reaction not being excessively reduced. Under these conditions, the substrate will accumulate, but when equilibrium is established at a higher concentration of substrate, a reaction rate can be measured, reflected as continued urea production. Substrate accumulation from this point of view is an adaptation to the abnormal enzyme.

There is a difficulty in accepting this argument. Substrate accumulation is a consequence of the defective enzyme, hardly an adaptation. In

the usual method of *in vitro* assay of the enzymes of urea formation, substrate concentrations more than ten times the optimal are routinely employed, and even under these conditions, the activity of the defective enzyme is still low, i.e., the reaction rate is still very low. Unless the activity is radically increased by such drastic increases of substrate concentration of 100-fold or more, this is still likely to be the case. It seems doubtful therefore that Scriver's explanation can be correct.

To summarize, despite the many suggestions, there is still no completely acceptable explanation of the formation of urea in relatively large amounts in disorders of urea formation.

12. Hyperammonemia in Conditions Affecting the Urea Cycle Other Than Primary Enzyme Errors of Urea Synthesis

Hyperammonemia resulting from any of the enzymatic disorders of the biosynthesis of urea, must be distinguished from other conditions in which plasma ammonia is raised, sometimes sufficiently so to cause clinical manifestation. Severe liver disease as a primary cause of acquired hyperammonemia may be excluded from consideration since it is readily distinguishable from urea cycle defects. However, there are a number of other conditions described with hyperammonia as a prime manifestation, which because they show some clinical and biochemical similarity to hereditary enzyme defects of the urea cycle, have been claimed to be urea cycle disorders.

12.1. Lysine Intolerance with Periodic Ammonia Intoxication

Colombo and his colleagues (C9, C10, C11) have reported in detail a female infant first observed at 3 months of age whose clinical features were remarkably similar to those manifest in children with urea cycle defects, i.e., vomiting, convulsions, apathy, episodes of coma, variable muscle tonicity, and mental retardation. This similarity led to an examination of the urea cycle enzymes in a liver biopsy, but no abnormality was discovered. The plasma amnio acids showed a significantly raised arginine and lysine level when the child was on a normal protein intake, but there was no aminoaciduria. The blood ammonia levels were high, varying from 100 μg to 600 μg/100 ml, according to whether the protein intake varied from 0 to 6 g/kg body weight per day. Blood urea was usually low or low normal, but rose when the protein intake was increased. Oral ingestion of lysine resulted in a marked increase in blood ammonia, and the infant became comatose. Similar tests with L-arginine and L-leucine resulted in only a small increase in blood ammonia, and the child remained well throughout. On the basis of these findings, Colombo *et al.* (C9), postulated that although no defect due to an abnormality of

an enzyme of the urea cycle was present, there was an indirect affect on the cycle because of inhibition of arginase by the high levels of lysine. That L-lysine and L-ornithine are potent inhibitors of arginase *in vitro* has long been known (H3, H4).

Colombo *et al.* (C9) offered two facts to support their hypothesis. Oral ingestion of lysine by the patient resulted in a marked decrease to zero levels of arginase in the patient's red cells, coincident with the rise in blood lysine to 12.6 mg from 4.4 mg/100 ml. No such decrease was found in a control child or in the parents of the affected infant. There was a moderate decrease when the affected child was given L-arginine or L-leucine. Second, a low activity, 22% of the mean normal, of L-lysine NAD oxidoreductase was found in the liver. The authors suggested that the low activity of this enzyme responsible for the first step in the metabolism of lysine accounted for the accumulation of the latter in the blood.

There is some difficulty in accepting this interpretation of the aetiology of the condition. Kirkman and Kiesel (K10) have shown that the suggested lysine intolerance from a deficiency of lysine dehydrogenase and from secondary inhibition of arginase by lysine only occurs at concentrations of lysine greatly exceeding those existing *in vivo*. Hyperlysinemia of even greater degree than that reported by Colombo *et al.* (C10, C11) has been described by Woody *et al.* (W6, W7) and by Ghadimi *et al.* (G3, G4) without any symptoms of hyperammonemia. If lysine inhibits arginase sufficiently *in vivo* to impair the urea cycle in the one case, it is difficult to understand why it should apparently fail to do so in the others. Since under normal conditions of assay, arginase activity is incomparably greater than any of the other enzymes of the urea cycle, speculative factors such as a much higher concentration of lysine intracellularly than in the extracellular fluid must be invoked to support the hypothesis that in lysine intolerance, arginase becomes the rate-limiting enzyme. It is surprising also that no specific mention is made of the levels of glutamine in plasma or urine. The hypothesis that lysine inhibition inhibits the urea cycle is still therefor unproven, although possibly correct. Although the red cell arginase of the patient was shown to have the same K_m values as control individuals, no similar comparison of the K_m values was made for the liver arginase. It is possible this was abnormal, and this would be sufficient to account for the deficient urea cycle activity under stress.

12.2. Familial Protein Intolerance

A syndrome of familial protein intolerance with intermittent mild or moderate hyperammonemia has been described by Perheentupa and his

colleagues (K6, P4). The infants presented with vomiting and diarrhea on weaning from the breast, with subsequent failure of growth. They developed an aversion to protein-rich foods in the second year of life. All had hepatomegaly, and some had splenomegaly in addition. The liver function tests were usually normal.

The plasma amino acid levels of these patients showed some significant deviations from the normal. There were reduced levels of lysine, arginine, leucine, and tyrosine, but alanine, citrulline, and serine were increased. There was an increased aminoaciduria, the ratio of amino acid nitrogen to total nitrogen varying from 3 to 8%, with an excess especially of lysine. This was confirmed by a measurement of the renal excretion of the amino acids which showed a marked increase for lysine, moderately so for arginine, and cystine only slightly if at all abnormal. The clearance of ornithine could not be calculated owing to interference by ammonia. The blood ammonia levels in the fasting state were within normal limits. The blood urea was always in the low normal range. After intravenous infusion with L-alanine, the blood ammonia rose from 50 μg to a peak of about 230 μg/100 ml. A similar rise was observed after 300 ml of milk. No such rise occurred in control subjects. The simultaneous infusion of arginine with alanine abolished completely the rise in ammonia, whereas the infusion of alanine with lysine resulted in an even higher and more prolonged rise in blood ammonia. This also was not observed in controls when similar infusions were given. The authors suggested on the basis of these results that there was a transport defect involving the basic amino acids. This led to a deficiency of arginine and of ornithine, although the latter could only be inferred since direct measurements could not be made. The lack of the two amino acids was accentuated by the low intake of protein. The deficit of these intermediates of the urea cycle resulted in a slowing down of the cycle and so to the transient hyperammonemia and protein intolerance. On this basis, the authors tried the effect of arginine supplement on their patients. The preliminary results suggested an improvement in tolerance to protein and an improved weight gain.

The activities of the enzymes of the cycle estimated in a liver biopsy obtained at laparotomy from two of these children were found to be similar to those of control normals. Furthermore, the K_m value for arginine of the liver arginase and the inhibition constant for L-lysine and L-ornithine were the same for the patient as those of the control subjects. This points to the fact that the hyperammonemia could not be explained on the basis of an abnormal arginase. There may still have been, however, a kinetic abnormality of one of the other four enzymes.

This condition of familial protein intolerance can be differentiated

from the protein intolerance manifested by children with inherited metabolic disorders of the urea cycle by the fact that postprandial hyperammonemia is relatively slight and that fasting blood ammonia is always within normal levels in the former, whereas in the latter, it is frequently above the normal.

12.3. A Cerebroatrophic Syndrome of Hyperammonemia

This condition has been described by Rett (R10, R11) and Rett and Stöckl (R12) in 22 children, all girls, the oldest of them 13 years of age, from a survey of 6000 mentally subnormal children. In all 22, the blood ammonia was raised from 2 to 5 times the normal the highest being 165 μg/100 ml. The blood urea was said to be normal in all cases, as was the plasma amino acids. Where liver biopsy was obtained, this was also normal. The brain was examined in 5 children who died. They showed cerebral atrophy but no Alzheimer Type II cells. A relationship between hyperammonemia and the cerebral changes of the syndrome was postulated and attention drawn to the similarity with some of the neurological manifestations of children with urea cycle defects. However, the cause of the hyperammonemia was unexplained, and it seems unlikely that these were examples of primary urea cycle disorders.

12.4. Hyperammonemia Associated with Hyperornithinemia

It has already been noted that the ornithine level in the serum is not raised in cases of liver ornithine transcarbamylase deficiency and the reasons for this have been discussed above. However, a very interesting condition of hyperornithinemia, hyperammonemia, and homocitrullinemia has been recorded by Efron (E3) and Shih *et al.* (S11) in a 3-year-old boy with mental retardation, infantile spasms, irritability, and intermittent ataxia. The child had always refused protein foods, including milk. These symptoms suggested ammonia toxicity. Plasma ornithine concentrations were very high, 12–14 mg/100 ml, more than ten times the normal level. The urine excretion was also raised, 14 mg/24 hours. There was surprisingly an increase in the excretion of homocitrulline. Reduction of protein intake to low levels resulted in a fall of plasma ornithine to 3–4 mg/100 ml, and the blood ammonia also fell to normal levels. When protein intake was increased to 2.0 g per kilogram body weight per day, plasma ornithine rose again to 9 mg/100 ml, and the blood ammonia to over 180 μg/100 ml, to the accompaniment of vomiting and ataxia. The serum transaminases were raised, especially during episodes of hyperammonemia.

The similarity of the clinical appearances to those found in enzymatic defects of the urea cycle suggested strongly that such a defect was pres-

ent in this child also. A liver biopsy was not examined, but in the serum, the ornithine transcarbamylase measured by the method of Reichard (R8) was actually increased rather than decreased and so apparently was the red cell ornithine transcarbamylase. Even if a defect of this enzyme had been detected in the liver, the very high levels of plasma ornithine would have distinguished it from the proved ornithine transcarbamylase defects so far reported, where the plasma ornithine level is conspicuously normal.

Shih *et al.* (S11) could find no explanation for the simultaneous presence of hyperornithinemia and hyperammonemia in their patient. However, it is possible that the hyperammonemia resulted from the very high levels of ornithine. It has long been known that ornithine is the most potent inhibitor of arginase (H6). The very high levels of ornithine could inhibit the urea cycle and so cause hyperammonemia. The difficulty is that hyperornithinemia due to a defect of liver ornithine-ketoacid amino transferase has been reported by Bickel *et al.* (B5a) in which severe hyperammonemia was not a feature. They investigated two sibs, a 6-year-old boy and a 3-year-old girl, who were both mentally retarded. There was a history of prolonged neonatal jaundice. The younger child was considered to have giant cell hepatitis, and a liver biopsy showed a precirrhotic condition. Both children had increased transaminases as well as signs of renal dysfunction, gross aminoaciduria, glycosuria, and, in one, also polyuria. It was concluded that the combination of brain damage and liver and renal tubular dysfunctions could be due to an underlying metabolic defect. Both children showed plasma levels of ornithine which were three times the normal. The liver ornithine transcarbamylase activity was normal in one of the children, and the blood levels of the enzyme were also normal in both. An assay of the liver ornithine ketoacid aminotransferase yielded levels of 0.2 and 0.3 unit per gram of liver compared with a range of 1–4 units per gram in control subjects of the same group (K4). The conversion of ornithine to glutamate semialdehyde and then to Δ'-pyrroline-5-carboxylic acid mediated by ornithine ketoacid aminotransferase appears to be a major degradative pathway for ornithine (K1, K3, R3), and a deficiency of this enzyme would account for the elevation of plasma ornithine levels in these two cases. The blood ammonia levels in both children were found to be at the upper limit of normal or only very slightly increased.

Thus there are two major differences in the two types of hyperornithinemia. The first is the high level of blood ammonia accompanied by the severe clinical manifestations of hyperammonia. The second is the much higher level of ornithinemia, 12–14 mg/100 ml compared with less than 2 mg/100 ml. It seems likely that the blood level of ornithine

found in Shih and Efron's case was high enough to inhibit arginase activity, and therefore the urea cycle, resulting in hyperammonemia. This explanation is similar to that suggested by Colombo *et al.* (C9, C10) to account for the chronic ammonia intoxication in an infant with lysine intolerance. Probably the hyperornithinemia in the case of Shih *et al.* (S11) was due to a severe defect in the liver ornithine ketoacid aminotransferase, although this was not assayed. It may be assumed that the level of ornithine attained in Bickel's cases was not sufficiently high to inhibit the arginase, or to inhibit it only to a slight extent. It is significant that the blood ammonia levels in Bickel's cases were not entirely normal, but at the upper limit of normal or very slightly increased, and it would have been interesting to measure the blood ammonia levels during ornithine infusions to test the hypothesis. The differences in the ornithine levels in the two types of hyperornithinemia may quite possibly be due to a different degree of enzyme deficiency, that in Shih's case being more severe than that in Bickel's patient.

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RAPID SCREENING METHODS FOR THE DETECTION OF INHERITED AND ACQUIRED AMINOACIDOPATHIES[1]

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1. Introduction

1.1. Nature and Scope of the Chapter

The two editions of the book entitled "The Metabolic Basis of Inherited Disease" edited by Stanbury, Wyngaarden, and Fredrickson (S48) have summarized our current knowledge of human genetic diseases which are characterized by known biochemical differences. This generalized concept of inherited metabolic disorders was first postulated by Garrod (G1, G2). From his pioneering studies of a number of metabolic diseases, such as cystinuria (K12), alcaptonuria (K9, L1, O4), albinism (F10, K10), and pentosuria (T5a), Garrod stated that the three conditions which define a disease as an inborn metabolic disorder are: (a) that the disorder must have a familial distribution in which one or more sibs are involved while parents and more distant relatives are normal; (b) that the patient accumulates or excretes a large quantity of a metabolite in comparison with the amount found in normal individuals; (c) that the accumulated or excreted metabolite, which is responsible for the clinical symptoms of the disease, results from a missing enzyme or reduced level of an enzyme which governs a single metabolic step.

Two new developments in clinical medicine have made the early recognition of inborn metabolic errors in the clinical chemistry laboratory of increased importance. First, there is the finding that utilization of dietary regimens deficient in the metabolites normally acted upon by the missing enzyme serves to ameliorate the more serious consequences of the disease, e.g., low-phenylalanine diets in cases of phenylketonuria (K11). Second, the availability of amniotic fluid early in pregnancy by amniocentesis (V1, V2) has made possible the prenatal diagnosis of genetic disease in those conditions in which the accumulated substance or missing enzyme is known (M10, M11, M12). This approach is presently being used in our laboratory to determine whether pregnant women, who have previously given birth to children with Tay-Sachs disease (S12), are carrying an abnormal fetus by analyzing the amniotic fluid for the missing enzyme, i.e., β-D-*N*-acetylhexosaminidase A (O6, S13). An early diagnosis in this situation is essential if the mother is to have a choice of ending the pregnancy or carrying the fetus to term.

Since in patients with inherited disorders the substance which accumulates in their biological fluids or tissues is present in manyfold concentration as compared to normal individuals, qualitative or semi-

quantitative procedures should ordinarily suffice for their detection. Emphasis of laboratory methodology for the screening of large segments of a population should therefore be directed toward being able to handle many samples with simple, rapid procedures that require a minimum of space and equipment. It is the main purpose of this review article to acquaint the clinical chemist with the more recent and pertinent literature in this field as well as to describe the methodology currently employed in the author's laboratory for the rapid screening of inborn errors of amino acid metabolism. However, such semiquantitative procedures should be considered only the initial step in the identification of a particular genetic disease, even when a positive result is obtained. In most instances it is essential that any positive finding be confirmed by the preparation of a suitable derivative, e.g., DNP-phenylalanine in phenylketonuria (B16), which is chromatographed in a different solvent system, by selective staining (H9), or by the use of a quantitative procedure such as ion-exchange resin column chromatography (B16, S5). Quantitative procedures are also essential for the detection of the carrier state, since in most recessive genetic disorders the level of an excreted metabolite, e.g., blood phenylalanine in phenylketonuria (K11, W22), may be only slightly above that of a normal individual or the enzyme level slightly below normal, e.g., Tay-Sachs disease (F16).

1.2. Publications Dealing with Amino Acid Methodology and Aminoacidopathies

The importance of this subject in clinical medicine is attested to by the large number of texts and review papers which have dealt with this topic in the last decade (A4, B1, B12, B13, B14, B18, B25, C15, E5, E9, E11, E12, F5, G8, G10, G11, G12, G21, H1, H10, H11, H17, H18, H19, H20, H21, J2, J8, K3, K4, K7, L4, M8, M25, N3, N4, N8, O2, O3, O7, P6, P7, P9, R1, R3, R9, S16, S18, S22, S25, S28, S34, S35, S36, S37, S41, S45, S46, S47, S48, S52, T4, T8, W2, W11, W22, W23, Z3). Articles appearing prior to 1965 utilized mainly paper chromatographic methods whereas those published in the last five years emphasized the use of thin-layer and high voltage electrophoretic techniques for the rapid screening of blood and urine for defects of amino acid metabolism.

2. Studies of Experimental Factors That Influence the Separation of Amino Acids in a Mixture

2.1. Choice of Absorbent Medium (Stationary Phase)

While thin layers of cellulose is the medium favored by most investigators in recent years for the separation of amino acids in bio-

logical fluids, the paper chromatographic method still has its proponents (B13, E13, G13, G16, L9, L10, O5, S19). The literature prior to 1959, employing paper as the stationary phase for the separation and the determination of the amino acids of human urine has been reviewed in the supplement by Jagenburg (J2).

Two of the more widely used paper chromatographic methods for the screening of amino acids in biological fluids have been those of Efron *et al.* (E13), which has been employed by Levy *et al.* (L9, L10) for whole blood and urine, and of Scriver and co-workers (S19). A similar unidimensional paper chromatographic procedure is being sold by Hyland Laboratories (H24) under the trade mark of "Chromatotest." The procedure using paper has the advantages of being cheap, readily available, and capable of handling a large number of individual samples simultaneously on a single sheet. It also permits the analysis of blood or urine samples collected on filter paper disks, which after autoclaving, are inserted into holes punched in the chromatogram (E13, L9, L10). Its main disadvantages are the prolonged time needed for solvent development (5–16 hours) and the unsatisfactory resolution of many pairs of clinically important amino acids—e.g., valine and methionine; glycine and serine.

An excellent discussion of the various commercially available absorbents suitable for the preparation of plates for use in thin-layer chromatography appears in Kirchner's (K7) book on the subject. The efficiency of chromatographic separation of a mixture of nine amino acids, on ten kinds of Whatman paper and on six kinds of cellulose powder thin layers, with butanol:acetic acid:water (4:1:5 v/v) as the developing solvent, was investigated by de Ligny and Remijnse (D5). They concluded that thin layers of cellulose powder gave superior separation to paper. Horton *et al.* (H16) and Wolfrom *et al.* (W20) found that acid-treated cellulose (Avicel) gave adherent layers on glass plates with excellent physical characteristics for thin-layer chromatography. Chromatographic analysis of 70 amino acids, indoles, and imidazoles using paper, thin layers of Avicel and cellulose was performed by Smith *et al.* (S38), who confirmed the superiority of Avicel or "microcrystalline cellulose" thin layers for amino acid separations. Jackson *et al.* (J1) have compared paper and cellulose thin-layer chromatography for the mass screening of amino acids and selected the latter procedure as the preferable one.

A number of investigators (F4, M14, P1, T9, V3) have suggested the superiority of mixtures of cellulose and silica gel to either one alone (F15, K13) for the separation of amino acids either chromatographically or by means of high voltage electrophoresis (F4). In particular, Turner

and Redgewell (T9), employing thin-layer chromatography, reported the mixed layer to give better separation of pairs with close R_f values, a greater sensitivity of various amino acids to ninhydrin, and a decrease in the time required for the preparation of suitable radioautographs with labeled amino acids. Van Sumere *et al.* (V3) stated that such a mixed layer and the use of monodimensional thin-layer chromatography with multiple development, using two solvent systems, allowed the separation of 17 out of a mixture of 22 amino acids.

Other authors (C13, K8, K14) have proposed ion-exchange cellulose or resins, either alone (K8) or mixed with cellulose (C13, K14), as suitable media for resolving mixtures of amino acids. Knight (K8) uses DEAE-cellulose paper in the free base form and two-dimensional chromatography, with a buffer solvent in the first dimension and a partition (organic) solvent in the second dimension as an efficient means of resolving amino acid mixtures. Copley and Truter (C13) and Kraffczyk *et al.* (K14) use combinations of thin-layer films of ion-exchange resins and cellulose in such a manner that the ion-exchange layer is utilized to remove salts from the sample and the amino acids are then separated by two-dimensional chromatography in the cellulose layer.

Of more academic interest is the report of Criddle *et al.* (C15) that kieselgur (silica gel G) is the best medium for thin-layer electrophoresis, and that of Petrovic and Petrovic (P19), who used a mixture of rice starch and gypsum as the most suitable absorbent for the separation of amino acids.

2.2. Choice of Developing Solvent

As is the case with a choice of the proper stationary phase, one is faced with choosing the best solvent system (or systems), when either two-dimensional or multiple development techniques are used, among the large number which have been proposed for the separation of amino acid mixtures. The more common developing solvents and R_f values for many biologically important amino acids are listed in the textbooks of Blackburn (B16), Stahl [see Brenner *et al.* (B24)], Smith [see Efron (E11)], Haer (H1), Heftman (H10), Kirchner (K7), Pataki *et al.* (P7, P9), Randerath (R1), Shellard (S25), and Smith (S37) and in the review articles of Lederer *et al.* (L4), Niederwieser and Curtius (N4), Opiénska-Blauth *et al.* (O7), Pataki (P6), and Kerkut and Shapira (K4). Of these, *n*-butanol:acetic acid:water (12:3:5) is the most widely used solvent system for the separation of amino acids in biological fluids (A2, B17, E13, H16, H24, J1, L9, L10, O8, S19, S37, S38). A list of 22 solvents suitable for the chromatography

of amino acids on cellulose layers has been compiled by Pataki (P7) and is reproduced in Table 1. Other solvent systems used for two-dimensional separation of amino acid mixtures are: methanol:chloroform:ammonia (25%) (2:2:1) 1st dimension and methanol:pyridine:water (20:1:5) 2nd dimension (B19, B26); isobutanol:dimethyl ketone:acetic acid:water (40:40:8:20) 1st and *n*-butanol:dimethyl ketone:ammonia (28%):water (40:40:8:2) 2nd (C6); isopropanol:butanone:1 *N* HCl (60:15:25) 1st and 2-methylpropanol-2:butanone:propanone:methanol:water:0.88% ammonia (40:20:20:1:14:5) 2nd (H5, H7, J7); *n*-butanol:acetic acid:water (4:1:5) 1st and phenol (saturated water) containing 0.1% ammonia and 0.17% cupron 2nd (J2); *n*-butanol:acetic acid:water (4:1:5) 1st and pyridine:isoamyl alcohol:water (7:6:6) 2nd (H15); *n*-butanol:acetic acid:water (4:1:5) 1st and pyridine:water (4:1) 2nd (S29), chloroform:methanol:ammonia (28%) (2:2:1) 1st and phenol:water (3:1) 2nd (F2); *n*-butanol:methyl ethyl

TABLE 1

SOLVENT SYSTEMS SUITABLE FOR THE SEPARATION OF AMINO ACID MIXTURES ON CELLULOSE-COATED THIN-LAYER CHROMATOGRAPHIC PLATES[a]

No.	Solvent	
1	Butanol:glacial acetic acid:water	4:1:5
2	Pyridine:methyl ethyl ketone:water	15:70:15
3	Methanol:water:pyridine	20:5:1
4	Butanol:formic acid:water	15:3:2
5	Propanol:8.8% ammonia	4:1
6	Ethanol:butanol:water:propionic acid	10:10:5:2
7	Pyridine:isoamyl alcohol:water	7:6:6
8	Isopropyl alcohol:water	4:1
9	Water-saturated phenol	
10	Chloroform:methanol:17% ammonia	20:20:9
11	Butanol:acetone:diethylamine:water	10:10:2:5
12	Isopropyl alcohol:99% formic acid:water	20:1:5
13	*sec*-Butanol:methyl ethyl ketone:dicyclohexylamine:water	10:10:2:5
14	Phenol:water	75:25 w/w[b]
15	Butanol:glacial acetic acid:water	63:27:10
16	Pyridine:water	4:1
17	*tert*-Amyl alcohol:methyl ethyl ketone:water	3:1:1
18	Propanol:2 *N* ammonia	4:1
19	Isopropyl alcohol:2 *N* ammonia	4:1
20	*sec*-Butanol:*tert*-butanol:methyl ethyl ketone:water	1:1:1:1[c]
21	Pyridine:glacial acetic acid:water	30:10:7
22	Methanol:pyridine:glacial acetic acid:water	80:4:1:20

[a] From Pataki (P7), p. 78 with permission Ann Arbor Science Publishers.

[b] Gas phase equilibrated with 3% aqueous NH_4OH.

[c] +0.5% diethylamine.

ketone:acetic acid (2:3:1) 1st and *n*-butanol:acetic acid:water (12:3:5) 2nd (P23, V6); *n*-butanol:acetic acid:ammonia (28%):water (10:10:5:2) 1st and isopropanol: formic acid (80%):water (20:1:5) 2nd (P23, V6); double development with pyridine:dioxane:ammonia (28%):water (35:35:15:15) 1st and double development with *n*-butanol:acetone:acetic acid:water (35:35:10:20) 2nd (K14); secondary butanol:formic acid (80%); water (75:15:10) 1st and *n*-butanol:34% ammonia (67:33) 2nd (W14); *n*-butanol:acetone: ammonia (28%):water (10:10:5:2) 1st and isopropanol:formic acid (80%):water (20:1:5) 2nd (P23); pyridine:acetone:ammonia (28%): water (45:30:5:20) 1st and isopropanol:formic acid (80%):water (75:12.5:12.5) 2nd (S44, W13); *n*-butanol:pyridine:water (1:1:1) 1st and phenol (88%):ammonia (25%):water (10:0.8:1) 2nd (S38). Szeinberg, Szeinberg, and Cohen (S57) employed isopropanol:water (3:1) as the first solvent and butanol:acetic acid:water (12:3.5) as the second solvent in a multiple development system for the unidimensional separation of an amino acid mixture on paper. Culley (C16) found double development with butanol:acetic acid:water (12:3:5) provided the best resolution of serum amino acids and one which minimized the streaking due to protein. One of the more difficult separations to achieve, even with two-dimensional thin-layer chromatography, is the separation of leucine and isoleucine. Jones and Heathcote (J7), Pillay and Mehdi (P23), and Bujard and Mauron (B26) have claimed that their solvent systems, as listed above, can achieve this separation.

While monodimensional development with butanol:acetic acid:water (12:3:5) is the favored procedure for the mass screening of inborn errors of amino acid metabolism (C16, E13, J1, L9, L10, S19), it has been pointed out by others that the resolution of a mixture of 20 or more amino acids, such as is found in blood, urine, and other biological fluids or tissues is not as good as that obtained with two-dimensional chromatography (J1, P24, W3). Plöchl (P24) reported that normal unidimensional chromatograms of whole blood filter paper disks showed 6–7 spots, whereas two-dimensional chromatograms yielded 11–14 spots. Wadman and co-workers (W3) have stated that "for the screening of disorders of the amino acid metabolism, two-dimensional methods are preferable because of their high resolving power." Two-dimensional chromatography has been criticized as being too cumbersome to handle large numbers of samples except at unwarranted expense in labor, space, and equipment (J1). However, Wadman *et al.* (W3) showed that on newly developed cellulose-layered DC alufolie (E. Merck A. G., Darmstadt), amino acids appear as such compact spots that good separations can be obtained on micro scale (5 × 5 cm) chromatograms in less than half a day. The present authors have verified these findings, as will be

TABLE 2
COLOR REACTIONS OF AMINO ACIDS ON CELLULOSE TLC PLATES

Amino acid	Color		
	Ninhydrin–collidine	Isatin–Cd, immediate	Isatin–Cd, >12 hr at room temp
1. Cystine	Gray	Pale pink	Light blue
2. Cysteine	Gray	Pale pink	Violet
3. Cystathionine	Gray	Pale pink	Violet
4. Cysteic acid	Violet	Yellow	Pink
5. Phosphoethanolamine	Violet	N.C.[a]	Very faint pink
6. Argininosuccinic acid (major anhydride)	Violet	Pink	Pink
6′. Argininosuccinic acid (minor anhydride)	Violet	Pink	Pink
7. Ornithine HCl	Violet	Deep pink	Deep pink
8. Asparagine	Brown	Pink	Deep purple
9. Homocystine	Violet	Pale violet	Gray/brown
10. Arginine	Violet	Pink	Deep pink
11. Lysine	Violet	Deep pink	Deep pink
12. 1-Methylhistidine	Gray/brown	Violet	Blue
13. Histidine	Gray/brown	Violet	Purple
14. Carnosine	Light brown	N.C.	Faint brown
15. Taurine	Violet	N.C.	Very faint pink
16. Glutamine	Violet	Deep pink	Deep pink
17. Aspartic acid	Green	Pink	Violet
18. Citrulline	Violet	Purple	Pink
19. Methionine sulfoxide	Violet	Light pink	Violet
20. Methionine sulfone	Violet	Gray	Pink
21. Serine	Violet	Pink	Pinkish purple
22. Glycine	Golden brown	Pink	Pink
23. Hydroxyproline	Yellow	Pink	Blue
24. Glutamic acid	Violet	Purple	Pink ring
25. Sarcosine	Dark gray	Pale violet	Pale pink
26. Threonine	Violet	Deep pink	Deep pink
27. β-Alanine	Green	Bluish gray	Blue
28. Alanine	Violet	Violet	Purple
29. Proline	Yellow	Blue	Deep blue
30. Tyrosine	Gray blue	Bluish gray	Green
31. Tryptophan	Light violet	Blue	Gray
32. Ethanolamine	Violet	Violet	Pale pink
33. γ-Aminobutyric acid	Violet	Pink	Pink
34. α-Aminobutyric acid	Violet	Bluish gray	Violet
35. β-Aminoisobutyric acid	Violet	N.C.	N.C.

TABLE 2 (*Continued*)

Amino acid	Color		
	Ninhydrin–collidine	Isatin–Cd, immediate	Isadin–Cd, >12 hr at room temp
36. Methionine	Violet	Pink	Pink
37. Phenylalanine	Brown	Violet	Deep blue
38. Valine	Violet	Bluish violet	Deep pink
39. Leucine	Violet	Pink	Pinkish violet
40. Isoleucine	Violet	Pink	Pink

[a] N.C. = no color.

described in Section 4, with the exception that the solvent systems of White (W13) were utilized to shorten the development time for the two-dimensional runs to less than an hour.

2.3. Detection and Identification of Amino Acids by Selective Staining

Methods for the location of the separated amino acids on the developed chromatogram are also available in various textbooks or review articles dealing with chromatographic techniques (B16, B24, H1, H10, K7, P6, P7, P9, R1, S25, S37, T4). The most widely used general staining reactions for the visualization of amino acids are the relatively unspecific ninhydrin (C11, T5) and isatin (S3, S26) reagents. The addition of divalent metal ions such as Cu^{2+} (M13), Co^{2+} (W14), Cd^{2+} (A9, H7, H8), Zn^{2+} (B4), and Ni^{2+} (S37) to either ninhydrin or isatin stabilizes the colored complexes of the individual amino acids. In the case of the ninhydrin–cadmium reagent, the color changes from the usual violet color, obtained with ninhydrin alone, to bright red-orange colors with almost all amino acids. The uniformity of colors and the sensitivity of the reaction, i.e., 0.5–1.0 μg of each amino acid, makes this reagent particularly suited for the quantitative determination of the individual amino acids in a mixture, e.g., protein hydrolyzate, by means of colorimetry (V4) or densitometry (H6, H7, K7, S25).

Unlike the ninhydrin–cadmium reagent, isatin–cadmium (H7, H13) is a useful polychromatic reagent. By reacting with individual amino acids to produce a wide variety of colors, both immediately and after standing overnight, it greatly facilitates the unambiguous identification of a particular amino acid (Table 2). In contrast to the effect of divalent metal ions, the addition of organic bases such as collidine (M13,

O7, P23, W19), pyridine (S37), and cyclohexylamine (H3) to a ninhydrin solution produces a polychromatic effect, which, while it facilitates the identification of individual amino acids, also reduces the sensitivity of the ninhydrin reaction. The colors obtained for forty biologically important amino acids with the ninhydrin–collidine reagent (W19) are also listed in Table 2. Stubchen-Kirchner (S54) had modified the ninhydrin–collidine reaction by spraying the treated plate with ethanolic KOH and using the following criteria as a basis for differentiation: (a) color of pigment in daylight, (b) color of UV fluorescence, (c) time and conditions (moisture) of development of the fluorescence, and (d) eventual changes in color. He claims to be able to differentiate a number of amino acids and other amino compounds, including leucine and isoleucine. Some investigators (J1, S19) have used a combination of ninhydrin, isatin, and 2,4-lutidine in acetone in order to better differentiate the individual amino acids. Shih and Madigan (S26) have proposed the incorporation of the color reagent, e.g., isatin, into the developing solvent as a simpler and more sensitive staining procedure.

Three other general amino acid color reagents which have not been widely used are the Folin reagent (0.2% sodium-β-naphthoquinone-4-sulfonate in 5% sodium carbonate solution), alloxan, and the chlorine–tolidine reagent. The Folin reagent (M27) is also polychromatic, forming light blue, grayish green, or pink spots on a white background with different amino acids; it has a limit of detection between 0.5 and 1.0 μg (P7). Alloxan (S3) gives pink or reddish pink colors with practically all amino acids, but the limits of sensitivity of most amino acids are generally less than those for ninhydrin. The chlorine–tolidine reagent is reactive mainly with the peptide linkage and procedures for the identification of amino acids with this reaction have been published by Barrolier (B3), Killilea and O'Carra (K5), Pataki (P5) and von Arx and Neher (V6). The latter procedure is the easiest to utilize for the detection of amino acids on chromatograms but requires higher concentrations of amino acids for detection than do the other general amino acid reagents. Most amino acids give weak bluish black colors with this reagent and the exact colors produced for some fifty-four individual amino acids with this reagent as well as with ninhydrin (F2), isatin (V6), and ninhydrin–collidine (M13) are listed in Table 25 of Pataki's textbook (P7).

Other reagents are relatively specific for only one or several amino acids. These include the ninhydrin-bicarbonate reagent (P3) for phenylalanine and aspartic acid; Ehrlich's reagent (S33) for tryptophan; the nitrosonaphthol reagent (A1) for tyrosine; the periodic acid reagent (S37) for serine, hydroxylysine, and ethanolamine; the nitro-

prusside–piperidine reagent (S15) for threonine; phosphate reagent (R7) for phosphoethanolamine; the ninhydrin–Ehrlich reagent (J1) for citrulline and hydroxyproline; the nitroprusside–cyanide reagent (W17) for cysteine, cystine, and arginine; the *o*-phthalaldehyde reagent (P10) for glycine, histidine, tryptophan, and taurine; the vanillin reaction (W4) for ornithine, lysine, proline, hydroxyproline, and sarcosine; the acidified permanganate reaction (W1) for cysteine, cystine, methionine, tryptophan, and tyrosine; the *p*-anisidine reagent (S6) for histidine and tyrosine; the nitroprusside–ferricyanide reagent (R5, V6) for arginine; the iodine–azide reagent (A10, S30, S30a) for cystine, cysteine, and methionine; the sulfanilic acid (Pauly) reagent (B11, B26, E1, F12) for histidine and tyrosine; the oxine–hypobromite (Sakaguchi) reagent (A1, J6, R5) for arginine; the iodoplatinate reaction (E1, T5, W17) for cystine, cysteine, and methionine or, alternatively for thin-layer plates, the iodoplatinate–starch reagent (N6) for methionine; and the phenanthrenequinone reaction (Y1) for arginine; the *p*-aminobenzoic acid reagent (O9) for tyrosine, tyramine, histidine, and histamine and the bromophenol blue reagent (P4) for histidine and its derivatives.

A number of investigators (M6, R13) have published procedures which eliminate cyanide as the reducing agent in the detection of cystine or homocystine. In the attempt to gain the maximum qualitative information from a single chromatogram, a number of authors have proposed the use of the multiple-dip technique. Smith (S37) has suggested that isatin should be applied first, followed by ninhydrin, then by Ehrlich's reagent, whose strong acid erases the previous colors with the Erlich colors appearing against a yellow background. After removal of excess acid with cold air, the *p*-anisidine or sulfanilic acid or Sakaguchi reagent can be applied to the chromatogram. Other multiple-dip sequences have been proposed by Easley (E1), Easley *et al.* (E2), Jepson and Smith (J6), and Hsia and Inouye (H21). An exhaustive list of spray reagents for thin-layer chromatography, including those for amino acids, has been collected by Waldi (W4).

The composition and conditions of use for ten selective staining reagents, when used in conjunction with two-dimensional solvent systems (H9), permits the unambiguous identification of seventy-six compounds of biochemical interest. Twenty-seven of these compounds may be identified specifically by a single reagent which is capable of producing a unique color (or fluorescence) sequence when the spot is viewed under successive conditions.

3. Preparation of Samples for Analysis

Body fluids or aqueous extracts of tissue contain, in addition to free amino acids, proteins, peptides, lipids, water-soluble organic com-

pounds, e.g., carbohydrates, urea, and inorganic salts. The presence of protein, lipid, and salts have a deleterious effect on the resolution of the individual amino acids in a mixture such as is encountered in the analysis of blood or urine. The investigator is then faced with the problem of how to deproteinize and desalt a biological sample without loss or modification of the free amino acids present in the original specimen and without complicating the procedure, so as to make it too unwieldly for routine screening purposes.

3.1. Free Amino Acids of Blood or Plasma

For the analysis of the free amino acids of blood, there are two prevalent methods of collecting the sample. The technique popularized by Efron *et al.* (E11, E13) recommends collection of the blood sample on a filter paper card (S & S No. 903 paper) in such a manner that an area of the paper becomes saturated and soaks through to the other side. The proteins are denatured by autoclaving the paper at 250°F for 3 minutes to prevent diffusion of the blood during subsequent chromatography. This method of blood collection was employed by a number of investigators for the mass screening of aminoacidopathies (A2, C16, K3, L9, P24, S55, S57). However, Adriaenssens, Vanheule, and van Belle (A2), Plöchl (P24), and Stuber (S55) eluted the amino acids from filter paper disks containing the dried blood with ethanol:water (60:40 v/v) and spotted the eluate either on filter paper or on thin-layer plates. Culley (C16) chromatographed punched-out paper disks containing dried whole blood on thin-layer plates with ethanol:NH_3 (28%):water (18:1:1) in order to elute the amino acids from the disks onto the thin-layer plate and after drying the plate for a few minutes at 70°, separated the amino acids with *n*-butanol:acetic acid:water (13:3:5) as the developing solvent. A similar procedure was published by Szeinberg and associates (S57) except that isopropanol:water (3:1) was used as the eluting solution prior to chromatographic separation.

Scriver *et al.* (S19) collected whole blood in heparanized capillary tubes which were sealed with "Plasticine," centrifuged, and broken at the junction of plasma and cells; the plasma was removed for analysis. Culley (C16) stated that "collecting blood in capillaries is advantageous because the 20 μl of serum which subsequently is chromatographed provides a stronger amino acid pattern than do the $\frac{3}{16}$ in. blood discs" and "allows better evaluation of amino acid levels."

Some investigators (E9, E11, E13, L9, S19) develop the chromatogram with an acid–alcohol solvent directly from the applied blood or plasma. Removal of protein prior to the application of the blood or plasma to the chromatogram can be accomplished by means of organic

solvent precipitation (A2, C2, D5, W13), Sephadex G-25 columns (M2), and ethanol–chloroform–water extraction (G17, N1).

3.2. Free Amino Acids of Urine

The analysis of urine samples for free amino acids presents even greater difficulties than does that of blood. While lipids and protein are virtually absent in most specimens, urine contains many more ninhydrin-positive compounds and higher amounts of organic compounds and inorganic salts than are present in protein-free blood or plasma filtrates. The ideal approach toward the analysis of urine for its free amino acid content would be a desalting procedure, without change of its original composition, and the employment of a rapid two-dimensional technique for maximum resolution (K14). Efron (E11, E13), Levy *et al.* (L9), Stuber (S55), Berry *et al.* (B13), and White (W13) applied either a filter paper disk saturated with urine or a urine sample directly to the chromatogram prior to solvent development. Other investigators (B21, C1, D9, D15, J2, K14, P22, R2, W3) have desalted the urine sample by means of absorption of the amino acids onto a cation exchange resin (Dowex 50W X8), followed by removal of anions and neutral substances with water and elution of the bound amino acids with 2 *N* ammonia, evaporation *in vacuo,* and solution of the dried material in one-tenth volume of water. Consden, Gordon, and Martin (C11) utilized electrodialysis for the desalting of biological fluids. Spinella (S44) has proposed the prior treatment of urine with an electrolytic desalter (Tobal) which utilizes an anion–cation exchange membrane. Frentz (F15) has recommended the use of ultrafiltrates of urine for the thin-layer chromatographic analysis of the urinary amino acids.

Electrolytic desalting converts some of the arginine to ornithine and causes a loss of between 10 and 30% of histidine, lysine, methionine, proline, and tyrosine (S50). Losses of certain amino acids also occur with the ion-exchange desalting procedure (C12). These include threonine, proline, methionine, leucine, isoleucine, ornithine, and arginine, and losses of between 7 and 37% occur for these amino acids when present in a synthetic mixture.

The authors prefer the extraction technique first proposed for the amino acid analysis of blood by Giri *et al.* (G17), in which the protein-free alcoholic extract is mixed with three times its volume of chloroform, which extracts the alcohol, leaving an aqueous layer at the top of the chloroform–alcohol mixture. Proteins are removed during the alcohol precipitation step while lipids and other organic substances, such as urea and carbohydrates, as well as appreciable amounts of inorganic salts, remain in the chloroform–alcohol layer. Since the volume of the water

layer is equal to the original volume of blood or plasma extracted, the concentrations of the individual amino acids in the eluate used for spotting the chromatogram should be the same as that in plasma. However, it is to be expected that the nonpolar amino acids would exhibit appreciable solubility in the chloroform–alcohol layer as compared to the more polar amino acids. The losses of the individual amino acids, present in a synthetic mixture, with this extraction procedure was determined by means of automated ion-exchange resin column chromatography with the lithium citrate buffer system of Perry *et al.* (P16). The results obtained in this study are listed in Table 3 and indicate an average loss of about 20% for all 17 amino acids, but considerably larger losses, i.e., up to 50%, for some of the amino acids. The same extraction procedure used for plasma can be utilized for other biological fluids including urine, cerebrospinal fluid, and amniotic fluid.

3.3. Free Amino Acids of Other Biological Fluids and Tissues

The free amino acids of cerebrospinal fluid have been determined by Molnar and Sztaricskai (M14) using two-dimensional thin-layer

TABLE 3
Quantitative Ion-Exchange Column Chromatographic Analysis of an Amino Acid Standard Solution

Amino acid	Untreated standard (μmoles)	Treated standard (μmoles)	Recovery[a] (%)
Aspartic	0.245	0.226	92.3
Threonine	0.254	0.232	91.3
Serine	0.254	0.244	96.0
Glutamic	0.264	0.230	87.2
Proline	0.254	0.238	93.8
Glycine	0.244	0.236	96.8
Alanine	0.286	0.228	79.8
Valine	0.257	0.174	67.6
Cystine	0.241	0.246	102.0
Methionine	0.256	0.164	64.0
Isoleucine	0.247	0.132	53.5
Leucine	0.254	0.132	52.0
Tyrosine	0.240	0.190	79.2
Phenylalanine	0.240	0.108	45.0
Lysine	0.220	0.240	109.0
Histidine	0.223	0.238	107.0
Arginine	0.217	0.238	109.0

[a] Percentage of individual amino acids recovered from the water layer of a synthetic mixture after alcohol–chloroform–water extraction.

chromatography on cellulose-silica gel plates. The free amino acids of feces have been analyzed with a thin-layer chromatographic technique by Tancredi and Curtius (T3). The levels of free amino acids in human amniotic fluids, fetal and maternal serum were determined in years prior to 1969 with paper chromatographic methods (S7, W18). Since then most studies of amniotic fluids have utilized quantitative column chromatographic methods for the analysis of their free amino acid content (E14, L8, S5). Protein hydrolyzates have been analyzed qualitatively for their released amino acids by means of circular paper chromatography and the use of ninhydrin and isatin as staining reagents (O8). Methods for the analysis of the amino acid content of protein and peptide hydrolyzates by thin-layer chromatography have been reviewed by Pataki (P7). Heathcote and Haworth (H6, H7) have published a two-dimensional thin-layer chromatographic technique for the quantitative analysis of the amino acids in protein hydrolyzates which agree with those obtained by ion-exchange column chromatography.

Adriaenssens *et al.* (A3) have described a simple screening method for the study of amino acids in tissues using frozen slices. Efron (E10) has described methods for the extraction of free amino acids from urine, plasma, spinal fluid, sweat, and cellular material. Saifer (S1) has made a comparative study of seven published procedures for extracting free amino acids from brain tissue and concluded that perchloric acid extraction is the simplest and gives the most consistent results.With minor modifications this extraction method can be applied to amniotic fluid (S5), brain and other tissues (A11, S1), plasma, urine, cerebrospinal fluid, etc.

4. Procedure for the Chromatographic Separation and Qualitative Identification of Amino Acids in Serum and Urine

The qualitative, or semiquantitative method, described here uses the extraction procedure of Giri *et al.* (G17), the microscale thin-layer chromatographic method of Wadman *et al.* (W3), the two-dimensional solvent systems of White (W13) and the selective staining procedure of Heathcote *et al.* (H9) for the separation and identification of all the amino acids usually found in plasma, urine, or other human biological fluids and tissues.

4.1. Preparation of Sample (G17, N1)

To remove NH_3 from urine, add approximately 150 mg of dry Dowex-50x-4 resin (S4) to 2.0 ml of urine in a stoppered tube. Shake vigorously and let stand for about 15 minutes with occasional shaking. Centrifuge for 10 minutes at 3000 rpm and decant supernatant into tube.

To 1.0 volume of serum or resin-treated urine, add 3.0 volumes of absolute ethanol in a centrifuge tube. Mix well and allow to stand for 10 minutes. Centrifuge any precipitated protein at 2000 rpm for 10 minutes. Decant off the ethanolic extract and add 3.0 volumes of chloroform. Mix well and allow to stand for 5 minutes. The aqueous layer on top of the chloroform–alcohol mixture contains the free amino acids and is pipetted into a separate tube. Aliquots of the extract of 0.10 ml and 0.20 ml are used for quantitative total amino acid analysis (Section 4.4). The remainder is evaporated to dryness *in vacuo* (40°) and the dried material is redissolved in 0.1 volume of water for use in spotting the plates for TLC amino acid analysis (Section 4.5).

4.2. Collection of Blood in Capillary Tubes (N2)

Blood is drawn by heel or finger puncture into two 100 μl capillary tubes (Micropets, Clay-Adams, New York). The lancet used for puncture should allow big drops of blood to be obtained. One end of each tube is sealed with plastic clay (Seal-Ease, No. A-2960, Clay-Adams) and the blood is allowed to clot. The capillaries are centrifuged at 2000 rpm for 5 minutes to remove cells. The tubes are cut with a file slightly above the level of the cells. The tube containing the serum is held horizontally, and 50 μl is drawn up into a calibrated micropipette. The serum is ejected into a 0.4 ml conical microcentrifuge tube, 150 μl of ethanol is added and the contents are well mixed in a Vortex mixer; 100 μl of chloroform is added and the contents are mixed with a Vortex mixer, centrifuged, and the top water layer is removed for analysis. For shipment to other laboratories, the capillaries after clotting and centrifugation should be sealed at both ends and placed in corrugated paper slots.

4.3. Screening Test for Increased α-Amino Acids in Urine

Hyańek and Cafourková (H23) have published a simple procedure for detecting an increase in the α-amino acid content of urine samples. A piece of filter paper is moistened with a 3% KOH solution, a drop of resin-treated urine (morning sample) is added and the paper is dried at room temperature. The paper is then dipped into a ninhydrin solution (15 mg ninhydrin is dissolved in 5 ml 96% ethanol and then mixed with 5 ml ethylene glycol and 0.1 ml acetic acid) and heated at 100° for 5 minutes. The intensity of the spot is compared to that of a normal urine and/or with standards of 2, 5, 10, 20, and 50 mg of glycine N per milliliter. If a positive result (>10 mg/ml) is obtained, then a quantitative determination is performed as described in Section 4.4.

4.4. Total (Quantitative) Amino Acid Analysis (S4)

4.4.1. *Reagents and Apparatus*

a. Citrate buffer (0.2 *M*, pH 5.0). Prepare by dissolving 21.01 g of reagent grade citric acid in 200 ml of freshly boiled water. Add 1 *N* NaOH to attain a pH of 5.0 (approximately 195 ml) and dilute the solution to 500 ml with freshly boiled distilled water. Add a few crystals of thymol as a preservative and store in a refrigerator.

b. Methyl Cellosolve (2-methoxyethanol), obtainable from Union Carbide Corp., S. Charleston, W. Virginia. This reagent must be free of peroxides, the presence of which may be detected by adding 2 ml of solvent to 4 ml of fresh 4% KI. The yellow color of free iodine indicates the presence of peroxides, which may be removed by shaking the solution with KOH pellets and then distilling. A colorless to light straw tint with the iodide reagent indicates absence of peroxides.

c. Cyanide–Cellosolve. Dilute 5 ml of 0.01 *M* KCN (0.65 g of reagent grade potassium cyanide per liter of freshly boiled distilled water) to 250 ml with methyl cellosolve. The solution is stable for at least 1 month at room temperature.

d. Ninhydrin–Cellosolve, 5% solution of ninhydrin in cellosolve. Prepare fresh just prior to use.

e. Ethanol, 60% solution in water

f. Glycine standard solution. A 10 mg/100 ml glycine-N solution is prepared by dissolving 0.536 g of reagent grade glycine in a 1 liter of solution, from this standard, solutions containing 2.50 and 5.00 mg/100 ml glycine-N are made by dilution with distilled water.

g. Evaporation caps, to fit 15 × 150 mm Pyrex tubes

4.4.2. *Method*

Pipette 0.1 ml and 0.2 ml of each aqueous extract and of the glycine-N standards (2.5, 5.0, and 10.0 mg/100 ml) into test tubes (15 × 150 mm). Dilute the volume to 1 ml with distilled water. Measure out 1 ml of distilled water as the reagent blank. Add 0.5 ml of citrate buffer to each tube followed by 1 ml of KCN–cellosolve and 0.2 ml of ninhydrin cellosolve.

Mix by tapping and cover with evaporation caps. Place tubes in boiling water bath for 15 minutes and then cool in cold water for 5 minutes. Add 5 ml of 60% ethanol. Mix. Measure the colors in a photoelectric colorimeter with a Klett No. 56 filter or in a spectrophotometer set at 570 nm against a reagent blank.

4.4.3. *Calculations*

Urine:

$$\frac{\text{OD unknown}}{\text{OD standard}} \times 12^* \times \frac{\text{24-hr vol}}{100} = \text{amino acid N (mg/24 hr)}$$

Serum and CSF:

$$\frac{\text{OD unknown}}{\text{OD standard}} \times 12^* = \text{amino acid N (mg/100 ml)}$$

4.4.4. *Normal Values*

Urine (adults) = 91–600 mg amino acid N/24 hr or 25.3 ± 9.4 mmoles α-amino acids (as glycine)/24 hr

Urine (children) = 0.7–2.0 mg amino acid N/lb

Serum = 4.2–6.2 mg amino acid N/100 ml or 0.37 ± 0.03 mmole α-amino acids (as glycine)/100 ml

CSF = 0.6–1.6 mg amino acid N/100 ml or 0.077 ± 0.016 mmole α-amino acids (as glycine)/100 ml

4.5. Thin-Layer Chromatography (W3, W13)

6.0×6.0 cm TLC plates are cut to size from 20×20 cm aluminum sheets coated with 100 μ cellulose (E. Merck, Brinkmann Instr., Westbury, New York, No. 5537/0025). About 1 mm of the cellulose layer is scraped off each edge of the small plates; 0.5–5.0 μl of the extract, equivalent to 2.0 μg of amino acid N, is spotted with a 5-μl Hamilton syringe, or with a Drummond micropipette, at a point about 1 cm in from both the bottom and left edges. The spot is kept as small as possible by applying the sample in small amounts and drying with a heat gun. Two glass-covered staining dishes (Fisher No. 8-812A) are lined with Whatmann 3MM paper, and the aluminum racks (Fig. 1) are positioned in the bottom of the trays so as to hold the filter papers in place.

For the 1st dimension run, the solvent used is pyridine:acetone: NH_4OH (28%):H_2O (22.5:15:2.5:10 by volume). About 25 ml of this solvent is used to saturate the paper and to cover the aluminum rack at the bottom of the tray. The tray is covered and equilibration allowed to take place for at least 30 minutes prior to chromatography. The thin-layer plates are quickly inserted in the notches of the rack, the tray is covered, and a weight is placed on the glass cover. The solvent is al-

* The value obtained with a glycine standard is multiplied by a factor of 1.2 in order to account for the losses that occur during the extraction procedure (Table 3).

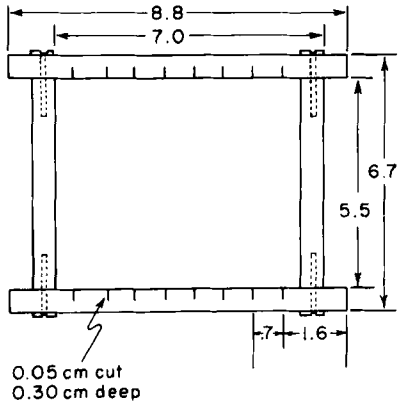

FIG. 1. Mechanical drawing of the aluminum rack placed in bottom of a staining dish to hold thin-layer microplates. Material: 0.6 cm stock bar aluminum; screws, 4-40 × ½ inch aluminum. Scale: full (centimeters).

lowed to ascend to the top of the plates, which takes about 10–15 minutes. The plates are removed and allowed to air dry at room temperature until solvent free. The plates are rotated 90° and placed in the tray of the second dish which was previously saturated with the solvent, isopropanol:formic acid:H_2O (27.5:6.25:6.25 by volume) in the same manner as described above for the first solvent. This solvent also takes about 15 minutes to reach the top of the plate. The plates are removed and dried with a hot air gun to remove all traces of the solvent. In general, it is advisable to leave the solvent in the dish between runs and to discard and replace it with fresh solvents so as to have the chamber saturated at all times.

4.6. STANDARD SOLUTIONS

Solution A. Contains 5 mmoles per liter each of leucine, phenylalanine, tryptophan, valine, proline, hydroxyproline, threonine, glycine, aspartic acid, and lysine in 0.1 *N* HCl.

Solution B. Contains 5 mmoles per liter each of isoleucine, methionine, tyrosine, alanine, glutamic acid, serine, arginine, histidine, and cystine in 0.1 *N* HCl.

The standard solutions are kept refrigerated when not in use. The standard solutions are neutralized with an equal volume of 0.1 *N* NaOH just before use.

Four microliters of each standard solution is spotted on a separate plate and chromatogrammed in the same manner as the unknowns. The unknown plates are then compared against the standard spots and

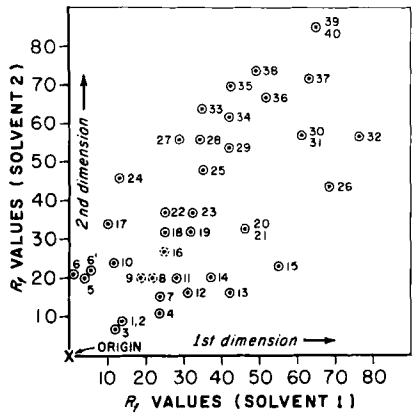

FIG. 2. Map of R_f values of amino acids commonly found in biological fluids or tissues as obtained with two-dimensional chromatography on cellulose TLC microplates. First dimension solvent: pyridine:acetone:NH_4OH (28%):H_2O (22.5:15:2.5:10 by volume). Second dimension solvent: isopropanol:formic acid:H_2O (27.5:6.25 by volume). R_f values of unknowns should be considered as only approximating those illustrated here and are best determined by relating their position on the chromatogram to more easily recognized spots, e.g., proline or hydroxyproline, or to standard solutions.

increases or decreases in any particular amino acid are noted. It is sometimes necessary to check any suspected increase by adding 2 μl of the standard, containing the amino acid in question, to the point of application containing 2 μl of the unknown prior to running the chromatogram. A number of amino acids which are present in urine or serum are indicated in Fig. 2 as dotted circles since they are too unstable to be included in the standard solutions.

4.7. Color Development

4.7.1. *Isatin–Cadmium Reagent* (H7)—(*Proline/Hydroxyproline*)

Dissolve 0.2 g of cadmium acetate in 20 ml of water to which 5.0 ml glacial acetic acid has been added (Soln 1). Dissolve 0.2 g isatin in 100 ml of acetone (Soln 2). Just prior to use, mix 1 volume of Soln 1 with 8 volumes of Soln 2. The plates are held at a corner with forceps and dipped into the color reagent. Color is developed by heating the plates at 70° for 10 minutes. Proline and hydroxyproline give blue spots. This reagent gives a variety of colors with different amino acids (Table 2).

A more sensitive test for hydroxyproline (0.1 μg/5 cm^2) is performed by first dipping the plate in 0.2% isatin in acetone, drying, and then spraying with Ehrlich's reagent (Section 4.7.5). Only hydroxyproline among 200 amino compounds gives a purple-red spot.

4.7.2. *Ninhydrin–Collidine Reagent* (W13)

Ninhydrin, 0.2% (w/v), is dissolved in ethanol and stored in a refrigerator. Just prior to use, 0.5 ml of 2,4,6-collidine is added to each 50 ml of the ninhydrin solution. Color development can be hastened by heating the dipped plate with a warm air gun. This reagent gives distinctive colors for a large number of amino acids (Table 2).

4.7.3. *Chlorine–o-Tolidine Reagent* (V6)

a. Sodium hypochlorite, 2% solution (Soln 1)
b. *o*-Tolidine, saturated solution in 2% acetic acid
c. Potassium iodide, 0.85% solution

Mix equal volumes of (b) and (c) just before use (Soln 2).

Spray the chromatogram lightly with Soln 1 and allow to stand at room temperature for 1–1.5 hours. The plate is then sprayed uniformly with Soln 2. The colors developed with this reagent for the amino acids commonly encountered in biological material are listed in Table 25 of Pataki's textbook (P7).

4.7.4. *Ninhydrin-Bicarbonate* (P3)—(*Phenylalanine/Aspartic Acid*)

The chromatograms are dipped into 0.2% ninhydrin dissolved in ethanol, dried at room temperature for 5–10 minutes, heated to 70° for 3 minutes, and sprayed with 1.0% $NaHCO_3$. The phenylalanine and aspartic acid regions appear as deep blue spots while the other amino acids remain purple and their intensities decrease on standing.

4.7.5. *Ehrlich's Reagent* (S37)—(*Tryptophan*)

Dissolve 1.0 g *p*-dimethylaminobenzaldehyde in 90 ml of acetone (Soln 1). Mix 9 volumes of Soln 1 with 1 volume of conc. HCl just before use. The plate is sprayed with the freshly mixed reagent and tryptophan gives a purple color within 20 minutes at room temperature.

4.7.6. *Nitrosonaphthol Reagent* (A1)—(*Tyrosine*)

α-Nitroso-β-naphthol 0.1% in ethanol (Soln 1). Mix 9 volumes of Soln 1 with 1 volume of conc HNO_3 just before use. The plate is sprayed with the reagent, allowed to damp-dry for 2–3 minutes at room temperature, and heated for 2–3 minutes at 105°. Tyrosine gives a red color which fades with excess heating.

4.7.7. *Periodic Acid Reagent* (S37)—(*Serine/Ethanolamine/Hydroxylysine*)

a. Periodic acid (H_5IO_6), 40% in H_2O–2 ml
b. Pyridine—2 ml
c. Acetone—100 ml

} Soln 1

d. 15 g ammonium acetate, 0.3 ml acetic acid, and 1 ml acetylacetone is dissolved in 100 ml ethanol (Soln 2).

The dry chromatogram is dipped in a fresh mixture of Soln 1. When the plate is visibly dry it is dipped in Soln 2. Substances yielding formaldehyde on periodate oxidation show a yellow-green fluorescence within 10–15 minutes followed by a visible yellow color within 30–60 minutes.

4.7.8. *Sodium Nitroprusside-Piperidine Reagent* (S15)—(*Threonine*)

The reagent must be freshly prepared.

e. Sodium nitroprusside, 5% in methanol—1 volume
f. Piperidine, 20% in methanol—1 volume
} Soln 3

The dry chromatogram is dipped in Soln 1 for serine (4.7.7). When dry it is dipped in Soln 3. Threonine produces a blue color on a pale blue background.

4.7.9. *Phosphate Reagent* (R7)—(*Phosphoethanolamine*)

Ammonium molybdate, 16 g, is dissolved in 120 ml of H_2O (Soln 1); 80 ml of Soln 1, 40 ml of conc HCl, and 10 ml of Hg is shaken with care for 30 minutes, and the excess Hg is filtered off (Soln 2). Conc H_2SO_4, 200 ml, is added carefully to the remaining 40 ml of Soln 1, followed by the addition of Soln 2. The cooled mixture is diluted with water to 1 liter.

This reagent reacts with phospholipids on the TLC plate immediately, without heating, to give blue spots on a white background. Phosphatidylethanolamine, lecithin, sphingomyelin, sulfatides, etc., produce a positive reaction in amounts greater than 2 μg. Phosphoric acid and its salts give no color in quantities up to 50 μg.

4.7.10. *Ninhydrin-Ehrlich* (J6)—(*Citrulline/Hydroxyproline*)

Use ninhydrin reagent without collidine (Section 4.7.2) followed by Ehrlich's reagent (Section 4.7.5) to detect citrulline and hydroxyproline. Citrulline gives a bluish pink color; hydroxyproline is pink.

4.7.11. *Nitroprusside-Cyanide Reagent* (W17)—(*Cysteine/Cystine/Arginine*)

Dissolve 1.5 g of sodium nitroferricyanide in 5 ml of 1 *M* H_2SO_4. Add 95 ml of ethanol and 10 ml of conc. NH_4OH and then filter (Soln 1). Soln 2: 0.4 *M* NaCN. Dip the chromatogram in Soln 1 and observe the color. Cysteine and other sulfhydryl (RSH) compounds are indicated by a bright red color on a yellow to green background. Arginine is orange on a yellow background and turns to blue gray. Spray the chro-

matogram with Soln 2. Cystine (and other R-SS-R disulfide compounds) appear as red spots. [For methods which have eliminated cyanide as the reducing agent, see references (M6 and R13).]

4.7.12. *o-Phthalaldehyde Reagent* (P10)—(*Glycine/Histidine/Tryptophan/Taurine*)

Dip chromatogram in 0.2% *o*-phthalaldehyde in acetone and heat at 50° for 10 minutes. Observe fluorescence without and with longwave UV light (365 nm). Glycine exhibited a green fluorescence in daylight with longwave UV light. Histidine and tryptophan showed yellow fluorescence under UV light. When the chromatogram was subsequently dipped into 1% alcoholic KOH and heated at 50° for 10 minutes, taurine gave a brown-red color (or red fluorescence), and glycine yielded a green color.

4.7.13. *Vanillin Reagent* (W4)—(*Ornithine/Lysine/Proline/Hydroxyproline/Sarcosine*)

Dip chromatogram into a solution of 2% vanillin in *n*-propanol and observe fluorescence. Ornithine gives a strong green-yellow fluorescence and lysine a weak fluorescence. Dip plate into 1% alcoholic KOH and allow to stand at room temperature. Ornithine gives an orange color, lysine a yellow color, and proline a salmon-pink color in about 30 seconds and then fade to yellow-brown colors. Proline, hydroxyproline, and sarcosine give pink spots on standing overnight.

4.7.14. *Permanganate Reagent* (W1)—(*Cysteine/Cystine/Methionine/Tryptophan/Tyrosine*)

Potassium permanganate solution. Add 5 ml of 2% $KMnO_4$ solution and 20 ml of 5% H_2SO_4 to 100 ml of distilled H_2O. Keep in a clean brown bottle. Spray the chromatogram with $KMnO_4$ solution until it has a uniform rose-violet color. After spraying with the acid-$KMnO_4$ solution, only cysteine, cystine, and methionine appear as white spots against a rose-violet background which turns brownish after about 15 minutes. The sensitivity of the acid-$KMnO_4$ reagent for the specific amino acids is about the same as that of ninhydrin.

4.7.15. *p-Anisidine Reagent* (S6)—(*Histidine/Tyrosine*)

a. *p-Anisidine*. To 10 ml of acetone, add 0.1 ml of conc HCl and dissolve 100 mg of *p*-anisidine in this mixture—1 volume
b. Amyl nitrite 1.0 *M* in acetone—1 volume
} Soln 1
c. Potassium hydroxide, 1.0% in ethanol (Soln 2)

Dip chromatogram in Soln 1. Air-dry and dip in Soln 2. Histidine and tyrosine give orange colors.

4.7.16. *Ninhydrin–Collidine–Ethanolic Potassium Hydroxide Reagent* (S54)—(*Leucine/Isoleucine/etc.*)

a. Ninhydrin–collidine. To a 0.4% solution of ninhydrin in isopropanol add 5% 2,4,6-collidine just before use (Soln 1).

b. Ethanolic potassium hydroxide—1% solution of KOH in ethanol (Soln 2).

The plate is sprayed with ninhydrin-collidine reagent and is allowed to remain at room temperature for up to 24 hr in order to bring out the color of the more poorly visible spots under fluorescent light, e.g., arginine and histidine. Phenylalanine and γ-aminobutyric acid fluoresce rapidly, i.e., right after the strips are dry, and glutamic acid after standing for a few hours. On the following day, the plate is exposed to water vapor for a few seconds and sprayed uniformly with the 1% alcoholic potassium hydroxide solution. The violet-blue color of most amino acids is converted into red or yellow to violet-red colors. The spraying is repeated until no blue hues are left, but too vigorous spraying should be avoided. Methionine (and its oxidation products) and leucine show a light blue fluorescence whereas isoleucine appears as a dark violet-red spot as do all the other amino acids which do not fluoresce with this reaction.

4.7.17. *Oxidation of Sulfur-Containing Amino Acids* (*Methionine/Cystine/Cysteine*) (W3)

To the dry sample spot, add 0.3 μl of 30% H_2O_2 containing 0.2 mg of ammonium molybdate per milliliter and bring to dryness before placing in the chromatographic solvent. Cystine and cysteine are converted to cysteic acid and methionine into methionine sulfone, and these amino acids will then appear in a different region of the chromatogram (Fig. 2).

4.7.18. *In Situ Conversion to Dinitrophenyl* (*DNP*) *Amino Acids* (P7)

a. Buffer solution (pH 8.8). Dissolve 8.4 g $NaHCO_3$ in 50 ml of distilled water. Add 1 *N* sodium hydroxide to pH 8.8 and dilute to 100 ml with distilled water.

b. 2,4-Dinitrofluorobenzene, 10% (w/v) solution in methanol

After the completion of the solvent development in the first dimension, e.g., *n*-butanol:acetic acid:water (12:3:5) or pyridine:acetone:NH_4OH

(28%):H_2O (22.5:15:2.5:10), the dried chromatogram is sprayed with the buffer solution. It is then oversprayed with the dinitrofluorobenzene solution. The chromatogram is sandwiched between two glass plates held apart by strips of polyethylene and kept at 40° for 1 hour in the dark. The chromatogram is cooled to room temperature and exposed to HCl vapors for 10 min, and the plate is dried. The yellow DNP-amino acids can then be separated with the "toluene" solvent, cf. Section 6.2.5.

5. Other Techniques for the Separation of Free Amino Acids in Biological Materials

5.1. High Voltage Electrophoresis

Smith (S37) has stated that "electrophoresis is a suitable procedure (for amino acid separations) as it is quick, it avoids the need to desalt and is also applicable to whole blood and urine." High voltage electrophoresis on paper has been extensively used for the separation of amino acids and peptides in biological fluids (A9, E10, F7, H12, J11, K2, N1, P18). Review articles on high voltage electrophoresis have been published by Criddle *et al.* (C15), Blackburn (B16), Efron (E10), Grassini (G23), and Ritschard (R3).

Farrelly and Watkins (F4) have used high voltage electrophoresis of unmodified urine or deproteinized serum for the rapid separation of fourteen amino acids in one direction on a thin-layer plate. Evered and Dando (E16) have employed low voltage electrophoresis for one way separation of amino acids on Whatman No. 1 paper using various buffer solutions. They stated that only the acidic and basic amino acids, β-amino acids, and cystine could be separated completely from a complex mixture such as blood or urine. Scherr (S10) and Stevens (S52) have also used low voltage electrophoresis for the unidirectional separation of amino acid mixtures on cellulose acetate strips.

Improved separation of amino acids in biological material is obtained by combining electrophoresis in the first dimension with chromatography in the second dimension either on paper (E10, P18, S34) or on thin-layer plates (A5, F1, F4, H13, K2, M22, M23, M24, N7, R3, S49, T6, W5). This method has been used mainly for preparing fingerprints or peptide maps of proteolytic digests of proteins. Compared to the high voltage equipment and cooling systems required for paper (K2), thin-layer electrophoresis and chromatography utilizes similar equipment, but with a saving both of time and in the amount of material needed for analysis (R3). The exact conditions under which the thin-layer electrophoretic and chromatographic runs are performed are listed in Table 23 of Pataki (P7), and the R_f values of various amino acids obtained with

this technique (N7) on cellulose layers are given in Table 24 of Pataki (P7).

The advantages of using electrophoresis for separation in the first dimension as compared to the usual two-dimensional chromatographic separation have been listed by Efron (E10) as follows: (a) The electrophoretic run is more rapid than chromatographic solvent development. (b) Electrophoresis eliminates the need for desalting by moving small ions off the paper. (c) Small amounts of amino acids are more discernible because round compact spots are formed. (d) There is better separation of certain amino acids, e.g., methionine from valine; cystathionine, argininosuccinic acid, and phosphoethanolamine are resolved from each other.

In the author's opinion, high voltage electrophoresis, in spite of its potential advantages is a technique which requires relatively expensive equipment of high potential hazard and is therefore unsuited for the large-scale screening of aminoacidopathies in most routine clinical chemistry laboratories.

5.2. Ion-Exchange Resin Column Chromatography

The literature on the application of ion-exchange resin column chromatography (M16) to amino acidurias prior to 1959 has been thoroughly documented by Jagenburg (J2) and Bigwood *et al.* (B14), and the more current literature by Blackburn (B16) and Peters and Berridge (P16a). The original Moore and Stein (M16) procedure has undergone a series of modifications by Spackman, Stein, and Moore (S43), Piez and Morris (P21), Hamilton (H2), Dickinson, Rosenblum, and Hamilton (D14), and Efron (E8). By means of single column cation-exchange chromatography (D14, E8) and a semiautomated system (Technicon Corp., Tarrytown, New York), all the physiologically important amino acids, with the possible exception of glutamine and asparagine, could be separated and analyzed quantitatively with sodium citrate buffer gradient elution. Recently, Perry and co-workers (P16) and Peters *et al.* (P17) have shown that the substitution of lithium citrate for the sodium citrate buffer system has the advantage of resolving asparagine and glutamine without loss of resolution of other acidic and neutral amino acids. When one is interested solely in the quantitative analysis of one or two amino acids present in a complex mixture, e.g., tyrosine and phenylalanine, the analysis can be performed in less than 45 minutes by the combined use of small, water-jacketed columns and single buffer elution pumped through the column at high flow rates (M4).

It is the author's opinion that ion-exchange column chromatography,

even with the short column, rapid-flow rate procedure (M4), is unsuited for the large-scale screening of segments of the population for defects of amino acid metabolism. Its main utility is for the confirmation, in a quantitative form, of possible abnormalities obtained with simple, rapid microscale chromatographic screening methods such as those described in Section 4 of this review article. For this purpose we have used the single column, cation-exchange, semiautomated Technicon system (S5) with the lithium citrate buffer system of Perry *et al.* (P16) as the preferred procedure for various biological fluids and tissues.

6. Preparation and Separation of Amino Acid Derivatives

It has been repeatedly stressed throughout this review paper that a presumed positive finding with respect to one or more amino acids should be confirmed with supplementary procedures such as high voltage electrophoresis-chromatography or cation-exchange column chromatography with buffer elution. Since many clinical chemistry laboratories do not possess expensive equipment of this nature, an alternative procedure consists in the preparation of suitable derivatives. These derivatives can then be separated and identified, in comparison with known standards, by means of relatively inexpensive thin-layer chromatographic equipment. As the amino acid derivatives are separated on different absorbent media and with different solvent systems than are the unmodified amino acids, the coincidence of an increased amount of an amino acid and of its derivative is good supportive evidence that the increase is a real one.

6.1. *N*-(2,4-Dinitrophenyl)-Amino Acids

The preparation and the chromatographic separation of dinitrophenyl (DNP) derivatives of amino acids has been described in review articles by Blackburn (B16), Biserte *et al.* (B15), and Randerath (R1), Brenner *et al.* (B24), Kirchner (K7), Pataki (P7), and Mills and Beale (M7). The preparation of such derivatives eliminates the interference of salts present in biological fluids during chromatography. The conversion can be performed in dilute solution, is simple and rapid and few interfering components have been noted (P7). The method is very sensitive, i.e., quantities of 0.2 μg can be readily recognized in transmitted light on two-dimensional chromatograms, and since the derivatives are colored their quantitative analysis by colorimetry is facilitated (M7). One disadvantage is that the DNP-amino acids are sensitive to light in the dissolved state and that all manipulations should be performed in subdued light.

6.2. Dinitrophenylation of Amino Acids in Urine or Serum (P2)

6.2.1. *Urine*

To 25.0 ml of freshly voided urine is added 5 *N* NaOH with stirring until it is just alkaline to phenolphthalein paper. The solution is filtered through filter paper, and to 20.0 ml is added 5 ml of buffer (8.4 g $NaHCO_3$ dissolved in about 80 ml of H_2O; the pH is adjusted to 8.8 with 5 *N* NaOH and diluted to 100 ml with H_2O). To this mixture is added 2.0 ml of freshly prepared 2,4-dinitrofluorobenzene (10% in ethanol w/v) and 40.0 ml of ethanol. Mix well and keep in a dark cabinet with occasional stirring for about an hour. The reaction mixture is cooled to room temperature and the pH adjusted with 5 *N* NaOH to about 12.0. The mixture is transferred to a 100-ml separatory funnel and extracted three times with 10-ml portions of ether to remove excess reactant; the ether phases are discarded.

6.2.2. *Serum or Plasma*

Deproteinize 2–5 ml of serum which has been diluted with an equal volume of water by adding 1 volume of 20% trichloroacetic acid. The precipitate is centrifuged and washed once with 10% TCA; the combined solutions are evaporated under vacuum to dryness, the residue is taken up in 20 ml of distilled water, and 5 ml of pH 8.8 $NaHCO_3$ buffer is added. The remainder of the procedure is the same as was described above for the urine sample.

6.2.3. *Ether-Soluble DNP-Amino Acids*

The alkaline solution is adjusted with 6 *N* HCl to the blue color of Congo red paper and extracted six times with 10-ml portions of ether. The ether extracts are combined and evaporated to dryness under vacuum; the residue is taken up in 1.0 ml of acetone or ethyl acetate.

6.2.4. *Water-Soluble DNP-Amino Acids*

The remaining water layer is extracted six times with 10-ml portions of *n*-butanol:ethyl acetate (1:1) and the combined organic solutions are evaporated to dryness under vacuum. The residue is dissolved in 1 ml of ethyl acetate:acetic acid:*n*-butanol (100:1:99). The extraction solvent should be completely evaporated from the layer before development of the chromatogram. This extract contains: α-DNP-arginine, α-DNP-cysteic acid, α-DNP-histidine, mono-DNP-cystine, ϵ-DNP-lysine, *O*-DNP-tyrosine, di-DNP-histidine. All other DNP-amino acids are included in the ether-soluble extract.

6.2.5. *Chromatography*

The water-soluble DNP-amino acids can be separated on silica gel G plates using *n*-propanol:NH_3 (34%) (7:3) as the developing solvent (B22, B23). Although DNP-arginine and ε-DNP-lysine are not completely separated, they can be detected by differences in the color produced by their reaction with ninhydrin. Dessauer *et al.* (D8) have employed four solvent systems for the two-dimensional separation of both polar and nonpolar DNP-amino acids on silica gel plates. The exact solvent systems employed, and the R_f values obtained for ether-soluble DNP-amino acids are listed by Randerath (R1). Munier and Sarrazin (M21) have employed cellulose layers without binders for bidimensional chromatography of DNP-amino acids. Good separation of a mixture of DNP-amino acids is obtained by using a "toluene" solvent [*toluene:chlorethanol:pyridine:5 N ammonia* (10:6:3:1)] in the first dimension and saturated ammonium sulfate:water:sodium dodecyl sulfate (100 ml:700 ml:0.576 g) in the second dimension. DNP-aspartic acid and DNP-glutamic acid which are not separated with this solvent system can be resolved if isobutanol:acetic acid:water (100:6:20) is used for the second dimension solvent. An alternative method for the separation of these two DNP-amino acids is the use of high voltage electrophoresis (F9, M15, M21) in which electrophoresis is carried out in the first dimension in pH 6.5 pyridine–acetic acid buffer and ascending chromatography in the second dimension with the "toluene" system. However, neither system successfully resolves DNP-leucine from DNP-isoleucine. Polyamide layers provide a simple and rapid absorbent for the separation of 31 DNP-amino acids including leucine and isoleucine and glutamic and aspartic acids. According to Wang and co-workers (T7, W7, W8, W9) the most useful solvent is benzene:acetic acid (80:20) in the first dimension and *n*-butanol:acetic acid (90:10) in the second dimension. Although DNP-methionine and DNP-phenylalanine are not resolved in this system, they can be separated by using formic acid (90%):water (50:50) as the solvent system for the second dimension. An excellent, earlier review article by Biserte *et al.* (B15), although confined to the use of paper chromatography for the preparation and separation of the DNP-amino acids, contains much experimentally valuable information.

6.2.6. *Visualization*

All DNP-amino acids, except for *o*-DNP-tyrosine and *im*-DNP-histidine, are yellow and are readily visible. They absorb longwave ultraviolet light (360 nm) and appear as dark spots on the chromato-

gram. Only those amino acids with free amino groups react with ninhydrin, these are: *o*-DNP-tyrosine (gray-brown), *im*-DNP-histidine (gray-brown), and ε-DNP-lysine (purple). The isolated DNP-amino acid can also be identified from its infrared pattern (K6).

6.2.7. *Quantitation*

The DNP-amino acids, after separation into individual spots on the chromatographic plate, can be eluted from the scraped off area by adding 4 ml of water to the material in a small tube. The tube is heated at 50° in a water bath for 15 minutes and centrifuged to clear the solution. The color is read against known standards at 360 nm. Direct estimation of DNP-, PTH-, and DANS-amino acids separated on the thin-layer plate can be performed by fluorescence and fluorescence quenching techniques (P8). It is also possible to convert unmodified amino acids, separated on a silica gel G chromatographic plate, into DNP-amino acids by *in situ* conversion as was described in Section 4.7.18. The DNP-derivatives can then be developed in the second dimension and the spots analyzed quantitatively.

6.3. 3-Phenyl-2-Thiohydantoins (PTH) Derivatives

Preparation and chromatography of the PTH-amino acids has been reviewed by Blackburn (B16), Randerath (R1), Beale (B7), Pataki (P7), Brenner *et al.* (B24), and Kirchner (K7). Although the phenylisothiocyanate reaction, first introduced by Edman (E3) and later modified by Sjöquist (S30, S30a) and Cherbuliez *et al.* (C4), has been widely utilized for N-terminal end group (E15, F11) and amino acid sequence (S29, S32) analysis of peptides and proteins, it is also suited for the preparation of the PTH-amino acids. Ilse and Edman (I1) found that practically all phenylthiocarbamyl amino acids, formed in the first reaction step with phenylisothiocyanate, are almost quantitatively converted into the corresponding PTH-amino acids if the reaction is carried out at pH 1 for 60 minutes at 80°. However, certain amino acids are not readily formed with the general procedure (C4, S30, S30a), and special methods are required for the preparation of PTH-tryptophan, PTH-serine, PTH-threonine, PTH-cystine, PTH-asparagine, and PTH-glutamine (P7).

Five solvent systems useful for the chromatographic separation of 32 PTH-amino acids on silica gel G thin layers with one-dimensional runs are listed in Table 44 of the textbook by Pataki (P7). According to Cherbuliez *et al.* (C3, C3a, C5), when three chromatograms are run simultaneously in one dimension on silica gel G plates with the following solvent systems: (a) chloroform:isopropanol:water (28:8:1),

(b) ethyl acetate:pyridine:water (7:2:1), and (c) chloroform:ethyl acetate:water (6:3:1), then out of 17 PTH-amino acids only PTH-asparagine and PTH-glutamine are not separated. A better multi-development, one-dimensional system is that proposed by Jeppsson and Sjöquist (J4) in which heptane:propionic acid:ethylene chloride (58:17:25) is employed as the first solvent and heptane:*n*-butanol:formic acid (75%) (50:30:9) as the second solvent on silica gel plates. This multisolvent system provides good separation of practically all PTH-amino acids, but the position of any particular amino acid on the chromatogram should be checked with known PTH-amino acid standards (Mann Research Labs, New York, New York). Wang *et al.* (W10) separated 16 PTH-amino acids with two-dimensional chromatography on polyamide layers. PTH-amino acids can be visualized by means of the chlorine-tolidine reaction as described by Brenner *et al.* (B23) except that the chlorine treatment is extended to 25 minutes (P7). A preferable detection method is the iodine–azide–starch reaction described in the review article by Beale (B7). With this procedure, the PTH-amino acids appear as white spots against a dark blue background within a few minutes after application, except for PTH-proline and PTH-hydroxyproline which may not be visible for several hours. A butylhypochlorite–iodide–starch reagent (M3) has also been recommended as a location agent for PTH-amino acids. Since in a number of solvent systems (B5), PTH-glycine is not well separated from monophenylthiourea, a reaction by-product, a simple method for the detection of the glycine derivative is useful. This can be accomplished by spraying the region of the plate where PTH-glycine should be present lightly with water and exposing the sprayed area to fumes from an open ammonia bottle. Amounts of PTH-glycine greater than 0.08 μg show a deep-red stable spot (P7). If the PTH-amino acids are chromatographed on a combined silica gel G (30 g)–Mn-activated zinc silicate (0.06 g) layer (P7) and the plate is examined with UV light (254 nm), the separated amino acids appear as dark spots against a greenish-fluorescent background. This is a useful procedure for determining the position of a particular amino acid on a chromatographic plate prior to choosing the most suitable second-dimensional solvent. Quantitative analysis of the PTH-amino acids can also be performed by means of UV measurements at 260 nm (S30) and by direct spectrophotometric measurements on the silica gel layer (P8, S32).

In the author's opinion, the use of PTH-derivatives for the separation and the identification of free amino acids, as a supplemental procedure to two-dimensional thin-layer chromatographic analysis, has no distinct advantage over the use of DNP-derivatives, but has a number

of decided disadvantages. These include the greater technical difficulties encountered in the preparation of the PTH-amino acids and the fact that a number of biologically important amino acids need special procedures for their preparation. The major usefulness of the phenylisosthiocyanate reaction is for the stepwise degradation of peptides and proteins for which Edman and Begg (E4) have developed an automated procedure.

6.4. Other Derivatives of Amino Acids

Other reagents which have been employed for the preparation of amino acid derivatives are: (a) 1-dimethylaminonaphthalene-5-sulfonyl chloride (G25) which yields dansyl- or DNS-amino acids or DNS-peptides (B7, B16, G24, G25, G27, K7, P7, Z1). Solvent systems for the separation of DNS-amino acids have been published by Arnott and Ward (A8), Deyl and Rosmus (D11), Morse and Horecker (M19), Seiler and Wiechmann (S24), Woods and Wang (W21), and Zanetta *et al.* (Z1). This reagent is about 100 times as sensitive as dinitrofluorobenzene for the detection of amino acids with UV light (360 nm) and may be determined quantitatively by fluorescence measurements (P8). (b) Bergmann and Bentov (B9) have used 2,4-dinitrofluoroaniline to convert amino acids or peptides to dinitroaminophenyl (or DNAP-) derivatives. Since they contain an aromatic amino group they can be diazotized and coupled to a dye for increased sensitivity and visualization (P7). Solvent systems for the separation of the DNAP-amino acids have been described by Deyl *et al.* (D12). (c) The separation of iodobenzene-*p*-sulfonylamino acids by thin-layer chromatography has been described by Cole and Fletcher (C9). (d) Thin-layer chromatographic separation of dinitropyridyl and nitropyrimidyl derivatives of all the common amino acids with six solvent systems in one dimension have been reported by Dibello and Signor (D13). (e) The original procedure of Satake *et al.* (S8) for the preparation of trinitrophenyl derivatives of amino acids and peptides was utilized by Nitecki *et al.* (N5) for the thin-layer chromatographic separation of amino acid mixtures. This latter procedure was applied by Philpott (P20) as a semiquantitative screening procedure for the amino acids in urine and plasma. (f) The derivatization of amino acids to their *N*-trifluoroacetyl (*N*-TFA) *n*-butyl esters was first investigated by Zomzely *et al.* (Z2). A procedure for their separation by means of thin-layer chromatography has been published by Mussini and Marcucci (M26). Gehrke and his co-workers (G5, G6, R4) have described micro methods for the sample preparation and the instrumental and chromatographic requirements for quantitative gas–liquid chromatographic (GLC) analysis of protein hydrolyzate

amino acids as their *N*-TFA *n*-butyl esters. The GLC method was adapted by them (Z3) to the quantitative analysis of amino acids in complex biological substances, e.g., blood plasma and urine. The results obtained were in good agreement with those determined by the more conventional automated, ion-exchange column chromatographic procedure. In some inborn errors of metabolism the enzymatic block not only results in the accumulation of an amino acid, but also in the formation of large amounts of other metabolites produced by alternative metabolic pathways. In the case of phenylketonuria, this results in the urinary excretion of such phenylalanine metabolites as phenylpyruvic acid, phenyllactic acid, *o*-hydroxyphenylacetic acid and phenylacetic acid. By means of the conversion of these organic acids to their trimethylsilyl derivatives, Vavich and Howell (V5) obtained urinary GLC patterns which were distinctive for phenylketonuria.

For the most part the derivatives described in this section have found their greatest utility for N-terminal end group analysis, for the fingerprinting of peptides and for the sequential analysis of protein amino acids (B7, Z1).

7. Interpretation of Amino Acid Data

In order to be able to detect an abnormal chromatogram of a particular biological fluid, it is necessary not only to have a clear idea of what a normal chromatogram of the fluid looks like, but also to be aware of the variations in the pattern which may occur within the normal range. The R_f values occupied by the amino acids most likely to be found in biological fluids and tissues, when analyzed with the procedure described in Section 4, are illustrated in Figs. 2 and 3. A typical pattern obtained for a normal adult urine sample is shown in Fig. 4, although it is likely that some of the amino acids which are present in readily detectable amounts in one sample, i.e., histidine, taurine, methyl histidine, etc., may be absent from another specimen. The urinary amino acid pattern not only varies among different individuals (B10, W15), but also in the same individual, with such factors as age, sex, and dietary regimen (B10, J2, R9). However, neither the total quantity nor the distribution of the amino acids in normal urine can be closely correlated with the dietary intake of protein (S51) apart from 1-methylhistidine, which is related to the amount of meat in the diet (D4) and β-aminoisobutyric acid, which may be genetically determined (H4). Differences from the normal urinary pattern may also be exhibited by patients who are pregnant (W6), with histidine and threonine being increased, or who are undergoing drug therapy. Normal infants may also exhibit a transitory aminoaciduria of acidic and neutral amino acids (D17) which

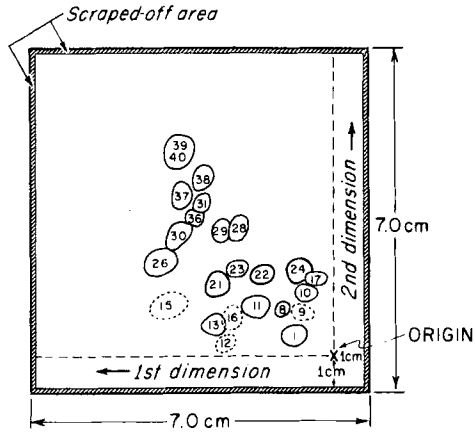

FIG. 3. Map of R_f values of the amino acid standard solution separated on TLC microplates as described in Section 4.5. The numbers correspond to the amino acids listed in Table 2, and the dotted circles to the position of some amino acids commonly found in biological fluids which are not present in the standard solution.

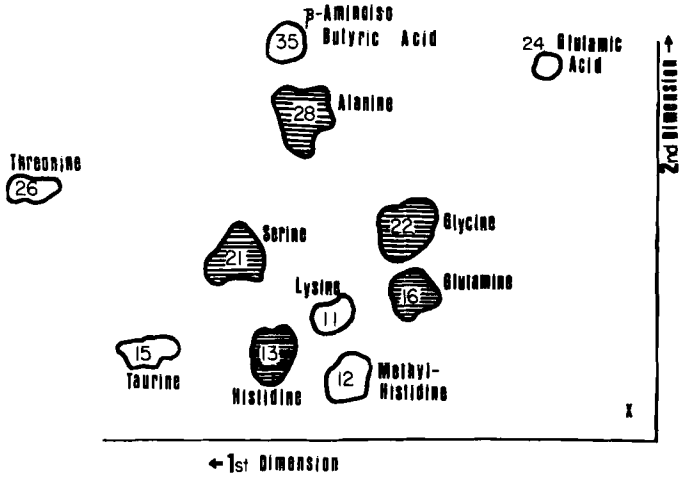

FIG. 4. Composite diagram of an amino acid chromatogram of urine from normal individuals. The specimen was applied at the origin (X) and developed as described in Section 4. The solvent systems employed were the same as those listed in Fig. 2, and the completed chromatograms were visualized with ninhydrin-collidine. Not all the amino acids shown need be present in any one sample. The numbers correspond to the amino acids listed in Table 2.

is due to incomplete maturation of transport mechanisms involved in amino acid reabsorption from the glomerular filtrate (M8).

The pattern can be influenced by the nature of the sample which may have been modified by enzymatic action or bacteriological contamination (E11). The pattern will also differ according to the extraction procedure used in the preparation of the sample for analysis (S1) and with the chromatographic technique employed for the separation and detection of the amino acid mixture. If a presumed "abnormal" amino acid pattern is observed in a urine sample, the patient should be taken off drug therapy for at least 3 days and a second sterile, morning urine sample, together with a blood sample, should be obtained and the determinations be repeated (E11). A positive finding in this instance should then be verified by a more quantitative technique, e.g., column chromatography (Section 5.2), gas–liquid chromatography (Section 6.4), or by the preparation and chromatographic analysis of amino acid derivatives (Section 6).

Abnormalities of the chromatographic pattern are not generally associated with those amino acids which are present in sufficient amounts in a normal biological fluid to show definitive spots on the chromatogram (Fig. 4). A moderate excess of a normal fluid would not in itself cause an abnormal pattern, but it is necessary that the volume of fluid applied to the chromatogram be referred to some measurable standard. Some standards which have been proposed for urine by various investigators are: (a) a volume related to a definite amount of total nitrogen; (b) a volume related to a definite amount of creatinine; and (c) a volume excreted in a timed interval, e.g., 2-second volume. The author prefers to relate the amount of applied sample to the amino acid-N content which is determined as described in Section 4.4. The amount of normal biological fluid to be applied to the chromatogram should be equivalent to about 2.0 μg of amino acid-N. Normal adults excrete about 200 mg of α-amino acid-N in 24 hours, and the amount is relatively independent of urine volume or dietary intake of protein.

7.1. Amino Acid Patterns of Normal Biological Fluids

The normal pattern of the urine is characterized by the presence of five amino acids which give prominent spots. These are glycine, serine, alanine, glutamine, and histidine. Moderate amounts to traces of lysine, threonine, glutamic acid, taurine, methylhistidine, and β-aminoisobutyric acid occur in some normal samples (Fig. 4). Soupart (S42) and Peters *et al.* (P17) have published data on the urinary excretion of the free amino acids by normal human subjects which provide a useful compilation of the current knowledge in this field (Table 4).

TABLE 4
QUANTITATIVE LEVELS OF FREE AMINO ACIDS IN NORMAL HUMAN AMNIOTIC FLUID, BLOOD PLASMA, URINE, AND CEREBROSPINAL FLUID (CSF)

Amino Acids[a]	Amniotic fluid[b] (mg/100 ml)	Plasma[c]		Urine		CSF[f]
		Newborns (mg/100 ml)	Adults (mg/100 ml)	Newborns[d] (mg/g creatinine)	Adults[e] (mg/g creatinine)	Adults (mg/100 ml)
Phosphoethanolamine	—	—	—	43.7	—	—
Taurine	1.00	1.76	0.83	427.5	59.2	0.073
Aspartic acid	0.22	0.11	0.22	Tr	3.4	0.100
Hydroxyproline	0.45	—	—	18.3	—	—
Threonine	2.03	2.59	1.94	25.0	14.6	0.370
Serine	0.39	1.72	1.18	46.2	26.7	0.270
Asparagine	0.41	—	0.57	—	7.0	—
Glutamic acid	1.57	0.76	0.86	7.4	2.9	0.106
Glutamine	3.25	11.16	8.30	64.2	49.3	6.360
Proline	1.97	2.13	2.71	9.2	—	0.007
Glycine	1.18	2.58	1.74	96.0	65.9	0.037
Alanine	3.26	2.94	3.07	28.5	12.8	0.248
Citrulline	0.11	0.28	0.53	—	2.6	—
α-Aminobutyric acid	0.10	0.15	0.17	Tr	1.8	0.040
Valine	1.65	1.60	1.99	4.7	3.8	0.207
Cystine (plus cysteic acid)	1.52	1.47	1.77	49.5	8.3	0.024
Homocitrulline	—	—	—	2.7	—	—
Methionine	1.25	0.44	0.32	Tr	4.6	0.045
Isoleucine	0.42	0.52	0.71	13.1	1.9	0.063
Leucine	0.87	0.95	1.32	3.9	4.1	0.168
Tyrosine	0.82	1.26	0.91	7.2	12.8	0.129
Phenylalanine	0.82	1.30	0.95	6.6	6.5	0.129
β-Aminoisobutyric acid	—	—	—	14.4	10.0	—
Hydroxylysines	—	—	—	6.5	—	—
γ-Aminobutyric acid	0.02	—	—	Tr	—	—
Tryptophan	0.06	0.65	0.98	—	11.1	—
Ethanolamine	0.12	0.32	0.01	110.4	—	0.057
Ornithine	0.63	1.21	0.92	2.6	1.3	0.057
Lysine	3.05	2.93	2.54	40.9	21.0	0.340
1-Methylhistidine	—	—	—	13.5	21.8	—
Histidine	1.23	1.19	1.24	27.9	89.3	0.344
3-Methylhistidine	—	—	—	18.6	26.8	—
Carnosine	—	—	—	15.8	—	—
Homocarnosine	0.10	—	—	—	—	—
Arginine	0.62	0.94	1.43	7.0	1.7	0.204

The amino acid concentrations of normal plasma or serum, as obtained with quantitative, cation-exchange chromatography has been compiled in the review article of Rosenberg and Scriver (R9). The extracellular and plasma free amino acids are in equilibrium with the whole body, and the process of cellular transport regulates the equilibrium in such a manner that the circulating plasma amino acid concentrations exhibit only minor fluctuations (F5). The eight amino acids present in highest concentration in plasma are glutamine, alanine, glycine, proline, valine, lysine, threonine, and serine. All the other amino acids usually found in protein hydrolyzates are also found in plasma (S42). Large amounts of taurine and smaller amounts of hydroxyproline have been reported in neonates and in young children.

The concentrations of the free amino acids present in amniotic fluid obtained early in pregnancy (8th to 22nd weeks of gestation) by transabdominal amniocentesis were determined by quantitative cation-exchange chromatography by Saifer *et al.* (S5). The seven most common (in descending order) were found to be alanine, glutamic acid-glutamine, lysine, proline, threonine, glycine, and valine. These seven amino acids, which comprise 70% of the total, are the same as those found in highest concentration in plasma. Of the 22 other amino acids found in amniotic fluid, both taurine and hydroxyproline are present in appreciable quantities (Table 4).

The concentration of an amino acid in cerebrospinal fluid (CSF) is considerably lower than its value in plasma except for glutamine (Table 4). Since the plasma:CSF ratios for the various amino acids are not uniform, they may originate as an efflux from brain cells under equilibrium conditions (L5). The eight amino acids which are present in the highest concentration are (in descending order) glutamine, serine, threonine, alanine, lysine, glutamic acid, valine, and histidine (F8). The amino acid composition of saliva, tears, sweat, and other biological fluids has been reported in the review article by Westall (W11).

7.2. Hereditary Disorders of Amino Acid Metabolism

A hereditary disease could presumably produce an aminoaciduria by five different known mechanisms (M20).

a. The metabolic disorder, resulting from a deficient enzyme, causes an increase in the plasma level of one or more amino acids, with a con-

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Footnotes to Table 4:

[a] Listed in order of peak elution from ion-exchange column as described by Saifer *et al.* (S5). Tr = trace amounts.

[b] Saifer *et al.* (S5).

[c] Dickinson *et al.* (D14).

[d] Armstrong *et al.* (A7a).

[e] Peters *et al.* (P17).

[f] Fisher *et al.* (F8).

TABLE 5: HEREDITARY DISORDERS OF AMINO ACID METABOLISM

Disease (genetics)	Abnormal enzyme (tissue)	Amino acids increased (blood)	Abnormalities (urine)	Additional observations	Clinical features (treatment)	Diagnostic tests	Pertinent references
Group I: Catabolic defect; initial low renal clearance; overflow aminoaciduria; detection preferable in plasma							
1. Phenylketonuria (recessive)	Phenylalanine hydrolase (liver)	Phenylalanine	Phenylalanine, phenylpyruvic acid, phenyllactic acid, *o*-hydroxyphenylacetic acid	Conc. of other amino acids, including tyrosine, is normal; bone marrow vacuolation	Mental retardation; seizures; eczema (responds to low phenylalanine diet)	$FeCl_3$ test; chromatography (elevated blood phenylalanine) phenolic acids (S39)	(A7, E5, E12, G12, H17, H18, H20, H21, L12, M8, O2, R9, S22, S41, W2)
2. Hypertyrosinemia or tyrosinosis (recessive)	*p*-Hydroxyphenylpyruvic acid oxidase (liver)	Tyrosine (+ methionine in the later stages)	Tyrosine, methionine, *p*-hydroxyphenylpyruvic acid (*p*-HPPA), *p*-HP-lactic acid, and *p*-HP acetic acid	Generalized aminoaciduria; renal glycosuria	Hepatic cirrhosis, renal damage (responds to phenylalanine and tyrosine restriction)	$FeCl_3$ test; chromatography (elevated blood tyrosine and/or methionine) phenolic acids (S39)	(E5, E12, G7, G18, H17, H18, H19, M8, P2, P14, R9, S22, S41, T4, W2)
3. Hyperhistidinemia (recessive)	Histidine α-deaminase	Histidine (+ alanine)	Histidine, imidazole, pyruvic acid	Histidine in CSF; decrease in plasma glutamine and glutamic acid; urocanic acid absent from sweat	Mental retardation, speech defect, seizures	$FeCl_3$ test; chromatography	(E12, G14, H17, H18, H20, L2, M8, R9, S41)
4. Branched-chain ketoaciduria[a] or maple syrup urine disease (recessive)	Branched-chain α-keto-acid decarboxylase (leukocytes)	Valine, leucine isoleucine, and alloisoleucine	Branched-chain ketoacids	Urine has color of maple syrup; ketoacids present in CSF	Mental retardation, seizures, cortical blindness, progressive rigidity and vomiting (responds to diet low in leucine, isoleucine, and valine)	Odor of maple syrup in urine; chromatography detects increase in ketoacids; 2,4-dinitrophenylhydrazine test is positive	(E5, E12, D1, G19, H17, H19, H20, M5, M8, R9, S23, S40, S41)
5. Hypervalinemia[a] (unknown)	Valine α-ketoisovaleric acid transaminase (leukocytes)	Valine	Valine		Hyperkinesia, hypotonia, vomiting, and retarded development (responds to a low valine diet)	Chromatography	(D3, E5, H19, M8, R9, S41)

6a. Homocystinuria[a] or methioninemia (recessive)	Cystathionine synthetase (liver, brain)	Homocystine (+ methionine)	Homocystine (30–300 mg/24 hr), methionine	Increased homocystine and methionine in CSF; plasma cystine decreased	Mental retardation, convulsions, thromboembolic complications, progressive paraplegia, mottled skin (responds to a low-methionine, high-cystine diet)	Chromatography	(E5, E12, G9, H18, H19, M8, R9, S11, S41)
6b. Hypermethioninemia	Presumably methionine activating system	Methionine	Methionine		Transient benign neonatal form	Chromatography	(H19, M8, S41)
7. Hyperglycinemias							
7a. Ketotic form	Propionyl-CoA carboxylase	Glycine (+ other amino acids)	Glycine, acetone	Possible NH_3 intoxication	Mental retardation, ketosis, vomiting, neutropenia, and osteoporosis (responds to low-protein diet)	Chromatography; blood NH_3 test, acetest stick (for acetone)	(E5, E12, H17, H18, H19, M8, N9, R9, S41, W24)
7b. Nonketotic form	Glycine oxidase (?)	Glycine	Glycine, homocitrulline, homoarginine	Decreased oxalate excretion	Gross physical and mental retardation, convulsions and impaired sexual and muscular development	Chromatography, quantitative oxalate analysis	
8. Hypersarcosinemia (recessive)	Sarcosine oxidase (?)	Sarcosine	Sarcosine	Ethanolamine	Mental retardation, hypertonia, dysphagia, and retarded growth	Chromatography	(H19, M8, R9, S41)
9. Hyperprolinemias							
9a. Type I (recessive)	Proline oxidase (liver)	Proline	Proline, hydroxyproline, glycine	Increased serum triglycerides, lower γ-globulin, ketosis on leucine loading	Mental retardation, seizures and kidney disease (treatment with low-protein diet)	Chromatography	(E5, E6, E12, H17, H18 H19, M8, R9, S41)
9b. Type II (recessive)	Δ^1-Pyrroline-5-carboxylic acid dehydrogenase	Proline	Proline, hydroxyproline, glycine, Δ^1-pyrroline-5-carboxylate	No nephritis	Mental retardation and seizures (treatment with low-protein diet)	Chromatography	

(Continued)

TABLE 5 (*Continued*)

Disease (genetics)	Abnormal enzyme (tissue)	Amino acids increased (blood)	Abnormalities (urine)	Additional observations	Clinical features (treatment)	Diagnostic tests	Pertinent references
10. Hydroxyprolinemia (recessive)	Hydroxyproline oxidase	Hydroxyproline (+ tyrosine)	Hydroxyproline index	After hydroxyproline load, no excretion of Δ^1-pyrroline-3-hydroxy-5-carboxylate or γ-hydroxyglutamic acid; decreased proconvertin index	Mental retardation, thrombopenia (treated by phenylalanine and tyrosine restriction)	$FeCl_3$ test chromatography	(E5, E6, E12, H17, H18, H19, M8, R9, S41)
11. Hyperpipecolatemia	Unknown	Pipecolic acid	Pipecolic acid (+ mild generalized aminoaciduria)	No impairment of lysine metabolism	Degenerative neurological disease with demyelination at all levels, hepatomegaly	Chromatography	(G4)
12. Urea Cycle Disorders							
12a. Hyperammonemia, Type I(unknown)	Carbamylphosphate synthetase	Glycine, NH_3, and glutamine	Glutamine, glycine	Protein intolerance; ammonia intoxication	Mental retardation; vomiting (treated with low-protein diet)	Blood NH_3 increased; chromatography	(E5, E7, F14, H4, M8, R9, S41)
12b. Hyperammonemia, Type II[a]	Ornithine transcarbamylase (liver)	Glutamine, NH_3	Glutamine (other amino acids are normal)	NH_3 and glutamine increased in CSF	Same as in 12a	CSF and blood NH_3; chromatography	(E5, E7, H4, L7, M8, R9, R15, S41)
12c. Hyperornithinemia	Ornithine transcarbamylase (?)	Ornithine	Ornithine (may be normal)	Ammonia intoxication	Same as 12a	Same as 12b	(R9, S27, S41)
12d. Citrullinemia[a] (unknown)	Argininosuccinic synthetase (liver)	Citrulline	Citrulline	Ammonia intoxication, normal blood urea N	Mental retardation, hepatomegaly, alkalosis (low protein diet)	Same as 12b	(E7, H17, H18, M8, M18, R9, S41)
12e. Argininosuccinic-aciduria[a] (recessive)	Argininosuccinase (liver, RBC)	Argininosuccinic acid (~4 mg %)	Argininosuccinic acid (>2.5 g/24 hr)	Ammonia intoxication Increased ASA in CSF, protein intolerance	Mental retardation, ataxia, seizures, hepatomegaly, (low protein diet should be tried)	Chromatography, monoamino, monocarboxylic acids increased	(E7, H17, H18, H19, H22, L6, M8, R9, S41)

13. Hyperlysinemia[a]							
13a. Type I (unknown)	Unknown	Lysine (+glutamine)	Ornithine, γ-aminobutyric acid and ethanolamine	Delayed conversion of injected lysine to CO_2	Mental retardation, hypotonia and seizures (restricted lysine diet)	Chromatography	(C10, D2, E12, G15, M8, R9, S41)
13b. Type II (unknown)	Lysine dehydrogenase (liver)	Lysine (+arginine)	Lysine	Ammonia intoxication, lysine intolerance	Mental retardation, coma, spasticity, vomiting and growth failure	Chromatography, blood NH_3	
Group II: Defect in catabolism; high renal clearance; detection preferable in urine							
1. Cystathionuria (unknown)	Cystathionase (liver)	Cystathionine	Cystathionine (>1.0 g/24 hr)	Cystathionine increased in CSF	Mental retardation, seizures and congenital anomalies (treated with pyridoxine)	Chromatography, positive urinary nitroprusside test	(E5, E12, F17, H17, H18, M8, R9, S41)
2. Hypophosphatasia	Alkaline phosphatase (serum)	Phosphoethanolamine (~0.4 mg/100 ml)	Phosphoethanolamine (>150 mg/24 hr)		Bone disease, craniosynotosis, and hypocalcemia	Chromatography	(B5, F13, H17, H18, M8, R9)
3. β-Aminoisobutyric aciduria	β-aminoisobutyric acid transaminase	β-Aminoisobutyric acid	β-Aminoisobutyric acid		Benign polymorphic trait	Chromatography	(G3, H18, H20, R9, S41, S56)
4. Hyper-β-alaninemia	β-alanine transaminase	β-alanine, γ-aminobutyric acid	β-Aminoisobutyric acid, γ-aminobutyric acid, and taurine	β-alanine and γ-aminobutyric acid increased in CSF	Mental retardation, seizures, and somnolence	Chromatography	(M8, R9, S20)
5. Carnosinemia	Carnosinase	Carnosine	Carnosine (20–100 mg/24 hr)	Carnosine and homocarnosine in CSF	Mental retardation and seizures	Chromatography	(P13, P15, R9, S17)
6. Hypertryptophanemia (unknown)	Tryptophan pyrolase	Tryptophan	Tryptophan	After a tryptophan load, urinary indoles are normal or decreased	Mental retardation, ataxia, dwarfism, photosensitivity, conjunctival telangiectasis (may respond to niacin therapy)	Chromatography	(B6, J5, T1)

(*Continued*)

TABLE 5 (*Continued*)

Disease (genetics)	Abnormal enzyme (tissue)	Amino acids increased (blood)	Abnormalities (urine)	Additional observations	Clinical features (treatment)	Diagnostic tests	Pertinent references
7. Methylmalonic aciduria[a]	Methylmalonyl-CoA isomerase or vitamin B_{12} coenzyme	Methylmalonic acid (+ glycine)	Methylmalonic acid	Methylmalonic acid increased in CSF, ketonuria, protein intolerance	Coma, extensor spasm, retarded development, metabolic acidosis (vitamin B_{12} treatment)	Chromatography; Acetest sticks	(O1, R9, R11, R12, S41, S53)

Group III: Defect in reactive transport site; high renal clearance; detection in urine only

Disease or trait (genetics)	Amino acid increased in urine	Amino acids poorly absorbed by intestine	Clinical features (treatment)	Diagnostic tests	Pertinent references
1. Cystinuria					
1a. Type I (kidney + gut)	Cystine + dibasic amino acids	Cystine and dibasic amino acids	Renal calculi usually containing cystine (treatment with high water intake or penicillamine administration)	Chromatography, cystine crystals in urine; urinary nitroprusside test is positive	(B6, E5, H17, H18, K12, M8, P12, R6, R8, T3a)
1b. Type II (kidney + gut)	Cystine + dibasic amino acids	Dibasic amino acids			
1c. Type III (kidney + gut) (recessive ?)	Cystine + dibasic amino acids	Normal absorption			
2. Hypercystinuria, isolated	Cystine	Unknown	Possibly related to familial hypoparathyroidism	Chromatography; urinary nitroprusside test is positive	(B25, R9)
3. Prolinuria or imminoglycinuria					
3a. Type I (kidney + gut) 3b. Type II (kidney only)	Proline (600 mg/24 hr), hydroxyproline and glycine	Unknown	Probably benign. Found in association with various diseases. Heterozygotes have glycinuria but no prolinuria	Chromatography	(M8, R9, S21, T2)
4. Hartnup Disease 4a. Type I (kidney + gut) 4b. Type II (kidney) (recessive)	Neutral monoamino, monocarboxylic acids (except imino acids, methionine and arginine)	Neutral amino acids (abnormal urinary indole pattern due to bacterial conversion of tryptophan)	Mental retardation and psychosis, episodic cerebellar ataxia, pellagralike symptoms and abnormal plantar reflex (nicotinamide therapy)	Chromatography shows increase of urinary monoamino, monocarboxylic acids	(B2, B20, E5, E12, H17, H18, H19, J5, M8, M8, P12, R9, T3a
5. Methionine malabsorption or Oasthouse urine disease (recessive ?)	Methionine (on high methionine diet) and smaller amounts of valine, leucine, tyrosine, and phenylalanine	Methionine (converted to α-hydroxybutyric acid and excreted in urine) phenyllactic acid, phenylpyruvic, acid	Mental retardation, seizures, hypernea, and edema, burnt sugar odor, diarrhea and sparse hair (methionine and phenylalanine restriction)	Chromatography, $FeCl_3$ test is positive (green color)	(E5, H14, H19, R9, S31, T3a)

6. Hyperglycinuria, familial					
6a. Type I (dominant ?)	Glycine (but not the imino-acids)	Glycine	No clinical pathology except for oxalate stone formation	Chromatography	(D10, E5, H17, H18, K1, P12, R9, W24)
6b. Type II (dominant)	Glycine + glucose	Normal glycine reabsorption	No clinical pathology	Chromatography; glucose-Combistix	
7. Tryptophan malabsorption (blue diaper syndrome)	Indigo blue and other indoles (amino acid excretion is normal)	Tryptophan	Hypercalcemia, retarded growth, febrile episodes, and irritability	Chromatographic separation and detection of indole derivatives (H21, S36)	(D16, R9, T3a)
8. Joseph's syndrome (familial)	Proline, hydroxyproline, and glycine	Renal transport defect of proline, hydroxyproline, and glycine	Mental retardation, seizures, hypotonia, status epilepticus, and retarded growth	Increased CSF protein, chromatography	(G20, J9, M17, R10, T3a, W12)

Group IV: Inhibition of transport process; high renal clearance; detection best in urine

Disease or trait (genetics)	Amino acids increased in urine	Additional observations	Clinical features (treatment)	Diagnostic tests	Pertinent references
1. Cystinosis[a] or Fanconi syndrome	Generalized aminoaciduria plus other substances, e.g., phosphate, glucose, uric acid, potassium; cystine not increased	Cystine deposits in liver, kidney, bone marrow, and eyes; decreased levels of phosphate, potassium, and uric acid in serum	Polyuria, albuminuria, glycosuria, acidosis, fever, dehydration, growth retardation, and vitamin D-resistant rickets	Chromatography; blood plasma and urinary determinations of potassium, phosphorus, uric acid, and glucose	(D7, F3, H17, H18, L3, P12)
2. Oculocerebrorenal syndrome or Lowe's syndrome (sex-linked recessive)	Marked aminoaciduria of the common amino acids	Proteinuria, alkaline urine, decreased ability to excrete NH_3 after a NH_4Cl load	Mental retardation, hypetonia, glaucoma, recurrent fever, rickets, and retarded growth	Chromatography; urinary protein analysis (Combistix)	(H17, H18, H19, L11, M8, P12, S14, T3a)
3. Busby syndrome	Generalized aminoaciduria		Retarded growth and pulmonary disease	Chromatography	(R14)
4. Wilson's disease or ceruloplasmin deficiency (autosomal recessive)	Excess secretion of glycine, serine, and cystine	Lenticular degeneration, positive copper balance, low serum ceruloplasmin levels, and defects in renal tubular reabsorption	Mental retardation, ataxia, extrapyramidal symptoms, and cirrhosis	Chromatography; low blood and high urine copper levels; low serum ceruloplasmin	(B8, H18, H19, P11, S9, W16)
5. Galactosemia (recessive)	Generalized aminoaciduria and galactose	Galactose and galactose 1-phosphate accumulate in blood due to deficiency of galactose-1-phosphate uridyl transferase	Mental retardation, cataracts, retarded growth, vomiting, jaundice, and liver enlargement (lactose restriction from diet)	Chromatography for amino acids and carbohydrates, reducing sugars (galatest)	(A4, H18, H19, H20, I2, K15, M8, T3a)

[a] The diagnosis of these diseases can be made prenatally (M10, M11, M12).

sequent "overflow" into the urine. The "overflow" type of aminoaciduria may be subdivided into the "threshold" group in which the amino acid and/or acids involved undergo normal renal tubular reabsorption but which results in the aminoaciduria when the absorption site is saturated. Examples of the "threshold" type are listed in Group I of Table 5 and include many of the more common examples of inborn errors of amino acid metabolism such as phenylketonuria (Fig. 5A), hyperhistidinemia (Fig. 5B), branched-chain ketoaciduria (maple syrup urine disease (Fig. 5C), citrullinemia, etc. These genetic disorders are best detected by analyzing the blood serum, or plasma, although additional useful information is obtained by simultaneously running the patient's urine sample.

The "nonthreshold" group (Group II, Table 5) constitutes those

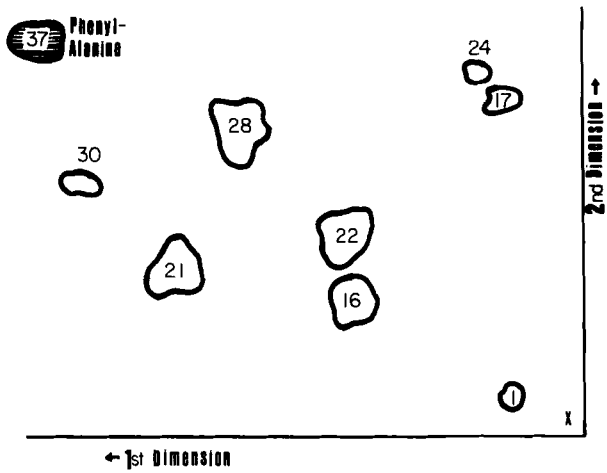

FIG. 5A.

FIG. 5. (pp. 188–192) (A) Diagrammatic representation of the urinary amino acid pattern from a case of phenylketonuria. Experimental conditions were the same as those listed in Fig. 4. (B) Diagrammatic representation of urinary amino acid pattern from a case of hyperhistidinemia. (C) Diagrammatic representation of urinary amino acid pattern from a case of branched-chain ketoaciduria (maple syrup urine disease). (D) Diagrammatic representation of urinary amino acid pattern from a case of cystathionuria. (E) Diagrammatic representation of urinary amino acid pattern from a case of cystinura. (F) Diagrammatic representation of urinary amino acid pattern from a patient with Hartnup disease. (G) Diagrammatic representation of urinary amino acid pattern from a patient with the Fanconi syndrome. (H) Diagrammatic representation of urinary amino acid pattern from a patient with Wilson's disease. (I) Diagrammatic representation of urinary amino acid pattern from a case of homocystinuria.

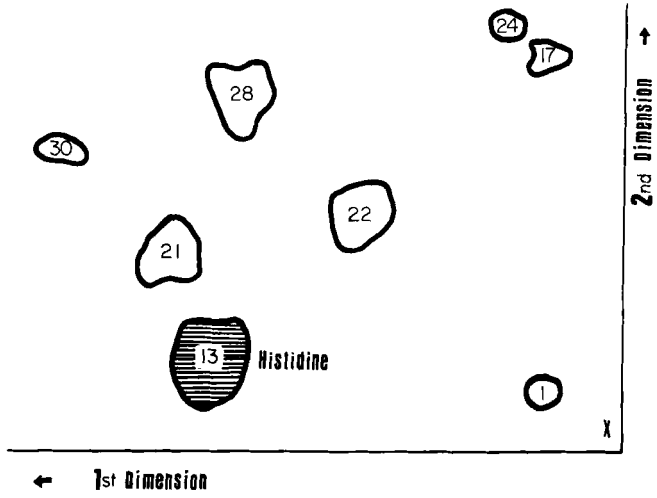
24
17
28
30
22
21
13
Histidine
1
X
2nd Dimension
1st Dimension

FIG. 5B.

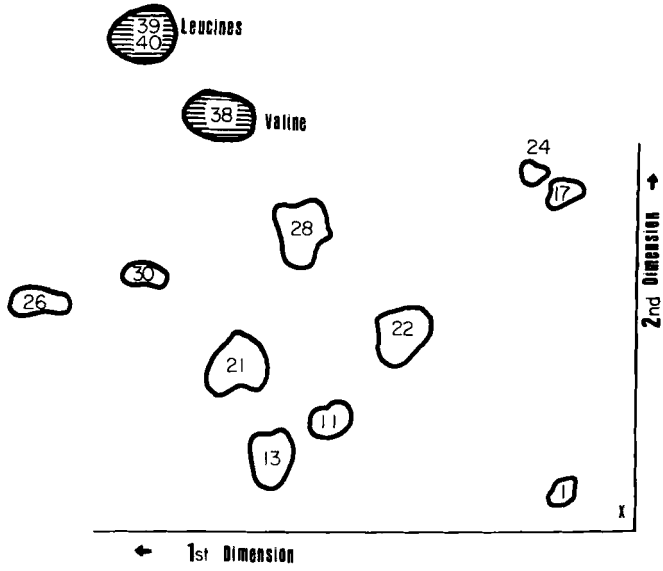
39
40
Leucines
38
Valine
24
17
28
30
26
22
21
11
13
1
X
2nd Dimension
1st Dimension

FIG. 5C.

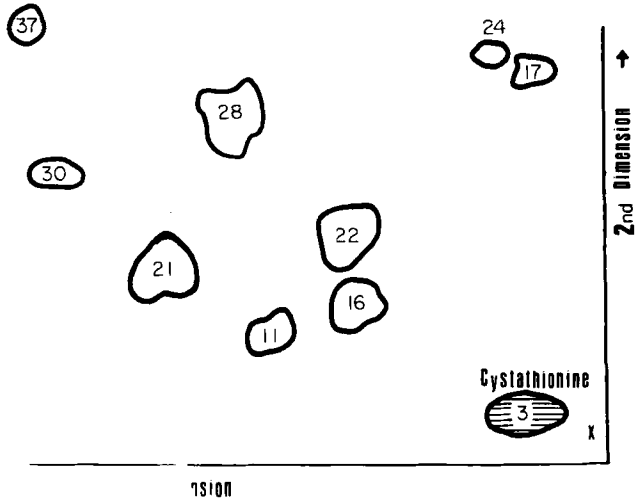

FIG. 5D.

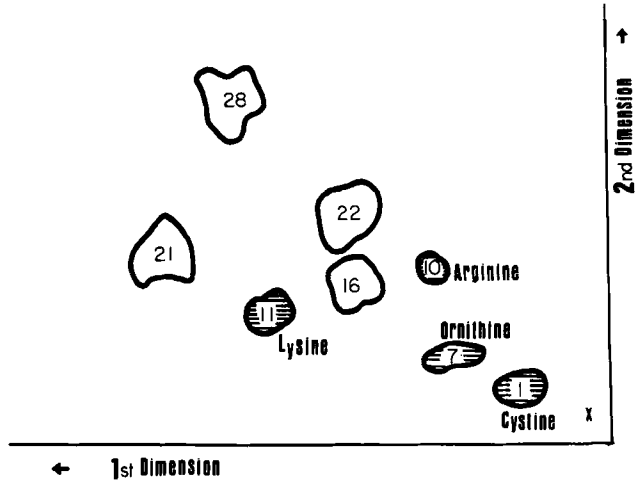

FIG. 5E.

amino acids whose excretion rate into the urine approximates that of inulin. Examples of such inherited metabolic disorders are cystathionuria (Fig. 5D), hypophosphatasia, β-aminoisobutyric aciduria, hyper-β-alaninemia, carnosinemia, hypertryptophanemia, and methylmalonic aciduria.

b. The excess excretion of a single amino acid as a result of its in-

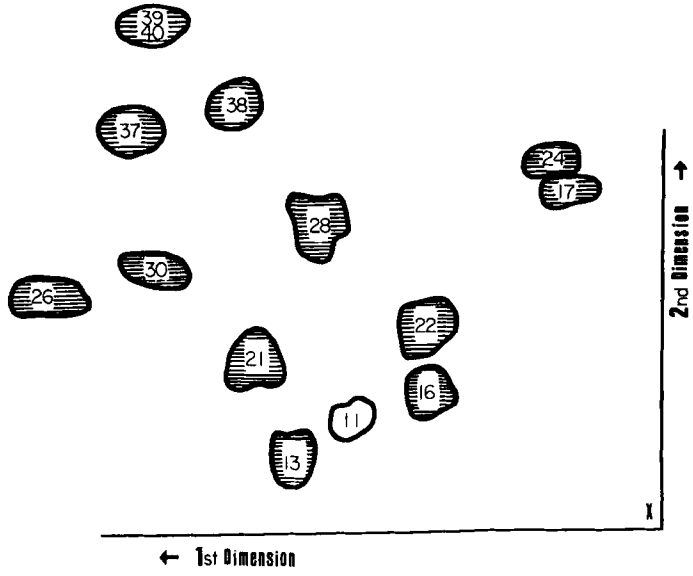
39
40
38
37
24
17
28
30
26
22
21
16
11
13
X
2nd Dimension
1st Dimension

FIG. 5F.

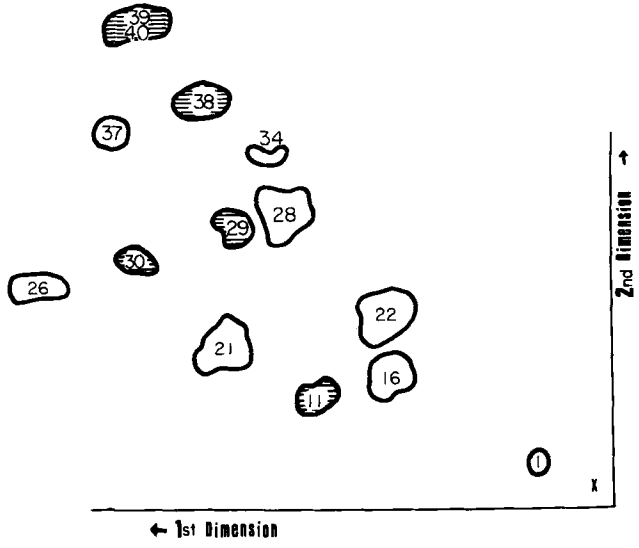
39
40
38
37
34
28
29
30
26
22
21
16
11
1
X
2nd Dimension
1st Dimension

FIG. 5G.

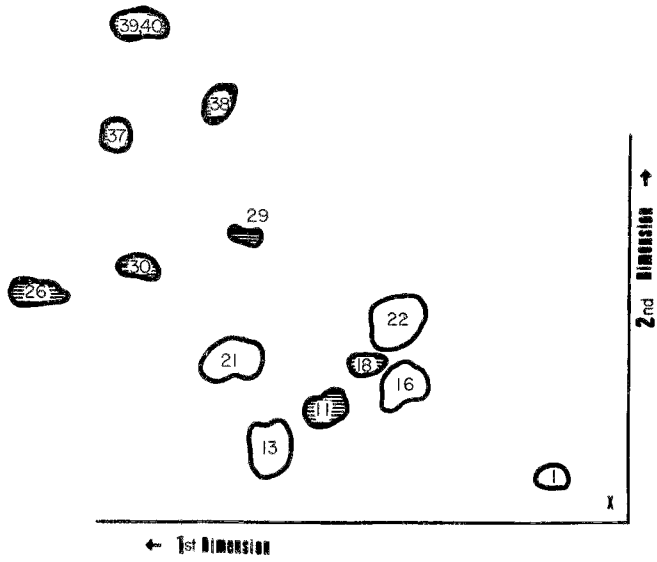

FIG. 5H.

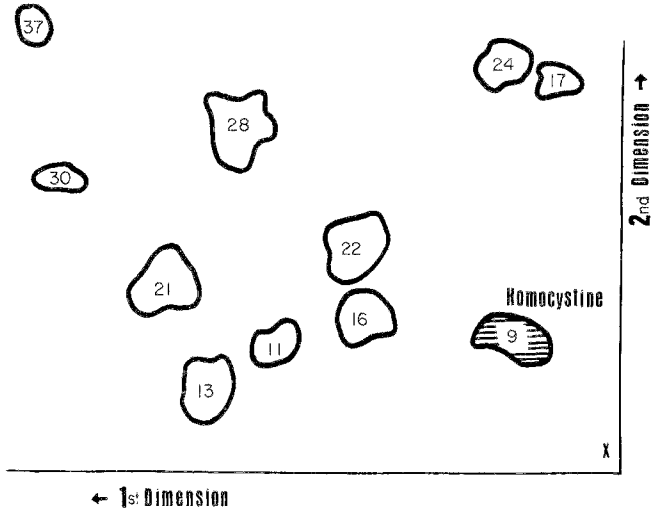

FIG. 5I.

creased plasma level could saturate a specific proximal tubular transport system and thus cause a secondary aminoaciduria of the renal type. Examples of this kind of hereditary disorder are listed in Group III of Table 5 and include cystinuria (type 1) (Fig. 5E) and prolinuria. These

disorders are best detected by testing the urine for excess excretion of a group of amino acids.

c. There is a specific amino acid dual transport defect both in the proximal renal tubules and in the jejunal cells of groups of amino acids which share a common transport site. This category includes such inborn errors of metabolism as Hartnup disease (type I) (Fig. 5F), tryptophan malabsorption, methionine malabsorption, Joseph's syndrome, and cystinuria (type II) as is listed in Group III of Table 5.

d. The metabolic disorder results in generalized and nonspecific proximal tubular damage. In this condition there is an increased excretion into the urine of other substances than amino acids such as glucose, phosphates, uric acid, and proteins (globulins) with a reduced capacity to secrete organic acids. Examples of such diseases include the Fanconi syndrome (Fig. 5G) and oculocerebrorenal (Lowe's) syndrome, whose characteristics are described in Group IV of Table 5.

e. The metabolic disorder results in the overproduction of metabolites which are nephrotoxic. Included in this category are galactosemia and Wilson's disease (Fig. 5H) (Group IV, Table 5) in which, respectively, excess galactose 1-phosphate and deposits of copper within tubular cells interfere with the transport mechanisms and cause the amino aciduria. A list of the known inherited secondary aminoacidurias has been published by Feigin (F4a).

7.3. Amino Acid Changes Resulting from Acquired Disorders

While kidney damage may be one of the by-products of a genetic disease, there are a large number of other acquired disorders which result in blood and urine amino acid aberrations. These include such conditions as protein and vitamin deficiencies, glandular malfunction, liver necrosis, muscular dystrophy, rheumatoid arthritis, xeroderma pigmentosum, and kidney damage (as a consequence of heavy metal, drug, or chemical poisoning). Feigin (F4a) has listed the amino acid abnormalities resulting from a number of acquired disorders (Table 6). Although the aminoacidopathy constitutes a secondary effect in these conditions, amino acid analysis often provides a distinctive pattern which may aid in their diagnosis or therapy. A case in point is the amino aciduria produced by lead intoxication. Earlier work in this field (C7) assumed that this phenomenon resulted from impaired proximal renal tubular reabsorption which yielded the constellation of effects known as the "Fanconi syndrome," i.e., generalized aminoaciduria, hypophosphatemia, and glycosuria, as did a variety of other genetic and acquired defects (L3). More recent work with lead-intoxicated rats (G22) showed that the aminoaciduria consisted of a large increase in histidine content

TABLE 6
ANALYSIS OF ACQUIRED AMINOACIDOPATHIES[a]

Disorder	Amino acid abnormalities	Comments
Kwashiorkor	Decrease in essential amino acids and tyrosine more than nonessential	Ratio of essential to unessential amino acids helpful in diagnosis even with subclinical levels of protein deficiency; abnormal phenylalanine tolerance test
Malnutrition	Low urinary hydroxyproline output	Increase following treatment helpful in assessing therapeutic response
Vitamin D deficiency	Generalized aminoaciduria	Occurs without evidence of hypocalcemia, hypophosphatemia, or increased alkaline phosphatase
Vitamin C deficiency	Generalized aminoaciduria	—
Vitamin B deficiency	Xanthenuria aciduria	—
Neuroblastoma, ganglioneuroma, ganglioneuroblastoma, carcinoid with liver metastases	Cystathionuria	May appear even with normal VMA levels; will cease with removal of neoplasm and may return with tumor recurrence; useful in following patient postoperatively
Functional parathyroid adenoma	Increased urinary hydroxyproline	Excretion returns to normal following removal of adenoma
Hyperthyroidism	Elevated plasma tyrosine; increased hydroxyproline excretion	—
Hypothyroidism	Decreased urinary hydroxyproline excretion	Levels of excretion change rapidly with appropriate therapy
Hypopituitarism in children	Decreased hydroxyproline excretion	Also seen in other forms of dwarfism but returns to normal following growth hormone therapy; one of earliest signs of therapeutic efficacy
Acromegaly	Increased hydroxyproline excretion	Falls to normal with therapy
Hyperparathyroidism	Increased hydroxyproline excretion	—

TABLE 6 (*Continued*)

Disorder	Amino acid abnormalities	Comments
Following renal transplantation	Generalized aminoaciduria or excretion of cystine and lysine	Aids in assessing tubular function following transplantation
Liver necrosis	Hyperaminoacidemia, aminoaciduria with tyrosinuria, leucinuria, and prolinuria	—
Muscular dystrophy	Generalized aminoaciduria, decreased urinary hydroxyproline excretion	No correlation with creatinuria
Hypophosphatasia	Urinary excretion of phosphoethanolamine	May appear in some apparently normal heterozygotes
Paget's disease	Increased hydroxyproline excretion	—
Fibrous dysplasia	Increased hydroxyproline excretion	—
Marfan's disease	Increased hydroxyproline excretion	Not present in many cases but helpful when it appears
Rheumatoid arthritis	Decreased serum histidine	Decrease not associated with salicylate administration or correlated with age, sex, or sedimentation rate
Xeroderma pigmentation	Mild aminoaciduria of cystine, cysteine, histidine, lysine, arginine, aspartic acid, glutamine, serine, and alanine	25% of relatives without skin lesions affected with aminoaciduria
Heavy metal poisoning (lead, mercury, cadmium, uranium, thallium)	Generalized aminoaciduria	—
Salicylate intoxication	Generalized aminoaciduria	—
Nitrobenzene poisoning	Generalized aminoaciduria	—
Degraded tetracycline	Generalized aminoaciduria	—

[a] From Feigin (F4a).

with lesser increases in the levels of glycine and valine and a decrease in that of tyrosine. The plasma showed a marked rise in glycine, which the authors postulated as possibly reflecting the defect in porphyrin synthesis or metal–amino acid complex formation, together with lesser increases in threonine and lysine. It is evident that the pattern of amino acid excretion for lead poisoning differs qualitatively from that found in cystinosis, which also is grouped as a "Fanconi syndrome" disorder.

Since the term "Fanconi syndrome" is difficult to define precisely (M8) and because "generalized aminoaciduria" should be thought of as a sign of renal injury, not as a diagnosis in and of itself (R9), it is hoped that a more quantitative approach to the analysis of acquired and inherited aminoacidopathies will serve to eliminate this term from the medical literature.

8. Treatment and Prevention of Aminoacidopathies

Rapid screening methods for the detection of inborn errors of amino acid metabolism will be of major benefit to the patient only if the diagnosis is made early enough to permit treatment, when feasible, which would prevent the more serious consequences of the disorder, e.g., mental retardation. Hsia (H19) has reviewed the literature to the beginning of 1966 and lists 39 disorders known to be associated with mental deficiency and the indicated treatment for 24 (or 62%) of them although not all are associated with defects of amino acid metabolism. Examples of inborn errors of amino acid metabolism in which restriction of the diet has been attempted more or less successfully (J8) are phenylketonuria (phenylalanine); maple sugar urine disease (leucine, isoleucine, and valine); isovalericacidemia (leucine); hypervalinemia (valine); homocystinuria (methionine); histidinemia (histidine); tyrosinosis (tyrosine); hyperglycinemia, without ketosis (glycine, serine, low protein); hyperglycinemia, ketotic form (low protein); hyperlysinemia (low protein); citrullinemia (low protein); other disorders of the urea cycle (low protein). Once the diagnosis has been made and the patient is placed on the restrictive diet (indicated in parentheses above) the clinical chemist must be prepared to provide quantitative analysis of the amino acid, or one of its metabolites, as a continual check on the efficacy of the therapy. For example, in the case of phenylketonuria, treatment of the patient may be followed by either serial blood phenylalanine determinations (M1) or by quantitative analysis of the phenylpyruvic acid content of the urine (S2).

Where no effective treatment exists at present for some inborn errors of metabolism, and where these disorders are associated with severe illness, mental retardation, or early death, the prenatal diagnosis of the diseased fetus by amniotic fluid enzyme analysis is now possible (M10, M11, M12). These new developments in the field of transabdominal amniocentesis, coupled with more liberal abortion laws, can serve to reassure families with a previous history of a fatal disease that their offspring will be physiologically normal. As applied to the field of amino acid disorders, enzyme analysis of cultured amniotic fluid cells has been used to diagnosis potential cases of homocystinuria (Fig. 5I) and

maple syrup urine disease (Fig. 5C). It has not as yet been demonstrated whether disorders of amino acid metabolism can be diagnosed by the finding of an amount of an amino acid (and/or acids) in the amniotic fluid above that present normally (Table 4). This is an important area of preventive clinical medicine which warrants further investigation.

9. Current and Future Research Trends in the Field of the Hereditary and Acquired Aminoacidopathies

A period of some 40 years elapsed between Garrod's (G1) classic description, in 1908, regarding certain genetic diseases being caused by inborn errors of amino acid metabolism, and the development of simple laboratory methods suitable for the clinical investigation of aminoacidopathies (C11, D6, M16). The number of hereditary aminoacidopathies being discovered have increased greatly from about 7 in 1948 to more than 40 in 1970. Even during the last two years spent in gathering material for this review article, two new inborn errors of amino acid metabolism have been reported. The first, reported in 1969 by Ampola *et al.* (A6), was found in a 47-year-old severely mentally retarded patient. The subject excreted 300 mg/24 hours of a sulfur-containing metabolite which increased 10-fold after cysteine loading. The substance was first detected in a urine sample by a positive cyanide-nitroprusside test, and the unknown spot was readily distinguished from cystine and homocystine by paper chromatography. Column chromatographic analysis of a urine sample showed a single abnormal peak while the other amino acids were within normal limits. The substance was not detected in deproteinized plasma. The unknown substance, which was somewhat unstable, was isolated in a relatively pure form by selective chromatographic elution from ion-exchange resin columns. The sulfur-containing amino acid was further purified by the use of high resolution ion exchange column chromatography, paper chromatography, gel filtration, and high voltage electrophoresis by Crawhall *et al.* (C14). They then converted the amino acid to a number of more volatile derivatives, i.e., *N*-trifluoroacetyl alkyl esters, and determined its structure by infrared and mass spectrometry. It was shown to be β-mercaptolactate-cysteine disulfide, an analog of cystine, and its structure was confirmed by partial synthesis. The compound was found to be excreted in small amounts by normal subjects after a cysteine load making it difficult to decide whether the increased excretion of this "presumed" normal metabolite is responsible for the patient's mental deficiency.

The second newly discovered inborn error of amino acid metabolism, pyroglutamic aciduria, was reported by Jellum and co-workers (J3) late

in 1970. The patient was a 19-year-old mentally retarded male who had other neurological symptoms of cerebral damage, chronic metabolic acidosis, and episodic vomiting. Biochemical studies showed a marked reduction in urinary urea excretion and the elimination into the urine of large amounts (up to 35 g/24 hours) of the abnormal metabolite pyroglutamic acid. The serum was found to contain increased amounts of glutamic acid derivatives. The metabolite was cochromatographed on silica gel G thin-layer plates, together with authentic pyroglutamic acid, eluted with methyl acetate and run in a mass spectrometer. The characteristic mass spectrum of pyroglutamic acid was obtained. Further identification of the abnormal metabolite as pyroglutamic acid was made by means of gas–liquid chromatography of an ethyl acetate extract of the urine subjected to methylation. Based on these findings, the authors concluded that the new inborn error indicated a defect in one of the early steps in the urea cycle which did not correspond with the three known defects of the Krebs urea cycle, i.e., hyperammonemia, citrullinemia, and argininosuccinic aciduria. The three papers (A6, C14, J3), quoted above, provide excellent examples of the modern experimental approach required for the identification of an unknown metabolite encountered in the investigation of a possible new inborn error of metabolism.

Unlike other disease entities, except for canine cystinuria, no other nonhuman inherited amino acid disorders have been uncovered. In place of animal studies, increasing emphasis is being placed on the use of cultured explants of tissues removed from patients with hereditary metabolic disease. Cultured explants of skin, leukocytes, and erythrocytes have been used to study the metabolism of such disorders as citrullinemia, branch-chain ketoaciduria, cystinosis, homocystinuria, and isovalericacidemia (S18).

Man ingests his protein episodically with an intake far in excess of his immediate needs. Yet this results in but little change in the plasma amino acid levels (R9, S18). Homeostasis of plasma amino acids results from their concentrative uptake and storage into the tissues and from their utilization in cellular metabolism. The processes of membrane transport, by which amino acid accumulation is achieved in all the body tissues, is subject to control by hormones, mutations, etc., but its characteristic feature is that it is a mediated phenomenon (C8). While at present, data on amino acid transport by the mammalian kidney point to several membrane systems for amino acid transport (S18), it is to be expected that future research will uncover mutant transport phenotypes for each amino acid.

The impact of the isoenzyme concept is first beginning to be felt in

the area of inborn errors of amino acid metabolism. An isoenzyme of tyrosine-α-ketoglutarate has been tentatively identified as the defective enzyme responsible for a particular form of tyrosinemia (F6). A number of aminoacidopathies, such as hyperphenylalaninemia, have been shown to consist of several clinical entities with more than one type of enzyme variant being involved (J10). Undoubtedly future research in this area will uncover many more examples of isoenzymes responsible for mutant phenotypes.

Work is presently in progress dealing with the detection and investigation of those amino acid disorders that affect catabolism of the carbon skeleton of amino acids (G26). Such conditions are often difficult to detect because the accumulated metabolites do not react with ninhydrin and methods for their detection are not available in most clinical laboratories. The greater availability of techniques such as gas–liquid chromatography should lead to the discovery of many more inborn errors of amino acid metabolism.

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IMMUNOGLOBULINS IN CLINICAL CHEMISTRY

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A vast publication explosion has followed the extensive work in the field of immunoglobulins. The object of the present review is to abstract in Sections 1–3 the basic knowledge needed to help us appreciate the clinical applications of immunoglobulins described in Sections 4 and 5 (the deficiencies), 6 (the increases), and 7 (the neoplasias). The word immunoglobulin we owe to Heremans (H13), and it refers to *that kind of protein in which specific antibody activity can be found.* The term is now preferred to γ-globulin because this can be confused with the commonest immunoglobulin class, γG-globulin, and also because immunoglobulins can be found with electrophoretic mobilities anywhere between the α_1 and the post-γ positions (see Fig. 12).

1. Immunoglobulin Structure and Identification

Thanks to Porter (P12) it is known that the basic immunoglobulin molecule (see Fig. 1) consists of two heavy chains (each molecular weight 55,000–75,000) joined to each other at the link region by a variable number of disulfide bonds (see Fig. 2) and then joined to two light chains. The basic units (7 S in the ultracentrifuge) can then be built into dimers (11 S), pentamers (19 S as for IgM), etc. The light chains (M.W. 22,000) are joined to the heavy chains by sulfhydryl bonds in all known

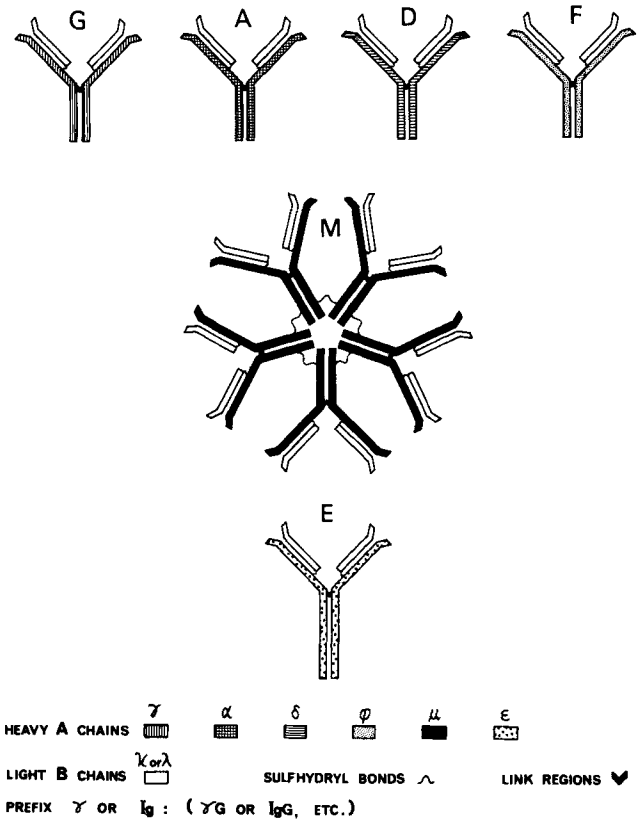

FIG. 1. Immunoglobulin structure. (IgF occurs transiently in the fetus and has not yet been found after birth.) Reproduced by courtesy of the *British Medical Journal* (H35).

immunoglobulins except for one subclass of IgA in which only covalent bonding exists (G19). Only one class of heavy chain can link to itself, and six classes (G, A, M, D, E, F) have been described. Only one of the two classes of light chain (K or L) is found in a given immunoglobulin, or indeed in a given plasma cell (T1). The known classes of heavy and light chains are designated by the appropriate small Greek letter, enabling formulas to be written thus, $\gamma_2\kappa_2$, $\alpha_2\lambda_2$, $\mu_{10}\kappa_{10}$, etc., and are indicated in Fig. 1. Fragments of immunoglobulin can be given similar formulas, e.g., $\gamma\kappa$ for a half-molecule of one γ-chain linked to one κ-chain; λ_2 for the dimer of λ-chains. Immunoglobulin molecules can then be designated in capital Roman letters using the prefix Ig or γ, e.g., IgGK, γAL, or IgMK. Subclasses are known in G (see Fig. 2), A (two), and M (two), and more may be described. They can be indicated by subscript numbers,

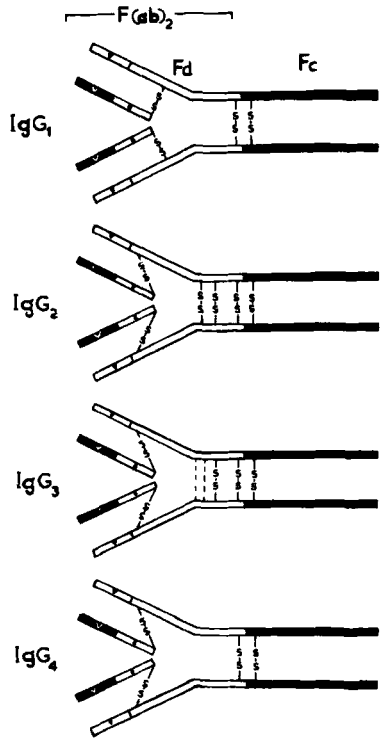

FIG. 2. The subclasses of IgG. The variable regions of heavy and light chains are indicated by V, the constant region of the light chains by C. After Frangione *et al.* (F6).

e.g., α_1-chain, IgG_3L, or γA_1K. The normal mixtures of immunoglobulins of a given class are now loosely called IgG or γA, etc., instead of the full term IgG globulins, etc. (see Table I, p. 233).

The portion of the basic immunoglobulin molecule below the link region (see Fig. 1) is called the Fc, because this was the fragment that was originally crystallized after being digested free by pepsin. Various Fc classes show marked variations in susceptibility to proteases. The various Fc portions distinguish the different classes of heavy chains and carry much of their class function. In IgG_1, IgG_3, and IgM_1, complement fixation requires the presence of the Fc. For all four subclasses of IgG, the Fc is essential for placental transfer. IgE cannot bind to mast cells without its Fc. Exposed determinants (by preliminary binding to sheep red cells, or by heat unfolding) of the Fc of IgG represent the antigen for rheumatoid factor. Variable proportions of carbohydrate become bound to the various classes of Fc by the Golgi apparatus of the plasma cell.

The basic unit can have two antibody combining sites, each shared by adjacent light and heavy chains, one in each arm of the molecule, and the whole of this portion above the link region is therefore called the $F(ab)_2$ piece, or if cut into single arms Fab-pieces (see Fig. 2). Each Fab consists of the Fd portion of a heavy chain and a whole light chain. The light chain and its equivalent portion of heavy chain each consist of some 212 amino acids. The 106 nearest the link region are relatively invariable for the particular subclass and carry the antigenic determinants for three K and five L subclasses. The other 106 amino acids show greater variation between different molecules, whereby individual antibody binding sites can arise, and are called the variable region. If Fudenberg's team are correct, a gene for the variable region can code a sequence which can be inserted into more than one class of heavy chain, etc. There may be 10 or more such genes for the heavy chain, called V_{HI}, etc., and it is known that one particular V_H has been inserted into both a μ and γ heavy chain in the same patient (W3). It also seems probable that within an inbred strain of BABL/c mice the same V_H gene binding phosphorylcholine has been isolated in five IgA paraproteins from different plasma cell tumors elicited in different mice by adjuvants (P13). Furthermore, the V genes result in the first amino acid sequences to be synthesized off polyribosomes producing heavy or light chains. It is thus conceivable that where the invariable subclass gene expression follows it could be switched from μ to γ, etc., within a given cell, where, however, only one class of light chain has been found to date. This has been considered at length because it enables us to understand: (i) how biclonal paraproteinemia can in some exceptional cases arise from what was initially a single cell line; (ii) how antibodies with the same activity might have similar V genes, e.g., cold agglutinins (see Section 7.7.3.); and (iii) how, if amyloid is due to deposition of V portions (with loss of the rest of the immunoglobulin molecule), a genetic variety would retain the same binding and the same clinical pattern of deposition and any variety would fail to react with even subclass-specific antisera, whose antigenic determinants reside in the remainder of the immunoglobulin molecule.

1.1. Antisera to Heavy Chains

The various classes of heavy chain can be recognized by antisera raised against them, and it is vital to understand these reagents.

Antisera to whole immunoglobulins will be directed against light chains and Fd + Fc heavy chains and to the V regions (the idiotypic antigenicity of any given molecule). Thus, while a myeloma protein offers an easy way of obtaining fairly pure antigen, much of the antiserum will

be idiotypic, and only one subclass of heavy chain will be represented. The antiserum would need to be adsorbed with light chain in order to use it as specific to heavy chain. In general, class-specific antisera depend on determinants in the Fc portions, and antisera raised against Fc will usually show less cross-reaction between the classes. Thus anti-γ antiserum is best prepared against IgG isolated from pooled human serum (representing all types) of which the Fc portions have then been prepared. Such an antiserum, termed anti-Fcγ, will specifically identify any IgG, and can be used to measure normal mixtures (Section 1.2). Although normal IgA and normal IgM are difficult to isolate, this is done by the best producers of antisera. The alternative is to use a large pool (12 or more) of paraproteins. Anti-Fcμ can then be prepared. As the Fc of α is so susceptible to digestion, for IgA the whole molecules have to be used, and adsorption then be carried out using $F(ab)_2$, etc. The reader is warned against the general use of anti-α or anti-μ raised against single paraproteins. For anti-D and anti-E, this has to be accepted at present, but such antisera (e.g., sheep) can be insolubilized and used to harvest a broader spectrum of IgD or IgE from normal human sera; then the complexes can be used in the same sheep to produce a broad antiserum. For radioimmunoassay of IgE this is most desirable, and a broad standard IgE can be similarly prepared by dissociating such complexes.

Antisera to heavy chain subclasses are similarly difficult to render specific (and yet broad). The antigenic areas seem to be largely in the link region and/or where the Fd is linked to the light chain (i.e., where the subclass differences exist, see Fig. 2), so whole molecules (preferably of a pool of subclassed paraproteins) have to be used. These points are emphasized because subclass assay for IgG will become desirable (see Section 5.9) and must therefore be dependable.

Idiotypic antisera are sometimes desired so that an individual myeloma protein can be followed below levels measurable by electrophoresis using the original protein as its own standard (H45) or used to identify a particular monoclone of plasma cells (W10). Here, of course, the antigen should be the idiotypic $F(ab)_2$, and absorption with much normal $F(ab)_2$ will be required.

It behooves the user to know how any antisera are achieved, against what antigens, and with what absorptions.

1.2. Radial Immunodiffusion

With standardized reactions between a class-specific antiserum and its immunoglobulin, it is possible to measure the level of that immunoglobulin in serum, etc. Turbidimetry of antigen-antibody precipitates is not recommended as too many variables influence the flocculence of the

precipitate. If the antigen is electrophoresed into antibody-containing gel, the eventual height of the precipitin "rocket" is proportional to its concentration (L1); however, this requires electrophoretic apparatus and consumes quantities of antiserum. The most economical assay at present utilizes radial immunodiffusion, wherein wells are made in antiserum-containing agar and filled with antigen so that precipitin rings form. The area of the precipitin ring is proportional to the concentration of antigen, provided that the unknown (e.g., mixed IgG of normal serum) is comparable to the standard (e.g., pure pooled IgG or a reference human serum) in both molecular size and antigenic constitution (see Section 1.1) and that the antiserum reacts equally with both (is a broad antiserum). Thus a myeloma immunoglobulin (an idiotype) cannot be accurately measured against a normal serum standard (H45). Similarly, macroglobulinemia cannot be assessed, and this is all the more so in that in neoplastic states the IgM may be in the form of 7 S and 19 S or even 28 S molecules, each of which will diffuse at a different rate (see Section 1.4). Secretory 11 S IgA diffuses to about 70% of the extent of an equivalent weight of 7 S IgA, but as this can be relatively constant under working conditions, salivary IgA (assuming it is all 11 S) can be measured against a serum standard, the result being multiplied by 1.4, or alternatively using a normal range directly expressed as percent serum (see Table 4).

A number of methods have been described (M3, M6, R12) but this author recommends his own simplified version, fully described in a Broadsheet (H34) giving details of standards: other relevant factors are mentioned below (Section 6.1).

1.3. Sephadex Immunodiffusion

First described independently by Grant and Everall (G18) and Hobbs (H19), this technique allows sizing of molecules at the same time as immunochemical typing. A method using Sephadex G-200 on microscope slides has been developed by Kershaw (C2). If the Sephadex is allowed to dry for only a short while at the end of the run, it can be stiffened to a degree enabling sharp-edged troughs to be cut with razor blades, the unwanted Sephadex being gently scraped toward the middle of the future trough, where it can be removed with a Pasteur pipette attached to a suction pump. This brings recognition of μ-chain, 7 S or 19 S IgM within the scope of any skilled technician using a minimum of apparatus. Such a poor man's ultracentrifuge also achieves typing, within 16 hours, so that polymerized IgG (viscosity syndrome) or mixed cryoglobulins (21 S reacting with anti-μ and anti-γ) can be distinguished more readily from macroglobulinemia (see Fig. 3). Sephadex G-150 is

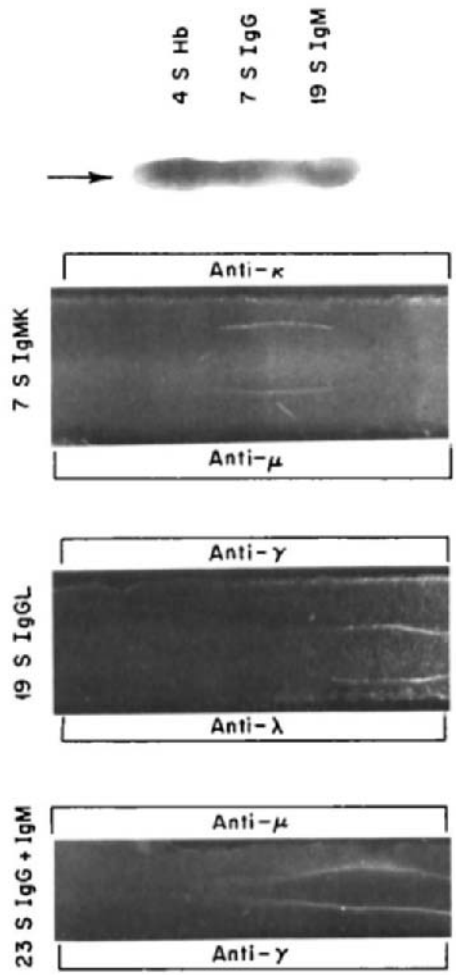

FIG. 3. Thin-layer chromatography in superfine Sephadex G-200 on microscope slides, followed by diffusion against monospecific antisera to identify proteins as indicated.

more suitable for recognizing tetramers of light chains, half-molecules, or heavy chains and for checking reduction and alkylation procedures. Sephadex G-75 can be used for monomers, dimers, and half-light chains.

1.4. CROSSED IMMUNOELECTROPHORESIS

First described by Ressler (R5), then developed by others (C7, H5), this technique has now been semiautomated and scaled down to 5 × 5 cm slides, thus economizing on antiserum (V1). After an initial electro-

phoretic separation horizontally in ordinary Veronal agar, the current is then switched vertically and the separated proteins are run into antiserum-containing agar, each producing its own "rocket," which can be measured against standards (see Fig. 4, p. 235). This technique has not yet been widely applied on a routine basis but will be of use in certain areas. When it is desired to estimate five proteins at once, e.g., five different molecular weights to visualize protein clearances (H8), an antiserum against just those proteins could be used, and serum and urine samples could be staggered by 5 mm, producing a paired series of peaks for easy comparison. Another use is where similar antigens occur of different molecular weights [invalidating radial immunodiffusion (Section 1.2)] but have separable electrophoretic mobilities; thus the β_1A/β_1C conversion of complement can be easily assessed, or 7 S and 19 S IgM be measured against relevant standards (H5), and this seems to be the only satisfactory method of measuring macroglobulinemia at present. By another variation of technique, just the difference between two samples (e.g., serum and plasma) can be visualized (K11).

1.5. Antisera to Light Chains

Now that it is known that three K and five L subclasses exist, we should not be satisfied with an antiserum raised against a single Bence Jones protein, for one such antiserum may react very poorly with another subclass. For general use, antigen pools are essential, and, as renal tubular damage is common and tubular proteins of similar size and charge contaminate such antigen pools, it is also essential to adsorb with a pool of the other class. Because K or L can have such small molecular weights and half-light chains occur, it is also advisable that insoluble immunoadsorbents be used, or else soluble complexes may be left behind. A full procedure has been well described (T2). This in general explains why commercial anti-light chain sera have been so bad. Claims have been made that antisera can be produced that will react only with free light chains; although this may be possible, the problems of idiotypes, molecular weight variation, and tubular protein contamination make it highly unlikely that measurements made of the serum content of free light chains have any absolute value. The clinical chemist is advised against relying on such estimates, and is advised not to quantitate K:L ratios or Bence Jones proteinuria by immunochemical methods. For us the best antisera to light chains are those that will reliably react with K or L (never with both), preferably both in the free state and within the immunoglobulin molecule. Such antisera can be produced and offer a most valuable means of establishing a monoclonal etiology for a paraprotein (see Section 7.1).

1.6. Detection of Bence Jones Proteins

In 1966, 79 urines containing different Bence Jones proteins were all examined by the 8 best methods then available (H20). Even the most sophisticated heat tests were negative in 33% of the urines, an intolerable false negative rate. I have also seen α_1-globulins (common in cancer patients) which yield false positives. While this was a remarkable observation in 1848, it is not satisfactory for medical diagnosis in 1971, and I no longer use heat tests. The best single screening test is Bradshaw's (B20), as confirmed by Hobbs (H20); cleared urine is carefully layered over concentrated hydrochloric acid, which can detect most globulins at 1 mg/100 ml (but even this can fail in 5% of Bence Jones proteinuria), and I always electrophorese a positive.

The only reliable test for clinical medicine is to concentrate the urine, subject it to electrophoresis, and then verify any narrow bands by immunoelectrophoresis by showing that they are due to a single class of light chain. This should be done for any patient if the clinician has strong reasons to suspect myelomatosis, etc.; otherwise Bradshaw's 95% reliable screening test is acceptable.

Many methods are available for concentrating urine. A convenient and rapid method is to (i) filter or centrifuge the urine; (ii) pass the filtrate/supernatant through ultrafilters to clear of bacteria (which can destroy Bence Jones in 4 hours) and small particles (we use a 20-ml syringe connected to a 25-mm diameter holder containing a 1 μ filter over a 0.22 μ filter); (iii) place 10 ml (if protein content is over 200 mg/100 ml, 1 ml will do) of ultrafiltered urine into a vertical collodion thimble, surrounded by buffer, connected to a vacuum pump (not above 12 psi). As the protein reaches the thimble side, a local self-wiping density gradient is formed and as little as 30 μl of concentrate can be recovered from the bottom with a capillary. Using ^{131}I-albumin, Millipore Swinnex filters and Sartorious thimbles, 90% recovery is easily achieved (without washing, scraping, etc.). The urine concentrate is electrophoresed alongside the donor's serum, whereby one can easily identify narrow components in the urine (see Fig. 14) at a higher concentration than in the serum, relative to albumin (and thereby cleared as though of lower molecular weight). It is even possible to realize that a Bence Jones protein overlies transferrin (β_1), because the β_1 is denser than it ought to be relative to albumin. When seen, these can then be checked against precious antisera, using immunoelectrophoresis.

2. Turnover of Immunoglobulins

The serum level of an immunoglobulin results from the input from plasma cells, and its subsequent distribution and rate of removal. Several

elegant mathematical models have been developed in studying these factors (R11). As far as is known at present, serum levels of any immunoglobulin above the 2 SD limit are achieved only by increased synthesis. For IgA and IgM the catabolic rate is usually a fixed fraction of the intravascular pool; i.e., the fractional catabolic rate (FCR) is constant and independent of the serum level so that the subsequent half-life ($T_{½}$) is also constant. IgG differs in that the FCR becomes higher with higher serum level so that the $T_{½}$ shortens.

2.1. IgG Globulins

IgG globulins, being 7 S in size, are distributed readily throughout the body fluids (50% are extravascular), and some 2–3 g are turned over daily.

Since the dominant subclass is IgG_1, and IgG_2 and IgG_4 behave similarly, these set the overall apparent catabolism of IgG. With a normal serum level (1000 mg/100 ml), the FCR is 6–7% and the $T_{½}$ is 22 days. If the level is lowered below 100 mg/100 ml in an otherwise normal subject, the FCR becomes 2% and the $T_{½}$ lengthens to 60 days. This is of value in replacement treatment of hypogammaglobulinemia due to inadequate synthesis of IgG. With elevation of the serum level, the FCR rises to a maximum of 18% when the normal mechanism protecting IgG from catabolism becomes saturated, at about 2500 mg/100 ml and above, shortening the $T_{½}$ to 10–13 days. This means that, when a myeloma IgG is reduced from 2.4–1.8 g/100 ml, a substantial decrease (some 40%) in IgG production has been achieved; with a similar reduction from 240 to 180 mg/100 ml, however, decreased synthesis of only some 20% has occurred. A model to explain this phenomenon has been proposed (B22) and modified (R11).

The IgG_3 subclass differs from the others in having a faster catabolism, FCR 17%, $T_{½}$ 8 days (S24). It is interesting that elevation of any one of the four subclasses increases catabolism of the others (R11).

Because IgG normally enjoys a long $T_{½}$, any factor increasing IgG catabolism will have a profound effect on IgG level. Thus, when (e.g., in the nephrotic syndrome or in protein-losing enteropathy), albumin is lost and the fall in serum level stimulates protein turnover in the liver, although proteins made by the liver show increased catabolism they also show increased synthesis. IgG, being largely synthesized eleswhere, suffers in the catabolism, which can rise to nine times the normal, and the FCR can rise to 63% (A4). The plasma cell synthesis rate cannot compete, and the serum level of IgG can be reduced to 100 mg/100 ml. At such a serum level, the daily loss of IgG in the urine can be as little as 0.5 g, and with highly selective proteinuria even the IgG filtered through the glomerulus each day cannot account for the increased turnover. This

catabolic hypogammaglobulinemia characteristically affects the usually long-lived IgG much more than the normally short-lived IgA or IgM, and a pattern of reduction of serum levels as shown in Fig. 10:8 is typical.

Hypo-IgG-globulinemia is also associated with myotonic dystrophy where the FCR can be 14% with a $T_{½}$ of 11 days (W12). This inborn error of IgG metabolism may be a loss of Brambell's protective intracellular carrier of IgG (B20) and some half of affected patients have subnormal serum levels of IgG. Rarely, similar endogenous hypercatabolism is found on a genetic basis and can affect several proteins (R11). It is therefore clear that a subnormal serum level of IgG may be the result of increased catabolism or decreased synthesis.

2.2. IgA Globulins

Estimates of the daily catabolism of IgA vary from 0.6 g (G7) to 2 g (S17), mainly because of different estimates of the normal serum level, as all workers agree on a $T_{½}$ of about 6 days. It also appears that the degree of ^{131}I labeling may have a bearing on how much IgA can enter the lamina propria pools (see Section 3.3) and become dimerized for secretion. The absolute total daily synthesis is thus not clear (probably 1.5 g), but the 7 S IgA of the serum is distributed similarly to IgG and has an FCR of 40%. Thus even the increased catabolism of protein-losing enteropathy increases the FCR only to 60%, and the serum level rarely if ever falls below the 2 SD lower limit of normal. Subnormal IgA levels are therefore almost always due to impaired synthesis. It is of interest to note here that subjects born without IgA, presumably sensitized by breast-feeding or kissing, often develop antibodies to IgA and thereby show increased catabolism and occasionally reactions to administered IgA (S32).

2.3. IgM Globulins

Although estimates of the $T_{½}$ of IgM vary from 5 days (B7) to 10 days (O4), Olesen used IgM cold agglutinins whose survival may have been influenced by repeated adsorption onto red cells, avoiding intracellular catabolism.

IgM, being 19 S, when it finds its way directly from the marrow or lymph into the bloodstream is only slowly distributed to tissue or joint fluids (B17, O4), some 75% being retained in the circulation. This shows an FCR of 36% with an estimated daily turnover of 0.4 g. The same remarks upon increased catabolism apply as for IgA (Section 2.2), so that a subnormal serum level of IgM is almost always due to impaired synthesis.

2.4. IgD Globulins

These globulins show a raipd turnover, $T_{½}$ 3 days, FCR 37%, with a distribution similar to IgM despite their 7 S size (R9).

2.5. IgE Globulins

These apparently have a very evanescent transit through the serum, presumably before becoming bound to tissue mast cells, etc. The FCR is 89% and the apparent $T_{½}$ is thus 2.4 days (W2). With the rapid removal of IgE followed by a rapidly increased synthesis following exposure to allergen, and the above catabolism, it seems that the best time to detect a raised IgE serum level is about 3–4 days after exposure.

2.6. Bence Jones Proteins

Turnover studies of Bence Jones proteins have been bedeviled by the use of tracer amounts given intravenously to normal men or mice. There are almost no studies of autologous labeled Bence Jones in myeloma patients with normal renal function. Tracer amounts are almost completely metabolized in normal animals, with very little appearing in the urine. Stopping of the urine flow or poisoning the tubules with maleate prolongs the $T_{½}$, and it is clear that such tracer amounts are filtered through the glomeruli and readsorbed by the renal tubular cells and presumably therein catabolized (R11). However, in the most severe tubular proteinuria, barely 1 g of protein daily is lost in the urine. Where patients with preexisting proteinuria acquire superadded tubular damage, the daily output increases by only about 1 g, so that this probably represents the maximum capacity of the tubules to readsorb proteins. In clinical myelomatosis, this load is often exceeded, and with urinary outputs of 10 g daily, catabolism of Bence Jones proteins becomes important only when renal failure compels retention. Thus while normal renal function exists, most Bence Jones protein is probably excreted in the urine; thus, following the daily output has been a useful guide to the management of the patient.

3. Known Functions of Human Immunoglobulins

3.1. IgG Globulins

This major class accounts for 73% of the immunoglobulins in normal serum and itself contains four subclasses in the approximate proportions, IgG_1 70%, IgG_2 16%, IgG_3 10%, IgG_4 4%. Individual functions for each class may yet be delineated; e.g., it is known that IgG_1 and IgG_3 fix complement, but since IgG_1 is the major component, what little is known of the main class function probably derives largely from the IgG_1. IgG

antibodies seem particularly to arise in response to soluble antigens, such as bacterial toxins, and usually appear after an initial response of antibodies in the IgM class. This may be so for three reasons. First, IgG antibodies have higher binding affinities than IgM antibodies. As IgG synthesis occurs, the identical IgG receptors on the surfaces of the proliferating antibody-forming cells will bind available antigen best, and thus deprive IgM precursors of antigen so that preliminary IgM antibody formation is switched off by subsequent IgG formation. Second, until IgM antibodies have lysed intact foreign cells, many soluble antigens are not readily available to IgG producers. Third, in the newborn, IgM production matures before that of IgG. Whatever explanations are correct, the neutralization of the soluble products diffusing away from invading organisms seems to be largely the prerogative of IgG antibodies. With isolated IgG deficiency two clinical patterns illustrate this function (Section 5.6). First, pyogenic infections of the tissue spaces occur, i.e., recurrent pneumonia, not bronchitis; organisms (e.g., streptococci, staphylococci) with soluble toxins have the advantage. Second, the hemolytic uremic syndrome occurs; here it is believed that a failure to neutralize toxins allows these to defibrinate the blood, and a fibrin mesh is formed (especially in the glomeruli) through which red cells are forced, thus acquiring their characteristic deformities (B21).

Isolated increase in IgG globulin is rarely seen as a normal response to the environment. It is characteristic of many autoimmune phenomena (H19) and may represent a continuous autogenous production of soluble antigens (see Fig. 10:1).

3.2. IgM Globulins

The low serum level of IgM globulin (only 7% of the immunoglobulins) perhaps reflects its potent ability to destroy foreign surfaces. It is generated especially in response to particulate antigens, and the more so when these are presented continuously and directly to the blood stream (see Fig. 10:3) (H27). It has been estimated that a single molecule of appropriate IgM antibody can become bound to a red blood cell, then fix and activate complement to a stage where a hole forms in the red cell membrane and lysis ensues. In this respect IgM seems to be about 7000 times more efficient than IgG. This power is better appreciated when it is calculated that such IgM antibody at 50 μg/100 ml could destroy half the red cells in the blood. Such a level could not be detected by ordinary methods for IgM and provides a challenge to a Coombs test even if anti-IgM is used; its contribution can also be missed in transplantation rejection unless very sensitive methods are available (W9). With isolated IgM deficiency, septicemia is common (Section 5.8): organisms can cross

TABLE 1
HUMAN IMMUNOGLOBULINS

Property	IgG	IgA	IgM	IgD	IgE
Molecular weight[a]	155,000	170,000	950,000	160,000	190,000
Sedimentation constants[a]	7 S	7 S	19 S	7 S	8 S
Heavy chains	γ_{1-4}	$\alpha_{1,2}$	$\mu_{1,2}$	δ	ϵ
% Carbohydrate	3	8	12	12	10
Mean serum level (mg/100 ml)	1000	260	100	3	0.03
$T_{1/2}$ (days)	24	6	5	3	2
Daily turnover (g/70 kg)	2.3	1.7	0.5	0.03	0.0014
Distribution:					
Intravascular	40%	40%	80%	73%	Yes
Other		Secretions			Mast cells
Placental transfer	+	0	0	0	0
Toxin neutralization	++++	?	+	?	?
Bacteriolysis	+	+	++++	?	?
Viral inhibition	+	+++	+	?	?
Reaginic	?	0	0	0	+++
Known function	Inactivation of substances crossing the tissue spaces	Protect body surfaces	Prevention of septicemia	?	Reagins

[a] 7 S IgM and >23 S complexes occur. Higher polymers can occur in all classes. In old serum, 10% IgG exists as dimer, 10 S. In serum, up to 15% IgA exists as dimer. In secretions, IgA is mostly a dimer together with secretory piece, 11 S, molecular weight 380,000.

the blood stream with impunity. Because of its large molecular size (19 S, M.W. 1,000,000), IgM in the circulation is largely confined to it. To be effective in the tissues, joints, or cerebrospinal fluid (CSF), it has to be made *in situ*. Finally about half the circulating small round white cells in the blood carry IgM-like sites on their surfaces (only 5% carry IgG sites) either as receptors or as an indication of their synthetic capacity. With all this evidence there seems to be little doubt that IgM has a major role in the protection of the circulation. It may also offer the second humoral line of defense in the gut (Section 3.3).

3.3. IgA Globulins and Secretory Immunoglobulins

Although its serum level is only 19% of all the immunoglobulins, IgA has a faster turnover (Section 2.2), so that its daily synthesis is almost up to that of IgG. Local antibody production in the gut was clearly shown by Davies in 1922 (D1). After Heremans discovered IgA in human

serum, Gabl and Wachter (G1) identified IgA as the major immunoglobulin in human saliva. Hanson and Johansson (H3) established that IgA in secretions contained additional carbohydrate antigen, now known as secretory piece, and Tomasi and Ziegelbaum (T10) showed that secretory IgA was an 11 S molecule of larger size than the 7 S IgA of the serum. It is now clear that major sites of IgA synthesis are the laminae propriae underlying mucous membranes (C18), e.g., throughout the gut and respiratory tract.

In man perhaps 60% of IgA is synthesized in such sites; although the bone marrow and other sites synthesize the rest, it may be that they are largely populated by IgA precursors, which largely arose in the laminae propriae and then migrated.

The 7 S IgA monomer released from cells under mucosae seems to be largely taken up by the epithelial cells, where it is dimerized and a secretor piece is added prior to its active secretion as 11 S secretory IgA (see Fig. 5). That secretion is active is evidenced by an apparent IgA clearance (across the membrane) some thirty times higher than for albumin, a smaller molecule (H12).

The secretory piece also protects the molecule from the digestive enzymes. It is therefore available right through to the feces, and such IgA coproantibodies are known to be vital in the defense of the gut against the enteroviruses, e.g., poliomyelitis (B10). The 11 S secretory IgA also seems able to fix complement (7 S IgA cannot). After holes have been made in the superficial membranes of gram-negative bacteria, the enzyme lysozyme (also available in the secretions) can then attack and break up the underlying polysaccharide backbone, and the whole system (11 S IgA, complement, and lysozyme) results in lysis of the bacteria (A1). It would seem that a little circulating 7 S IgA can gain access to the subepithelial pool and become secreted (S23). The subepithelial pool of 7 S IgA can also travel backward, via the lymphatics, to gain the bloodstream, or directly via the portal veins. It is therefore not surprising that a mainly isolated increase in serum IgA-globulin level can result from infections of the gut and respiratory tract (see Fig. 10:2). Since secretory IgA can also be found in tears, sweat, milk, and the genitourinary tract, infections in these areas can also elevate the IgA, proportionate to the area involved. Isolated deficiency of IgA (Section 5.7) is commonly associated with recurrent respiratory tract infections and gut diseases; however, some subjects enjoy a normal life, probably because they compensate with local IgM production (the second line of humoral defense): relatively high levels of IgM are found in their saliva and jejunal juice. Where IgA and local IgM are both deficient (H16), attempted compensation may be seen as lymphoid nodular hyperplasia. The overall evidence admirably collected by Heremans (H15) shows

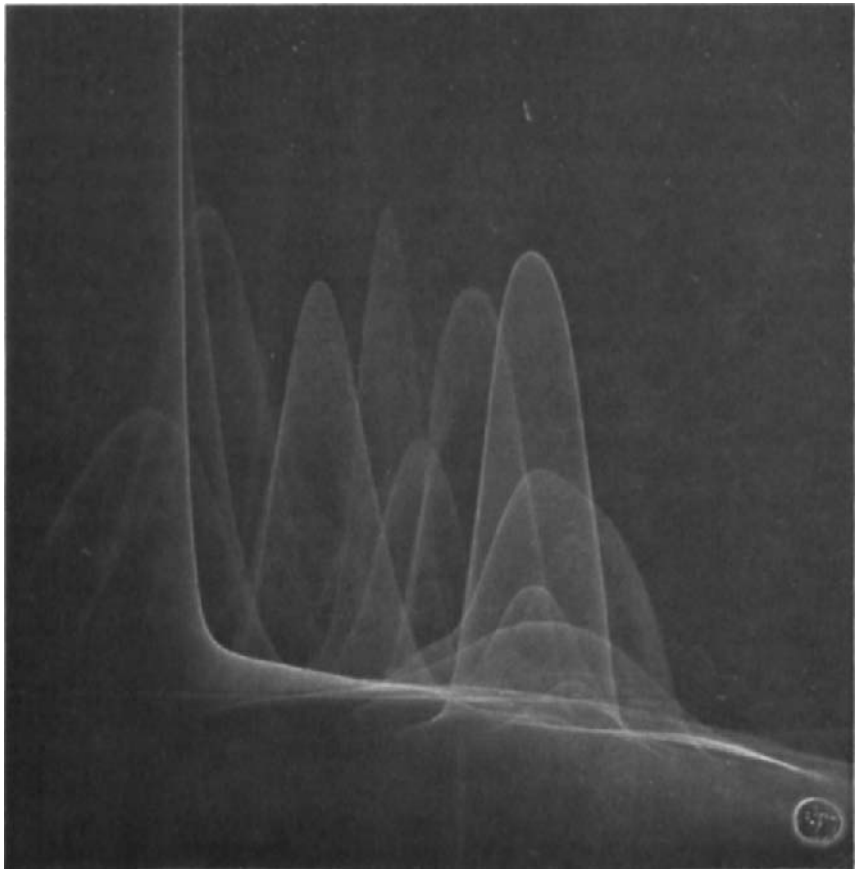

FIG. 4. Electrophoresis of serum in agarose along the bottom toward the anode on the left has been followed by electrophoresis vertically into agarose containing antihuman serum. The areas under the peaks can be made proportional to concentrations of many of the proteins. Satisfactory resolution can be achieved in 18 hours on 5 × 5 cm standard glass slides using automation (V1).

that IgA plays a major role in contributing to an antiseptic slime over the surfaces and passages of the body. In this role, IgA appears to show regional localization (O3) and regional disorders of IgA are becoming recognized, e.g., α-chain disease initially confined to the jejunum (S8) and possibly celiac disease (H40).

That IgM can apparently be cleared across the membranes at a rate similar to IgA (H12) also implies its active secretion in the gut, and its role as the second line of humoral defense has been stated. IgM may have its own secretory mechanism and secretory piece, and indeed this may also be true of IgE and IgD. It would nevertheless seem that the

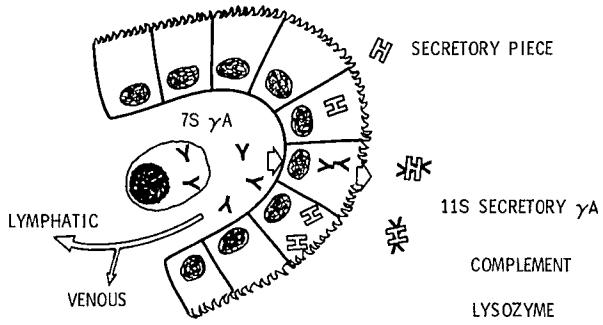

FIG. 5. The concept of secretory IgA. Locally synthesized 7 S IgA (which can leak back) is largely taken up by epithelial cells, dimerized, and to it is added secretory piece prior to its secretion as 11 S secretory IgA. Courtesy of the *British Journal of Hospital Medicine* (H33).

IgA system is our latest and most sophisticated evolution. It provides a stable complement-fixing lethal antibody outside the body, of which only trace amounts ever seem to leak back (T6), and a 7 S noncomplement-fixing information system on the inside. In contrast IgM and IgG can be lethal, but can also backfire by fixing complement within us. The role of serum 7 S IgA still awaits complete understanding, but it is known to provide the chief defense mechanism against certain viruses (T7).

3.4. IgE GLOBULINS

In the best hands, antibodies that are reaginic in man have so far been identified as belonging to the IgE class (I3, J2). These antibodies bind to sites (probably mast cell membranes) in the capillaries and tissues (especially in the nasopharynx and bronchi) (see Fig. 6). When the allergen subsequently becomes bound to the antibodies, this triggers the release of the amines (histamine, serotonin, bradykinin, slow reacting substance, etc.) which mediate the local anaphylactic reaction, i.e., immediate hypersensitivity. This could have a useful function in shaking off helminths that try to become attached, and narrowing the bronchi should make their entrance more difficult, so that IgE antibodies may play a role in defense against helminths.

Some people, however, find such reactions a great nuisance. These may be those who have an imbalance, e.g., a poor vocabulary of IgG and IgA antibodies with excessive production of IgE (K2). The object of densensitization courses of immunization in these patients is to try and increase the response in IgG antibodies which would have higher binding affinities and compete effectively to deprive IgE sites of antigen. Because IgE normally has a very low serum level (250 pg/100 ml), any increase in reaginic antibody will be readily seen as an increase in total

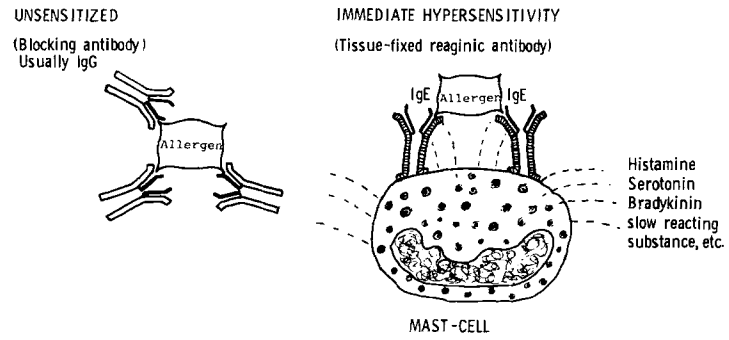

FIG. 6. Diagram to show how, when allergen becomes bound to mast-cell fixed reaginic antibody (IgE), the vasoactive amines mediating immediate hypersensitivity are discharged. The mast cell is capable of becoming recharged, etc. Excess IgG-antibodies can block the access of allergen, which acts only when bound to two IgE molecules.

serum level of IgE (a rise above 1000 pg/ml is significant). With subsequent rapid binding to tissue sites, such elevations may be transient and are best sought on the third day after suspected exposure to an allergen. With elegant techniques (W8) it is possible to identify not only the IgE, but also the allergen to which it is directed. With a given serum, the amine-releasing ability (i.e., reaginic activity) is directly proportional to its IgE content. In different individuals, however, there are varying amounts of competitive antibodies so that the same high level of IgE may have a variable final effect. Even in the same individual the IgE level does not per se indicate susceptibility, e.g., during desensitization the IgE level often rises yet is often nullified by IgG antibodies. After many months a desensitized patient may in fact have no increase of IgE on exposure to the allergen, i.e., IgG affinity claims all the antigen and the patient is clearly nonreaginic for that antigen. At present it seems fair to say that if a patient's symptoms are due to reaginic hypersensitivity, a high serum level of IgE should be demonstrable at some time, and ideally that IgE should be shown to be directed against the suspected allergen. Severity of symptoms, however, is not readily correlated with a spot IgE level (a high level may even be asymptomatic), and it seems that the biological assay of amine release may offer the best guide to reaginic status in an individual patient.

3.5. IgD GLOBULINS

Very little is known about IgD, and no clear function has yet been attributed to this class. Apart from IgD myeloma, personal assays in over 2000 sera have provided no clinically useful information.

In a nutshell, *IgG protects the body fluids, IgA protects the body surfaces, IgM protects the bloodstream, and IgE mediates reaginic hypersensitivity.*

4. Secondary Immunoglobulin Deficiencies

Following Bruton's (B25) recognition of the syndrome of *agammaglobulinemia* (AG) (easily diagnosed by simple serum electrophoresis), Giedion and Scheidegger (G5) realized that an apparently normal electrophoretic γ-globulin could be inadequate. Rosen introduced the term *dysgammaglobulinemia* (DG) (which should be reserved for this situation, as in myelomatosis the γ-globulin does not usually look normal). Barandun (B4) then introduced the term antibody deficiency syndrome (Antikörpermangelsyndrom) to cover these immunoglobulin defects, conveniently split into two main groups by the simple screening procedure of serum electrophoresis.

The antibody deficiency associated with certain conditions is believed to result somehow from those conditions, to which it is therefore called secondary. Secondary AG and DG are 10–100 times commoner than the primary forms, which mostly occur on a genetic basis and are considered below (Section 5).

Pedantry introduced the term hypogammaglobulinemia (although we are all quite happy to talk about anemia). Deficiency of IgG can be arbitrarily defined as severe when the serum level is less than 200 mg/100 ml (20% MNA, see Section 6.2 and Table 4), or moderate when from 200 to the -2 SD lower limit of normal for age, and in this review I use the terms severe AG and moderate AG to describe these. During the first 6 months of life, infants commonly show levels below 20% MNA, so that in this period 10% MNA (100 mg/100 ml) is better used as the arbitrary limit (M11). Although these definitions are arbitrary they are useful in that it is known that within one year 70% of patients with severe AG will suffer severe infection (H19) and that without treatment 65% may die (M11). Moderate AG is complicated within a year by severe infection 40% (H19), but as this is so common (H25, M11) my present policy is chiefly to observe such patients: some will earn their γ-globulin treatment, others can manage with supportive measures such as early treatment of any infection.

Strictly speaking, the term dysgammaglobulinemia can be used only when antibody deficiency has been established by challenging the patient with a series of antigens with known reliable normal ranges of responses (H32). In practice most cases of DG are associated with severe deficiency of IgM and/or IgA, and for convenience secondary DG here will indicate

patients with subnormal levels of IgM and/or IgA who had IgG levels within the normal range.

Incidence of Secondary Immunoglobulin Deficiencies

Among 20,000 new patients screened by serum electrophoresis and confirmed by serum IgG measurements, severe AG was found in 130, and moderate AG in 445 (H25). From the yearly rates above, it can thus be said that infection in at least 1.3% of all hospital patients is predisposed to by secondary AG.

Among 11,000 new patients screened by serum immunoglobulin estimations, secondary DG was found in 112 (H25). Thus secondary immunoglobulin deficiencies occur in some 4% of hospital patients, and are likely to be the cause of symptoms in at least 2%. That it is indeed so common is confirmed by the large numbers of patients reported by other workers: 100 (B11), 118 (C5), 70(A5). Two patterns of immunoglobulin deficiency predominate (see Fig. 10). Pattern 8, low IgG with IgA and IgM within normal for age, is typical of prematurity or delayed maturity (the term physiological hypogammaglobulinemia is avoided, as it fails to distinguish these), marrow disorders, and catabolic hypo-IgG-globulinemia. Pattern 9 is typical of toxic DG and antibody deficiency secondary to lymphoid neoplasia (see Table 2).

4.1. Prematurity

As first described by Hitzig (cited in H37) the premature baby can develop severe AG. This is because the mother's gift of IgG is largely transferred across the placenta in the last trimester (H37) (see Fig. 7). Thus babies born before 22 weeks of gestation will have severe AG, and those born before 34 weeks will develop severe AG within 2 months (Y1). It has been shown (H37) that a single prophylactic dose of γ-globulin

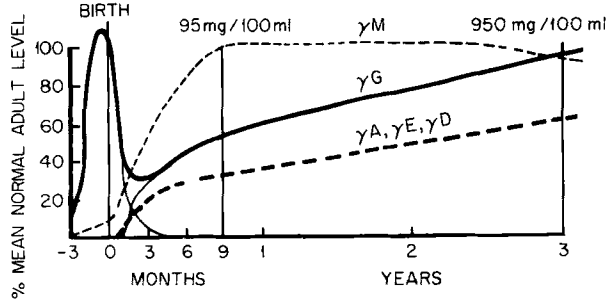

Fig. 7. Maturation to adult serum immunoglobulin levels. IgA, IgD, and IgE do not gain full maturity until adolescence. Courtesy of the *British Journal of Hospital Medicine* (H33).

TABLE 2
SECONDARY IMMUNOGLOBULIN DEFICIENCIES[a]

Predominantly Pattern 8 of Fig. 10

Prematurity
- At birth before 22 weeks gestation
- Soon after birth before 34 weeks gestation

Delayed maturity
- Slow starters
- Maternal allo-antibodies (familial)
- Incomplete Bruton's etc. (familial)
- Maternal agammaglobulinemia
- Maternal IgG paraproteinemia

Marrow disorders
- 40% of hypoplasia
- 10% of extensive bony metastases
- 50% of myelosclerosis
- 20% of paroxysmal nocturnal hemoglobinuria

Catabolic hypo-IgG-globulinemia
- 90% of nephrosis
- 55% of protein-losing enteropathies
- 50% of severe malnutrition
- 50% of myotonic dystrophy
- 5% of thyrotoxicosis
- Thoracic duct fistula
- Idiopathic
- High levels of corticosteroids
- Diazoxide treatment

Predominantly Pattern 9 of Fig. 10

Toxic dysgammaglobulinemia
- Prolonged uremia
- Gluten-sensitive enteropathy
- Diabetes mellitus without proteinuria
- Following severe infection
- Rubella *in utero*
- Cytotoxic therapy

Malignant immunocytomata
- **68**% IgG—myelomatosis
- **28**% IgA—myelomatosis
- **18**% Bence Jones only—myelomatosis
- **11**% IgD—myelomatosis
- **14**% IgM—myelomatosis
- **17**% No paraprotein myelomatosis
- **5**% Waldenström's macroglobulinemia

TABLE 2 (*Continued*)

Lymphoid neoplasia
10% Reticulosarcoma
12% Mycosis fungoides
9% Hodgkin's disease
34% Lymphosarcoma
40% Giant follicular lymphoma
60% Chronic lymphatic leukemia
Spindle cell thymoma

[a] Percentages in roman type refer to deficiencies with moderate AG; percentages set in boldface type refer to those with severe loss of normal IgG.

given to premature babies can reduce the subsequent infection and death rate, and in any event such babies should be carefully followed up with immunoglobulin estimations for up to 6 months of leaving hospital. Although cot death surveys are reported negative (B2), I have personally found severe AG in three such instances, where a premature birth had not been carefully followed (see also S28). The more rapid maturation of IgM and salivary IgA is reassuring as to eventual normality, which is the reward for a small effort spent at this period, as against years of hard work and eventual failure in congenital AG (see Section 5.1).

4.2. Delayed Maturity

Up to 4% of babies, though born at full term, fail to replace the maternal IgG in time, and 1% suffer severe AG (H25). Very rarely this is because the maternal IgG itself was too low (H48), although surprisingly good placental transfers, against the gradient, can be achieved by mothers with serum levels of 200 mg/100 ml (H19, H37). Another established cause is maternal antibodies against the infant's allotype of IgG (F13). The claim that they represent genetically incomplete expression of the very rare Bruton's disease (S21) falls far short of the observed incidence, although in IgA-deficient families this delayed maturation of IgA is seen (Section 5.7). Finally delayed maturation of IgG is recorded (L8, R1) where a maternal paraprotein presumably influenced catabolism and synthesis. All these elegant phenomena, however, do not seem to account for the majority of babies showing delayed maturity, who might more simply be described as slow starters. Most catch up within a year, although a few have taken up to 3–5 years. In some, prophylactic γ-globulin treatment is worthwhile until a self-maintained IgG level shows that the patient is coping alone; and in all, careful observation is indicated.

Valuable findings are normal maturations of serum IgM and IgA

(H48, S20) or salivary IgA (S12) and normal lymphocyte transformation, which helps to distinguish this common disorder from the much rarer primary deficiencies (Sections 5.1, 5.3). Again the effort can be finally rewarded with a normal healthy child.

4.3. Marrow Disorders

In mammals it would seem that some 60% of antibodies are synthesized in the bone marrow (A8), in which some 66% of the plasma cells are producing IgG (D7). It is therefore not surprising that AG can be found with diseases severely affecting all the red marrow of the bones (see Table 2). In paroxysmal nocturnal hemoglobinuria a further factor could be IgM antibodies against IgG (K1).

4.4. Catabolic Hypo-IgG-globulinemia

This has already been considered in Section 2.1; it is included here and in Table 2 to remind the reader that this is a common cause of IgG deficiency. Although it accounted for 20% of all the moderate AG and 17% of all the severe AG found among hospital patients (H25), it did not often result in infection. This was presumably because the quality and experience represented in the remaining IgG was good, and it was largely in young children with less experience that the few infections did occur. This is fortunate, as replacement therapy with γ-globulin would have to be at 3–9 times the usual dosage, a level that I have not yet needed to advise.

High dosage corticosteroids can increase IgG catabolism and can also impair synthesis (R11). No one would deny that such treatment can reduce serum hyper-IgG-globulinemia; however, in over 100 patients I have never seen this result in severe AG, and in moderate AG in only 3. Diazoxide, a drug used on a long-term basis to treat idiopathic hypoglycemia of childhood, has been shown to induce IgG deficiency (B1), but among the 18 cases in the literature (W13) and a further 6 cases observed by me, only 9 developed moderate AG, none developed severe AG, and none seemed to become prone to infection.

4.5. Toxic Dysgammaglobulinemia

In this group the immunoglobulin deficiency is thought to result from circulating factors suppressing immunoglobulin synthesis. Renal failure (H19, H25) celiac disease (A10, B16, H38), diabetes mellitus (B11, H25), severe infection (H25), and rubella *in utero* (H25, P9, S22) can have this result. Immunosuppressive (cytotoxic) treatment produced severe AG in only 1 of 54 patients, although DG resulted in another 4. Thus while such treatment is well known to predispose to severe infection

(especially when combined with corticosteroids), the serum immunoglobulin levels do not explain this.

In general, toxic DG first produces a lowering of the IgM level, then the IgA level, and finally the IgG level. The sequence usually takes months and cannot be explained by differences in catabolism, and only twice have I seen acquired IgA deficiency precede IgM deficiency. It would seem that IgG respresents predominantly secondary responses to antigens, and as such is harder to delete, whereas IgM primary responses can more readily be inhibited.

4.6. Antibody Deficiency Secondary to Lymphoid Neoplasia

The same sequence of suppressed synthesis, IgM, then IgA, then IgG to result in pattern 9; Fig. 10 is characteristic of the commonest predisposition to immunoglobulin deficiency, lymphoid neoplasia. The sequence may take years as shown in Fig. 8, where the highest incidence and most severe DG and/or AG is found with the neoplasias compatible

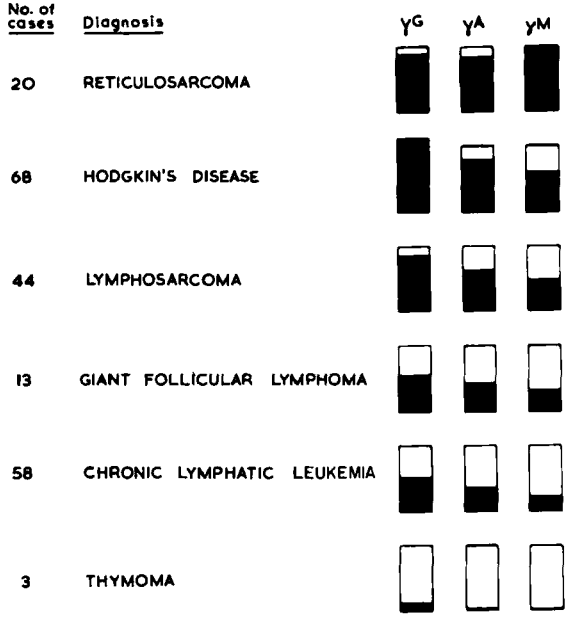

Fig. 8. Immunoglobulin deficiency in 206 patients with malignant reticuloses. The white boxes indicate 100% mean adult serum levels; the black areas, the mean levels found in patients with malignant reticuloses. In general, deficiency becomes more severe with longer duration of disease, and affects IgM more than IgA, more than IgG. Reproduced by courtesy of the *Proceedings of the Royal Society of Medicine* (H25).

with longest survival. It is known (F1) that the most severe AG is found in patients who have had chronic lymphatic leukemia the longest. Thymoma may also preexist for up to 9 years before severe AG develops (W15), although conversely thymoma can emerge in patients known to have preexisting AG (P6).

The more rapidly fatal reticulosarcoma hardly has time to affect serum immunoglobulin levels. Of the patients reported in Fig. 8, 18% have had severe pyogenic infections, the incidence correlating well with the IgG level (H25). However a further 10% have had proven candidiasis or pneumocystis carinii which could not be correlated with IgG deficiency but was related to IgM deficiency (H25) and impaired cellular immunity (L2).

Malignant immunocytomata (see Section 7) also are associated with antibody deficiency. The incidence of severe AG or severe DG (IgA and/or IgM levels less than 20% MNA) is given in Table 2, and in myelomatosis correlates well with the incidence of infection (H21, H28). In macroglobulinemia however, some 30% of patients suffer excessive infection and few have severe AG (H21); indeed many have a serum IgG level above normal. The mechanism of infection here is that excess IgM can blindfold the neutrophiles and impair phagocytosis (P1, P4), possibly by saturating their immunoglobulin-complement receptor sites, because thoroughly washing the cells restores normal function.

In the majority of patients there is a clear picture of lymphoid neoplasia preceding the secondary antibody deficiency. In about half the patients with IgG-myelomatosis (about 20% of all those with malignant immunocytomata), it is clear that the high serum level of IgG paraprotein increases the catabolism, and lowers the serum level, of the normal IgG (S18). However, in the other 80% of malignant paraproteinemia, and in the other lymphoid neoplasias, it has been established that decreased IgG synthesis is responsible (A4), and it has already been noted (3.2, 3.3) that subnormal IgM and IgA are nearly always due to impaired synthesis.

How does this suppression of normal immunoglobulin synthesis occur? Simple displacement of the normal plasma cells by neoplastic cells seems unlikely, because in the marrow this would also result in leukoerythroblastic anemia; this in fact is found in fewer than 5% of these patients. Marrow occupation would also depress IgG first (Section 4.3), whereas this is usually the last to fall. Furthermore, IgA is largely derived from the gut, which is rarely directly involved by tumor. It has also been suggested that the neoplastic cells misappropriate available amino acids at the expense of normal plasma cells. Immunoglobulin deficiency is, however, unusual with most other types of neoplasia, and even among the

reticuloendothelial malignancies it cannot be correlated with the extent of the tumor. These theories also fall down with localized tumors which can be associated with immunoglobulin deficiency. In two patients, removal of an apparently solitary myeloma was found to restore immunoglobulin levels to normal (H19). In two other patients with severe hypogammaglobulinemia (IgG 160, 140 mg/100 ml) and gross splenomegaly, splenectomy was followed by recovery of IgG levels (500, 800 mg/100 ml). In these two cases, which are very much the exception, histology revealed giant follicular lymphomatosis of the spleen, quite distinct from the hyperplasia typical of adult hypogammglobulinemia. Neither patient has yet shown metastases at two and three years, respectively. The findings in the above four patients suggest that reticuloendothelial tumors can release some humoral substance that can inhibit the synthesis of normal immunoglobulins.

In a minority of patients there is a clear picture of long-standing antibody deficiency preceding the development of lymphoid neoplasia. It seems that the humoral defect results in overstimulation of the cellular mechanisms of immunity to a degree increasing the risk of mutation. Evidence that immunoglobulin deficiency can itself sometimes be primary to lymphoid neoplasia has been reviewed elsewhere (F12).

5. Primary Immunoglobulin Deficiencies

Secondary deficiencies are 10–100 times commoner than primary deficiencies (Section 4). Among the primary immunoglobulin deficiencies, it is also clear that DG is some 6–12 times commoner than AG (H25). In general the primary deficiencies are more severe than the secondary (see Table 3).

The term dysgammaglobulinemia (DG) is a practical one, covering patients with apparently normal electrophoretic γ-globulin who have antibody deficiency, and thereby reminding us to measure immunoglobulins in suspected cases. For lack (S10) of any coherent classification of DG, needed because of clinical differences in the known varieties, these have been numbered (I–VII) chronologically (H44). In 6 of the 7 known types a characteristic pattern of immunoglobulin deficiency is found (see Table 3); e.g., I have never seen an IgA $< 10\%$ MNA with a normal IgM that was known to be acquired; when the IgM is $<10\%$ MNA on an acquired basis, IgA will usually have become subnormal; the pattern of type III, however, is more commonly secondary in origin (see Table 2), and such causes have to be excluded. Type VI reminds us that where the clinical story is strong enough, normal immunoglobulin levels should not deter us from investigating the formation of specific antibodies (see H32).

TABLE 3
PRIMARY IMMUNOGLOBULIN DEFICIENCIES

Deficiency	% MNA[a]		
	IgG	IgA	IgM
Hypogammaglobulinemia			
Bruton type	**<10**	**<1**	**<1**
Late onset	2–30	10	5
Combined immune deficiency			
Swiss type	**Falling**	**<1**	**<1**
Thymic dysplasia / Achondroplastic	Falling	<1	<1–60
Atypical	Falling	<1–150	<1–300
Dysgammaglobulinemia			
I	40–100	**<10**	**<10**
II	**<20**	**<10**	70–2000
III	**<40**	50–200	50–200
IV	50–300	**<10**	50–800
V	50–200	50–200	**<10**
VI	**50–170**	**50–170**	**50–170**
VII	**<20**	200–400	**<10**

[a] The most constant patterns are shown in boldface type.

5.1. HYPOGAMMAGLOBULINEMIA, BRUTON TYPE

Bruton's infantile sex-linked hypogammaglobulinemia (S10) has an incidence of 1 per 100,000 live male births, and it now seems possible to recognize the female carrier state (F14). Female homozygotes are not described, but may occur now that affected males are surviving longer.

As the boy uses up his mother's gift of IgG globulin (usually after 6 months), infections begin to occur and undue susceptibility becomes evident between one to two years of age. Some 80% of the infections are respiratory, and with successive episodes, permanent residual damage ensues. Whether or not diagnosis is made before this happens has a bearing on subsequent response to treatment, for no amount of γ-globulin can correct bronchiectasis.

Usually the invading organisms are pyogenic (staphylococci, pneumococci, etc.), but others, such as pneumocystis carinii, are found in some 15% (B28). Gastrointestinal, skin, and eye infections, meningitis and septicemia, and infection of several systems also occur, each with an incidence of about 15%. Tuberculosis occurs in some 5%, a higher rate than expected in normals. Most virus infections such as vaccinia, measles, mumps, varicella, and rubella are dealt with normally. A notable exception is infective hepatitis, which can be rapidly fatal or

followed by cirrhosis in these boys and seems to be independent of γ-globulin treatment.

Atopic allergy can occur (R10). Recorded complications include arthritis (G16), malignant disease (M11), dermatomyositis (R10), a neurological syndrome (M11), and amyloid; some 19% of patients experience reactions to immunoglobulins.

Serum electrophoresis shows very little if any γ-globulin. Estimation of immunoglobulins reveals IgG < 10% MNA (100 mg/100 ml), and IgA and IgM < 1% MNA. A few boys show higher levels than these. Isohemagglutinins are usually absent or low. These persisting low levels of IgA and IgM are valuable pointers (S20). Salivary IgA also fails to achieve adult levels by 6 weeks of age (S12). Neutropenia is not uncommon (M11), but lymphocyte counts are normal. Lymphocyte transformation following PHA or other nonspecific stimuli is initially normal but not well sustained thereafter, and there is no production of mRNA in response to specific antigens. On the other hand, the synthesis of DNA following PHA or specific antigens, to which the subject has been sensitized, is in fact indistinguishable from normal (C15). Since to take on the appearance of a plasma cell requires mRNA synthesis, the above findings would seem to explain why lymph node biopsy reveals an absence of plasma cells, and indeed of germinal centers.

The retention of DNA synthesis is compatible with the normal cellular immunity of these subjects. Delayed hypersensitivity tests are usually within normal limits, and homograft rejection eventually occurs (R10). Slight quantitative reductions or delays in these responses only emphasize that the observed reactions usually represent a combined or balanced effect of cellular and humoral immunity (S25).

With adequate treatment (0.025 g of IgG globulin per kilogram), the mortality has been markedly reduced.

Non-sex-linked infantile agammaglobulinemia (P3, S10), has a sporadic incidence of about 4 per million born (M11). It is probably autosomal recessive, affects both sexes, and is otherwise indistinguishable from Bruton's, except that the prognosis seems worse in that 45% presenting in infancy die within 6 months (M11).

5.2. Acquired Hypogammaglobulinemia

When primary (R10), the term hypogammaglobulinemia of late onset has been proposed (S10), as an autosomal recessive inheritance is established in some cases (W15). However discordance in identical twins suggests that the disease can be truly acquired (C20, G13). The incidence is about 1.5 per million living of either sex [equally affected over 10 years of age (M11)].

It is characterized by infections as in Bruton's disease, although a spruelike syndrome with diarrhea, steatorrhea, and other evidence of malabsorption seems to be commoner in adults [some 50% in the United States experience (R10)]. Furthermore, as a longer, more insidious deficiency has existed, many may present with complications, so that hepatosplenomegaly and lymphadenopathy are found in over 20% of adults. Frequently these organs and the lungs are involved with non-caseating granulomata, and this has been called sarcoidosis. However the granulomata are absolutely uniform in size and remain a mystery (R10). Hypersplenism and autoimmune diseases, such as disseminated lupus erythematosus and Coombs-positive hemolytic anemia, are common.

Serum IgG levels are often not as low as in Bruton's disease, although a team (M11) collected seventy adults with levels below 200 mg/100 ml. Lymph node biopsy shows abiotrophy of the follicles, rather than disorganized morphology as in Bruton's disease, and may also show granulomata or even amyloid. Thymoma should be sought.

More than 20% die within ten years, despite γ-globulin treatment. In one patient the granulomatous lesions have responded to corticosteroids (R10). Occasionally spontaneous recovery is observed (M11). It seems likely that when thymoma occurs it may be a complication of the long-standing humoral deficiency (4.6), for in more than 17 patients to date there has been no recorded improvement following thymectomy.

5.3. Combined Immune Deficiency

Since the first description (G9) this condition can now be divided as follows: (i) Swiss-type agammaglobulinemia (H17) also occurring in other Caucasians (M11), Negros and Navajos (R10), and Mennonites (H11); (ii) thymic dysplasia (M18); (iii) thymic dysplasia with some immunoglobulin synthesis (H32); (iv) achondroplasia lethalis (G2, H32); (v) acquired in adulthood (K8).

In (i)–(iv), symptoms usually come on within 6–12 weeks of birth, usually as persistent moniliasis, followed by intractable watery diarrhea and pneumonia with a typical pertussoid cough. Serum immunoglobulins indicate gradual loss of the maternal IgG with (i) no detectable IgA, IgM; (ii) and (iv) transient IgM; (iii) variable levels of immunoglobulins. In all cases isohemagglutinin titers are 1 in 8 or less. Lymphopenia is the rule, severe in (i) and (iv), less severe in (ii) and (iii), and lymphocyte transformation is virtually absent (DNA uptake usually $<$ 2% of normal controls). This can be detected at birth, and if a matching donor (1 in 4 of siblings) is available, infusion of donated bone marrow might be considered in an attempt to correct the stem cell failure. How-

ever, with such a cause of immunoglobulin deficiency, lymphocytes should be tested, for γ-globulin replacement alone is of no avail.

5.4. Dysgammaglobulinemia Type I (Deficiency of IgA, IgM)

Giedion and Scheidegger (G5) were the first to show that an electrophoretically normal γ-globulin could be associated with antibody deficiency. In their patient the serum immunoglobulin pattern showed a persistent absence of IgA and IgM with an apparently normal IgG.

Over 20 patients (children and adults) are now recorded with a male to female ratio of 4:1. For most acquired antibody deficiencies, the rule is a fall in IgM, then in IgA, and finally in IgG. This is the pattern to be expected in adults (B5, G6), and a slow acquisition in childhood has also been observed (B27). In many such cases it may simply represent an incomplete expression of hypogammaglobulinemia of the sex-linked or non sex-linked varieties. It has also been recorded (G25) in a patient with 13–15 trisomy syndrome due to translocation. Patients with this serum immunoglobulin pattern seem to fall into two main groups, probably depending on the quality of the IgG-globulin.

In one group where the IgG is shown to be largely inert, the symptoms and onset are similar to Bruton's disease. Two boys died of measles (L7): in one boy the pattern was acquired between 6 and 10 years of age (B27): twin girls presented at 11 years of age (D2): onset occurred at 23 years in a negress (G6).

In the other group, all the patients have been adults with a history of susceptibility to infection commencing between 19 and 29 years of age. These patients have presented with diarrhea, some evidence of malabsorption, and infestation with *Giardia lamblia*. Jejunal biopsy and radiology have revealed nodular lymphoid hyperplasia (H16). This syndrome is also seen with severe hypogammaglobulinemia in surviving adults (K4). Cases with isolated IgA deficiency where the absence of isoagglutinins has raised a suspicion of inadequate IgM are also described.

The first group, mainly children with infections, shows persistent IgA and IgM deficiency. It is unusual to see any rise in IgG with infections, this being a useful pointer to its dubious quality. Responses to tetanus, pertussis, and diphtheria vaccines are poor, although some response usually occurs in TAB. Unlike the first case described, most others have shown isohemagglutinins. Lymphocytes have been normal in numbers and in transformation to PHA. The response to dinitrofluorobenzene was normal (D2), but other delayed hypersensitivity reactions have been variable, as has homograft rejection. The lymph nodes have varied from normal through primary follicles with no germinal centers (D2), to absence

of primary follicles and no lymphoid tissue in the gut (L7). Usually plasma cells are present and the thymus is normal. It seems worth excluding congenital rubella, as one infected infant survived to 10½ months with a similar clinical picture (P9).

The adult group have shown serum IgG levels from 25 to 50% MNA with IgA and IgM deficiency. The chief contrast with the children has been very low or absent isogglutinin titers. Cellular immunity appears to have been normal; the nodular lymphoid hyperplasia seems to be a compensation for the humoral inadequacy; indeed splenomegaly is also found in half these adults.

The prognosis seems somewhat more hopeful than in Bruton's disease. One male had survived recurrent infections from birth to the age of 25 with only supportive measures (B5).

5.5. Dysgammaglobulinemia Type II (Deficiency of IgG, IgA)

Since the first description (I4), this condition has been well reviewed by Stiehm and Fudenberg (S27), who however mistakenly called this type I.

Over 20 patients (children and adults) were recorded, with a male to female ratio 4:1. In some, inheritance was clearly sex-linked recessive (R10), sometimes with Bruton's disease in sibships and only a transient production of IgM (S27), suggesting an incomplete expression of Bruton's disease. In others the genetics are unknown or the disease may have been truly acquired.

The typical picture is of recurrent respiratory tract infections in a male infant soon after birth, generally in association with enlarged tonsils, cervical adenopathy, and enlargement of liver and spleen (S27). In anyone referred for a second tonsillectomy, this is the first diagnosis. Pyogenic organisms are usual, though extensive verucca vulgaris and pneumocystis carinii are recorded. Pancytopenia and hyersplenism are not uncommon, and neoplastic infiltrations may occur (R10). Serum shows a persistent IgG and IgA deficiency with a raised IgM, tending to be high following infections and sometimes falling to normal with γ-globulin therapy. The IgM appears to be functional in most cases with normal isoagglutinins, but in some these are absent. The response to soluble antigens (diphtheria and tetanus toxoids) is poor. Lymphocytes are normal in numbers and in transformation to PHA. Delayed hypersensitivity is usually normal but can be excessively delayed or be lacking altogether. Neutropenia can be a troublesome complication. In the boys the lymph nodes show no follicle formation and no typical plasma cells; instead there is infiltration with plasmacytoid cells which are PAS positive and produce IgM. This infiltration can become so extensive as to appear neoplastic. The thymus is usually normal. In the adults, lymph

node follicles may appear normal with surrounding plasma cells producing IgM. In infants it is important to exclude rubella (S22).

The prognosis is about the same as Bruton's disease.

5.6. Dysgammaglobulinemia Type III (Deficiency of IgG)

First mentioned by others (H13, S11), this has been studied in detail (H32). After exclusion of known causes (catabolic, etc., Section 4.4), isolated IgG deficiency is occasionally found in infants, children, and adults. The genetics are unknown. Some are sporadic without known consanguinity. Others are found in families with other immunological abnormalities (B29). It is very similar to Bruton's disease. In six of the author's cases, the accent has been on pyogenic infections of the lungs and soft tissues. There has been no diarrhea or malabsorption, no sinusitis, and no septicemia. During episodes of pus formation the patients have been very febrile and ill, with only a slow amelioration following correct antibiotic treatment. Some patients develop the hemolytic uremic syndrome.

The only positive findings have been severe serum IgG deficiency and subnormal levels of antibodies to tetanus toxoid and diphtheria toxoid following adequate challenge. Serum and salivary IgA and the response to oral poliomyelitis vaccine (presumably mainly in the IgA class) have usually been normal, as have serum IgM and isohemagglutinins. Neutrophile function, lymphocyte transformation, and the responses to vaccinia and childhood virus infections were all normal. This seems a milder disease than Bruton's and does well on γ-globulin prophylaxis or even only with prompt treatment of infections.

The tissues, gut, and bloodstream seem to be adequately protected. It seems that pyogenic organisms can flourish when they gain access to the lung parenchyma or tissue spaces. Their exotoxins are apparently not neutralized and allowed to exert their full effect, the patients seeming particularly ill at such times. It would seem that the chief function of IgG globulin is to protect the tissue spaces from the soluble products of invading organisms.

5.7. Dysgammaglobulinemia Type IV (Deficiency of IgA)

First recorded by Heremans (H14) and first associated with predisposition to infection in studies of ataxia telangiectasia (F5, P8), this has an incidence of about 1 in 500 of either sex, but is rare over the age of sixty.

5.7.1. *Associated Diseases*

Collected results from many published papers and a large personal experience enable the following estimates of the incidence of IgA defi-

ciency: 80% in ataxia telangiectasia (much less in France, where it was first noted, see M14); 3% in malabsorption syndromes (gluten-sensitive, tropical sprue); 3% in children with recurrent upper respiratory tract infections.

IgA deficiency has also been observed in 7 patients with central nervous system (CNS) disorders and in 5/7 patients with partial deletions of chromosome 18 (S26). The association with ataxia telangiectasia is not complete and probably represents a coincidence of genes, exemplified by the presence of either defect alone in relatives of propositi. It is of interest that other CNS defects are recorded with IgA deficiency, and partial deletions of chromosome 18 are also associated with mental retardation. It seems, however, that the predisposition to infection is closely correlated to IgA deficiency, so that ataxia telangiectasia per se does not justify separate classification as an antibody deficiency syndrome (S10).

There is also a significantly increased incidence of IgA deficiency in patients with autoimmune or potentially autoimmune disorders, and usually it is not clear which came first. It can be argued that autoimmunity is a complication of immune imbalance subsequent to inborn IgA deficiency (H24). With inborn absence of IgA, exposure to normal human colostrum, plasma, and saliva can result in the production of antibodies to IgA. By the time such patients are discovered the etiological mechanisms are often obscured and IgA treatment is out of the question. The incidence of IgA deficiency is known to be 1–4% in the following conditions: Still's disease, systemic lupus erythematosus, rheumatoid arthritis, Sjögren's disease, warm hemolytic anemia, megaloblastic anemia, idiopathic pulmonary hemosiderosis, thyrotoxicosis, and cirrhosis.

5.7.2. *Genetics*

The inheritance seems heterogeneous. A few are clearly autosomal dominant (H24, S30). Most are probably autosomal recessive (G14). The relationship to CNS disorders, other immune deficiencies, and chromosome 18 merit further study. Because of the heterogeneity, the definition of IgA deficiency is difficult. Serum IgA $< 1\%$ MNA is safe, and $<10\%$ MNA is acceptable if increased IgA turnover can be excluded. Regional deficiencies of secretory IgA may exist.

5.7.3. *Clinical Features*

While some cases may be asymptomatic, the majority are not (B26, H24). There is often a failure of the normal protective action of IgA globulin in the respiratory and alimentary tracts. Recurrent sinusitis, bronchitis, and otitis media are the most common symptoms. While diarrhea may not be prominent, malabsorption can often be established, and

in such cases jejunal biopsy usually shows total villous atrophy, or may even reveal nodular lymphoid hyperplasia (G23). The mucosa and the malabsorption may respond to antibiotics (C3), gluten-free diet (C19), or fresh plasma (G23). Unusual presentations have included hemorrhagic varicella, peculiar infections, and glomerulonephritis (H24). Patients may also present with autoimmune diseases. On the whole the picture is less striking than in Bruton's disease, but these patients often represent a stubborn minority attending pediatric, chest, gastroenterological, hematological, rheumatoid, and other clinics.

Serum IgA globulin is $<10\%$ MNA and mostly $<1\%$ MNA. Saliva and jejunal juice also have undetectable IgA (one exception is recorded, see S33); often an increased level of 19 S IgM (and sometimes 7 S IgM) is found. The serum levels of IgG and IgM are variable, though a given patient usually shows elevations of either IgG or IgM. In the author's experience of some 40 patients, those who show such elevations in the serum are all symptomatic; indeed this suggests an attempt to compensate for impaired immunity. In a similar manner, patients may show enlargement of lymph nodes, liver, and spleen. Biopsy invariably reveals scanty lymphoid follicles with infrequent germinal activity (i.e., impaired humoral immunity), often with marked paracortical hyperplasia and reticulum cell hyperplasia (i.e., compensating cellular immunity). In some cases this is so marked as to overlap with neoplasia, and we have treated such a patient with vincristine, with a good initial response. Applying strict criteria for age (B12), there is no convincing evidence of thymic hypoplasia. The white cell picture is usually healthy, and lymphocyte transformation to PHA and delayed hypersensitivity are normal in most cases (H32). In progressively worsening patients (especially in ataxia telangiectasia), lymphocyte transformation may later become subnormal. Deficient cellular immunity has clearly been established early in one patient (T4).

On the whole this is a mild immune paresis, sometimes asymptomatic, with few patients requiring IgA or fresh plasma therapy. The lack of older subjects and the mortality of five out of twenty-four propositi within two years, indicate that it may take a serious course (H24). Many autoimmune complications occur: sinopulmonary infection may be well controlled with tetracycline prophylaxis.

From the above and from what is known of secretory IgA globulin (H33, T8) it seems that the chief function of IgA globulin is to protect the respiratory passages and the gut. Since most viruses gain access via these routes, and for herpes simplex there is strong evidence that, in serum, IgA antibodies are the most important (T7), it may be that autoimmune disorders arise from inadequate protection against viruses.

5.8. Dysgammaglobulinemia Type V (Deficiency of IgM)

Mentioned in 1962 (W6), IgM deficiency was later associated with septicemia (H43); since then independent cases have come to light (S31).

Patients with undetectable serum IgM are rare, but those with <10% MNA are common (H32). The male to female ratio is 4:1, and the deficiency is also seen in many boys with the Wiskott-Aldrich syndrome.

Some preponderance of IgM deficiency among male relatives (H43) and in families with other immune pareses (B29, G6, S1, W6) indicates genetic origins. Borderline serum IgM levels (?heterozygotes) seem common in parents of propositi (B27, H43).

More than one episode of proven septicemia is now possible in this antibiotic era, and it was during the investigation of such patients that an association was found between septicemia and IgM deficiency, whether primary, secondary, or physiological, in the first 6 weeks of life. Professor J. F. Soothill (personal communication) has found that, in hypogammaglobulinemia subjects, the total level of IgM did not correlate well with septicemic episodes. When the quality of IgM globulin was also taken into account by measuring isohemagglutinin titers, there was a very good correlation between absence of isohemagglutinins and septicemia.

In cases without detectable IgM globulin it is usual to find no isohemagglutinin, and we have now proved septicemia in 10 of 29 patients with idiopathic isolated IgM deficiency. There was no history of recurrent infections, just a sudden septicemia "out of the blue." The only other finding of note was persistent splenomegaly in four patients. In two adults who had splenectomy, the spleens were reported as showing congestive splenomegaly. More striking were the postoperative courses: one patient has had three episodes of septicemia; the other has had two episodes. It seems that the splenomegaly was compensating for the IgM deficiency.

It is important to measure the serum immunoglobulins on several occasions, as transient suppression of the IgM level may be due to the toxemia of septicemic infection. The many causes of secondary falls should be excluded, and a family study be undertaken. No other abnormalities have yet been found; lymphocyte transformation is normal. Isolated IgM deficiency combined with cellular immune deficiency is recorded (S4).

Sudden death from septicemia may occur at any time. In such subjects all acute illnesses should be taken very seriously.

The evidence for the role of intravascular IgM globulin has been reviewed (Section 2.3), and it seems that the chief function of IgM globulin is to protect the bloodstream.

5.9. Dysgammaglobulinemia Type VI (Deficiency of Quality)

Hobbs and Citron showed that normal serum immunoglobulin levels in a man 38 years old could be associated with a severe antibody deficiency syndrome corrected by γ-globulin treatment (H19). Previous workers had shown that deficiencies of antibodies to measles virus (M19), to vaccinia virus (K3), and to other viruses (L10) in the presence of apparently normal γ-globulin, could result in overwhelming infections. However, at those times cellular defects of immunity had not been excluded; these now seem a more probable explanation in many cases (H4, N1).

A 6-year-old girl and a woman 35 years old have had recurrent staphylococcal infections, sometimes with septicemia. Both have repeatedly been shown to have no detectable antibodies against staphylolysin or leucocidin, as well as having subnormal responses to a few other antigens. Both became free of infection and acquired antistaphylococcal antibodies following γ-globulin treatment. In the case of a young man with repeated infections from 2 months of age, yet with normal immunoglobulin levels, it has been shown that the lack of antileucocidin etc., prevented the patient's neutrophiles from killing phagocytosed staphylococci. This defect was corrected passively by γ-globulin treatment, and later by active immunization (D3).

The patients in this group have usually had to earn their full investigation. Three patients had had 213, 5, and 35 hospital admissions, respectively, before starting γ-globulin treatment. Serum and secretory immunoglobulin levels have always been within normal limits, and in one patient always near the lower limits. A useful clue has been a failure of increase of antibody and immunoglobulin levels in the expected manner following severe infections. Antibody deficiency was general in one patient, it was only shown to a few antigens (staphylococcal, brucella) in a second, and it seemed specific to staphylococcal antigens in a third. This little-explored group may thus vary from general to specific immunological unresponsiveness. In our limited experience, lymphocyte and neutrophile functions were normal, with normal delayed hypersensitivity to vaccinia, candida, and tuberculin.

Two adults are well on γ-globulin prophylaxis. Repeated immunization against staphylococcal antigens helped one man until his death at 20 years from pseudomonas infection (D3). A girl was well on γ-globulin treatment for 6 months: when this had been discontinued for a further 6 months, against advice, she died from her third proven staphylococcal septicemia.

Now that subclasses of immunoglobulins are being assayed, such patients may well be found to have subclass deficiencies. In two of our

latest patients, a pure subclass deficiency has been found, affecting IgG_1 only (with compensatory increases in the others to yield a normal total IgG).

5.10. Dysgammaglobulinemia Type VII (Deficiency of IgG, IgM)

This serum immunoglobulin pattern has been associated with proven antibody deficiency (C4). The five recorded patients were all boys (B27, D2, S1). Two parents had IgM deficiency, and one boy in a sibship suggestive of Bruton's disease.

One boy was asymptomatic at and up to ten years of age, and another had recurrent pneumonia from the age of ten. The others had recurrent infection from six months onward, one surviving hemorrhagic varicella.

The serum immunoglobulin pattern has been constant in these cases, and isoagglutinins were absent in four of the boys. The responses to oral poliomyelitis vaccine have been poor: Since this oral vaccine usually evokes IgA antibodies, this suggests that the patients' IgA may be largely inert. Subnormal responses to diphtheria and TAB are recorded. Lymphocyte transformation to PHA and delayed hypersensitivity were normal where tested. The tonsils, adenoids, thymus, and spleen were normal except in one case with lymphopenia, where tomography did not reveal any thymus, and tonsils, adenoids, and lymph nodes could not be detected (S1). The prognosis seems similar to that for Bruton's disease.

It should be noted that Arabian lymphoma of the small intestine, presenting as intractable diarrhea and malabsorption in teenagers of Arabian stock (though also found in Pakistanis) will be associated with serum IgG and IgM deficiency with an apparently marked increase in IgA. The latter is due, however, to polymerized heavy chains only, i.e., α-chain disease (7.6.5) and should not be confused with the present immunoglobulin pattern.

6. Polyclonal Immunoglobulin Patterns

The first real attempt to quantitatively survey serum levels in disease was reported in 1960 by Heremans (H14). In 1965, McKelvey and Fahey (M9) followed up, and to date over 300 papers have appeared; it is not feasible to name all the contributors. In this section we will consider the factors that affect results and the normal ranges and then select and substantiate those areas where clinical value emerges as judged from personal experience of some 50,000 measurements over 5 years.

6.1. Factors Influencing Immunoglobulin Levels

What follows largely applies to fresh serum levels measured against standard serum (thawed once), although other fluids will be considered below.

6.1.1. *Precision*

Immunoglobulins of high purity and of only one molecular size can be prepared; these can be calibrated directly by their ultraviolet absorption at 280 mm (E 1%, 1 cm: 7 S IgG = 13.8; 7 S IgA = 13.4; 19 S IgM = 13.3). Such preparations deteriorate rapidly and also tend to aggregate in pure solution, so they must be used fresh, preferably within 4 hours of isolation. For use as absolute standards, they should (a) represent the variation in their class expected in the sera to be tested—e.g., IgG should be from a normal serum pool representing all four subclasses and normal allotypic variation; (b) be measured using antisera raised against themselves or adequately representative antigen (Section 1.1). A single myeloma (monoclonal) globulin cannot be used here as a standard or as an antigen to raise antiserum because it represents a single idiotype (Section 1.1); conversely myeloma sera cannot be reliably measured against normal standards (H45). With such precautions, a pool of sera from normal adults can be calibrated against pure absolute standards; normal ranges are given below (Section 6.2).

6.1.2. *Reproducibility*

A single estimation by most current Mancini methods can show a 2 SD variability of less than ±10%, and the mean of duplicate estimations can approach ±3% (H34). Our own week-to-week quality control has remained within ±9% over 5 years.

6.1.3. *Individual Variation*

In a given normal subject this usually remains within ±20% over years.

6.1.4. *Genetic Factors*

Apart from the obvious deficiencies and familial hyperglobulinemia, studies in healthy twins indicate that the actual normal levels of IgG and IgA have only a small genetic contribution, i.e., a realizable potential.

6.1.5. *Environment*

Germfree animals have very low levels of immunoglobulins, an observation indicating that the environmental challenge is largely what maintains even normal levels. Natives of underdeveloped countries typically run higher IgG and IgM levels than do the British (see Fig. 9), but after having lived here for some years come down closer to the British levels (about 140% MNA) (C11, H33). The residual elevation can be called *racial* and presumably reflects genetic survival value in their countries of origin. IgA levels, however, are all the same, presumably because there

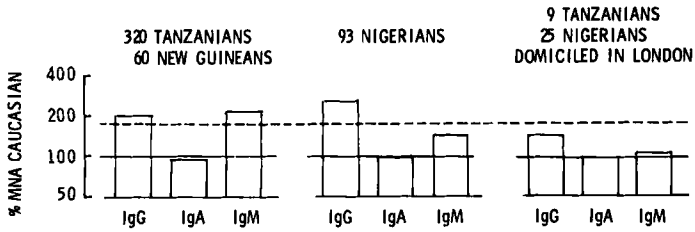

FIG. 9 Racial factors and mean serum immunoglobulin levels. In their native environment IgG and IgM are raised. Domiciled in London for over 2 years only IgG is slightly raised. Genetic potential seems less important than environment. MNA = mean normal adult. Native Nigerian data are adapted from (T13). Courtesy of the *British Journal of Hospital Medicine* (H33).

is little difference in the leak-back from the gut, where antigenic challenges are always at a high level. IgE levels are usually much higher in areas with high rates of helminth infection.

6.1.6. *Sex*

Females above the age of 7 years have an IgM level some 20–30% MNA higher than males (A3, R7).

6.1.7. *Age*

The maturation of serum immunoglobulin levels is shown in Fig. 7, and normal ranges throughout childhood are given below (Section 6.2). It has been claimed that senility is associated with raised levels of IgG and IgA (H1), but we could not confirm this in London.

6.2. NORMAL RANGES (SERUM, PAROTID SALIVA, JEJUNAL JUICE, CSF)

6.2.1. *Serum*

Using a pool of serum from normal adults (54 males, 53 females, Caucasian, London), our calibrations against pure fresh absolute standards yielded mean values of IgG 947, mg/100 ml; IgA 248 mg/100 ml; IgM 94 mg/100 ml; IgD 3 mg/100 ml; IgE 250 pg/ml. Because at present the absolute amounts of immunoglobulins are disputed (often because of inappropriate selection of the standards or the false assumption of a 16% N content), such a pool of normal sera can itself be used as a standard, calling it 100% MNA (mean normal adult), and this has been done throughout this review to enable better comparison of published work. MNA serum stored in aliquots at −20°C and thawed only once, on the day of use, also provides an excellent working standard. Over a hundred papers have given normal ranges for serum immunoglobulins,

but very few have allowed for the *log-normal frequency distribution* (first stressed in G15, H37), which is seen for all classes. Because of the small (sex) difference for IgM only, it is convenient in clinical practice to combine males and females; Table 4 gives our ranges as % MNA. Throughout Figs. 7–11 the 100% MNA line is used, and the variations in diseases are shown on a log scale. It can be shown that 1 week after contracting various infections the mean levels of IgG, IgA and IgM of a group of subjects will be highly statistically significantly elevated above those found before the event, or above those of matched controls (Z1). In an individual patient, however, a spot serum elevation becomes significant only if above the 2 SD limits. As % MNA, these are very similar and are therefore indicated by the broken lines at 175% MNA in Fig. 10.

Where a disease group shows a mean level above these limits or some definite pattern of response, there may be some useful clinical application. As stated in Section 3.2, an increase of IgM by only 50 μg/100 ml could have serious results and be undetectable by gross measurement; i.e., normal results do not necessarily exclude important immune reactions.

Elevation of serum immunoglobulins above normal has thus far been

TABLE 4
2 SD Log-Normal Ranges for Immunoglobulin Levels as % MNA (Mean Normal Adult) (Sexes Combined)

Site	Age	IgG	IgA	IgM
Serum	0–2 weeks	50–95–180	>1	1–7–20
	2–4	39–72–131	1–2–8	6–15–30
	4–6			10–22–52
	6–12	21–40–78	3–8–20	15–35–72
	3–6 months	24–46–89	5–12–30	20–50–91
	6–9	30–52–90	8–15–36	35–70–15
	9–12	31–58–110	12–20–38	45–90–200
	1–2 years	31–65–139	15–35–66	40–90–170
	2–3	37–70–158	17–42–71	
	3–6	54–100–170	20–50–110	
	6–9		28–62–125	
	9–12		34–72–130	
	12–15		40–88–150	
	>15 (Adult)	54–100–170	45–100–172	50–100–180
			γD	
	Adult		30–100–500	
Parotid saliva		<0.2	0.8–6[a]	<2
Jejunal juice		0.1–1.0	1.4–11[a]	1–12
Cerebrospinal fluid		0.1–0.3	<0.5	<1

[a] As compared directly to 7 S IgA of serum. Multiply by 1.4 (11 S diffuses 70% of 7 S) and 2.48 (milligrams of 7 S IgA in 1% MNA) to convert to milligrams of 11 S secretory IgA per 100 ml.

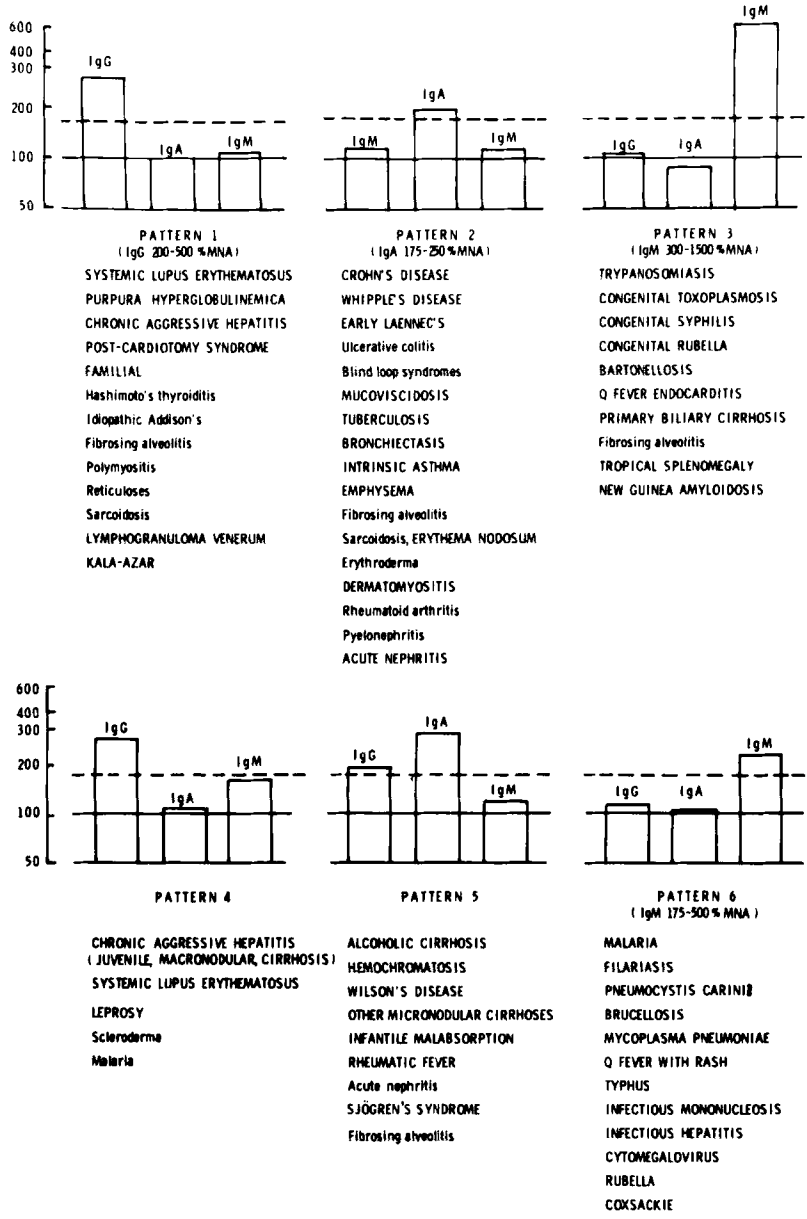

FIG 10. Serum immunoglobulin patterns drawn to log scales of % mean normal adult. Broken lines indicate 2 SD limits. Over half the patients with diseases in capitals will have levels above those shown. Courtesy of the *British Journal of Hospital Medicine* (H33).

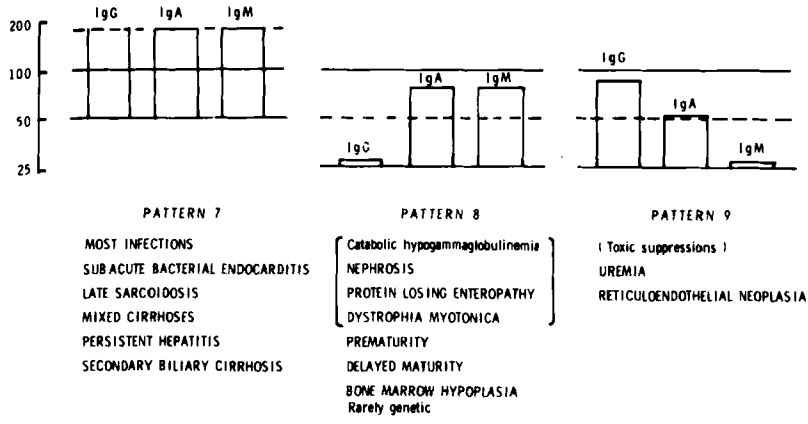

FIG. 10 (*continued*).

shown to result only from increased synthesis of immunoglobulins. Serum levels can be reduced both by decreased synthesis or increased catabolism. Serum immunoglobulin patterns only offer a crude window through which to view some immunological events, but can be useful in certain contexts, and sometimes fluids other than serum offer more useful information.

6.2.2. *Parotid Saliva*

Cannulation of Stensen's duct or cupping of its orifice with a Kirby sucker provide useful ways of collecting parotid saliva free of other saliva. Mixed saliva shows very wide variations in volume and protein content, so that normal ranges are valueless except perhaps in a 6-week-old infant in which one is trying to establish absence of IgA (S12). Parotid saliva offers a tighter normal range (see Table 4), neonates achieving adult levels within 6 weeks (S12); I do not use lemon juice or other substances to provoke a flow. In studying the individual clinical patient, I have as yet found parotid saliva useful only in (i) establishing normality; (ii) confirming the absence of IgA (although there are claims of dissociation, IgA being present in saliva but not in the serum, I know of no convincing proof this dissociation occurs, and have not seen this in now over 100 patients with IgA deficiency); (iii) noting compensation by secretion of IgM (see Section 3.3). Our normal ranges for jejunal juice (see Section 6.8) and CSF (see Section 6.10) are also included in Table 4.

6.3. INFECTIOUS DISEASES

Most generalized infections provide multiple antigenic challenges through many routes, and it is not surprising that it is typical for all the

immunoglobulins to show elevations of about the same percentage of MNA a week or more after the onset (see Fig. 10:7) (Z1). Because this is so nonspecific, it is of little clinical value other than to indicate that the patient is making a broad immune response, and thereby probably to exogenous antigens. In long-standing sarcoidosis or liver disease, this nonspecific pattern may also ensue, possibly owing to gradual broadening of the antigenic challenges. Where the challenge is mainly directed at mucous surfaces, IgA may predominate (see Fig. 10:2), or where the bloodstream bears the brunt, IgM may predominate, as in tropical parasitemia (M5). Marked IgM elevation is also seen with idiopathic tropical splenomegaly, whether this occurs in New Guinea (W4) or Nigeria or Tanzania (Corbett, Bennett, and Hobbs, unpublished observations), although blood stream trypanosomiasis and malaria have been excluded. No parasite has been implicated in the idiopathic amyloidosis which is also endemic in New Guinea (in areas unaffected by idiopathic splenomegaly), yet which is also associated with massive IgM levels (Cooke, Anders, Champness, and Hobbs, unpublished observations). It is rare to see an isolated IgG response to infection (see Fig. 10:1), and even where this occurs, as in lymphogranuloma venereum or with nodular leprosy [23 New Guinea patients yielded mean levels similar to those of Fig. 10:4, (Cooke, Anders, Henry, and Hobbs, unpublished observations)], this may be because the actual disease is manifested by a superimposed immune reaction. In kala azar it may be attributable to localization of the parasite in the bone marrow, which in man is the major site of IgG synthesis.

6.4. Perinatal Infection

Immunoglobulin estimations are valuable in detecting not only infection acquired *in utero,* but also that acquired in the first 6 weeks of life.

The fetus is normally protected from antigenic challenges and so is born with very low levels of IgA and IgM. When organisms gain access, especially after the 18th week, the fetus can manifest increases in both or either. In 1956, Koch, Schlagetter, Schultze, and Schwick (K5) first showed how elevation of IgM in the cord serum could be useful in detecting intrauterine infection, such as congenital syphilis. This has now been confirmed many times with all kinds of infections, including toxoplasmosis, cytomegalovirus, or rubella, and elevation of IgM and/or IgA should always be fully investigated. Its positive value is not always diagnostic of infection. Other stimuli, such as maternal allotypic proteins or intrauterine transfusion (H41), can elevate IgM and IgA. Its negative value is also not foolproof: significant IgM elevation was found in only 18% of 88 infants claimed to have congenital rubella (M8).

During the first 6 weeks of life it may be very difficult to diagnose infection. The baby may already have the respiratory distress syndrome, so that superadded infection can be missed. The temperature responses are labile and neutrophilia is the rule, though the degree of left shift in the neutrophiles can be very useful (X1). By carefully establishing IgM normal ranges for each week the 2 SD upper limit is relatively so low up to 6 weeks that elevations due to infection easily stand out (see Fig. 11). Thereafter a rise can be lost within the 2 SD limits. There was also an excellent correlation between increased leftward shift in the neutrophiles and significant IgM elevation.

For many months we have now routinely kept all cord sera so that they can be screened (a) for elevations indicating intrauterine stimulation and (b) as a baseline for any subsequent elevation which might establish that postnatal infection has occurred.

6.5. Liver Diseases

Sherlock (S14) states, "Patterns are not diagnostic of any one disease but only give suggestive evidence," basing her conclusions on the work

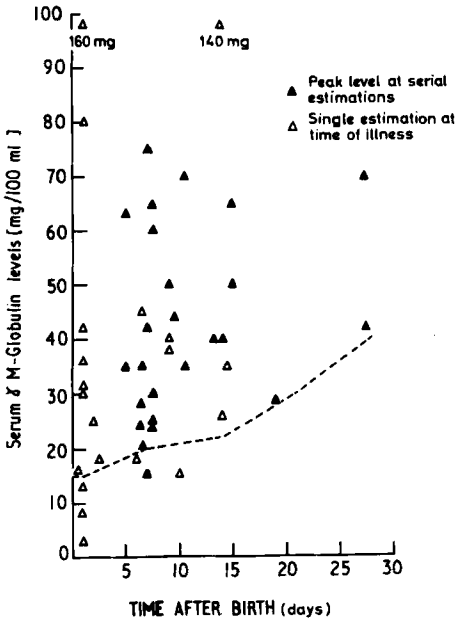

Fig. 11. Serum IgM levels in 45 consecutive neonates in whom proof of infection was obtained. The broken line indicates the 2 SD (logarithmic) upper limit for uninfected controls in the same unit. From Yeung and Hobbs (to be published). Courtesy of the *British Journal of Hospital Medicine* (H33).

of Feizi (F4). When one considers the difficulties of categorizing liver diseases, it is astonishing that 18 other papers on immunoglobulins in liver diseases (see Table 5) agree that distinctive patterns are usually associated with diagnoses equivalent to (i)–(iv) below. The simplified histological differentiation into chronic aggressive or persistent hepatites (D4) supports our own results.

(i) Dominant elevation of IgM (Fig. 10:3) occurs with *primary biliary cirrhosis*. In a few cases this disease overlaps with chronic active hepatitis, and a mixed pattern of IgG and IgM may be seen. Either is distinct from the nonspecific pattern (Fig. 10:7) seen with secondary biliary cirrhosis, etc. (H23). Isolated elevation of IgM (only otherwise seen with acute hepatites) is also an easier and more reliable (95%) dis-

TABLE 5
SERUM IMMUNOGLOBULIN PATTERNS IN LIVER DISEASES[a]

References	Patterns				
	Fig. 10:3 in primary biliary cirrhosis	Fig. 10:4 in chronic active hepatitis	Fig. 10:5 in Laennec's cirrhosis	Fig. 10:6 in acute hepatitis	Normal IgM in simple biliary obstructions
(H14)	2/2	1/1	0/5	6/8	1/1
(T9)	0	0	8/8	4/7	0
(P2)	18/19	0	0	0	0
(M9)	2/2	0	7/7	0	0
(L3)	0	0	17/19	16/19	0
(M16)	0	0	5/5	0	0
(L9)	0	0	0	33/55	0
(H23) and since	22/22	34/35	64/66	41/42	24/24
(G22)	0	13/13	—	0	0
(B19)	0	—	12/12	0	0
(A7)	0	1/1	3/3	2/2	—
(G10)	0	0	0	19/20	0
(G10a)	0	13/13	23/25	0	9/9
(B14)	0	0	0	62/66	0
(W14)	0	0	0	36/40	12/15
(Z1)	0	0	25/25	37/37	0
(A2)	0	0	38/38	0	0
(B13)	11/13	0	0	0	36/36
Totals:	55/58	62/63	202/213	256/296	82/85
	95%	98%	95%	86%	96%

[a] The number of patients with diagnoses equivalent to those shown and the number with each given pattern are indicated. The incidence of the given patterns in other categories of liver disease is not shown, but was mostly below 5%. 0 indicates that no such categories were included in the references or that they could not be identified.

tinguishing test than is mitochondrial antibody. The latter is found in some 30% of nonalcoholic chronic liver diseases; since primary biliary cirrhosis represents less than 5% of these there could be 6 false for every genuine positive result. In practice the two tests together are most reliable, but we tend to screen using the immunoglobulin levels.

(ii) Dominant IgG elevation (Fig. 10:4) occurs with the *macronodular chronic aggressive hepatitis* classically described in young women (usually Australia antigen negative and smooth muscle antibody positive), the only liver disease where prednisone significantly affected survival (C16). The IgG level also correlated well with the progress of the patient, falling with improvement.

(iii) Dominant IgA elevation (Fig. 10:5) occurs with the *micronodular Laennec type of cirrhosis,* so typically resulting from the uniform toxic damage from alcohol, iron (hemochromatosis, sideroblastic, and sickle cell anemia), or Wilson's disease. Since the liver is of gut origin, it is not surprising that its commonest reaction is IgA in type, and thereby the ingress of IgG or IgM producing cells as in (i) and (ii) could reflect autoimmune aggression.

(iv) Dominant elevation of IgM (Fig. 10:6) can also be found soon after admission with *acute hepatites.* Later less striking elevation (150% MNA) of IgG can follow. This seems to be true whether Australia antigen (ca. 60%) or Paul Bunnell (ca. 10%) antigen can be detected, and is also seen with rare hepatites due to rubella or cytomegalovirus. All this suggests that we should not anticipate a single etiological agent for infective hepatites, but rather a common etiological mechanism, e.g., similar to serum sickness.

(v) Shulman and Trowell (unpublished observations) following up 15 of our patients in whose sera Almeida has identified Australia antigen by electron microscopy, and who have *persistent hepatitis,* are finding that significant elevations of IgG and IgA develop, with IgM usually sticking at about 200% MNA. Persisting raised immunoglobulin levels with persistent hepatitis are recorded by others (B14, G10a), who also found measurements of value in indicating 21 of 36 blood donors implicated in the transmission of serum hepatitis. However, today screening for Australia antigen is possible. Two of our patients now show aggressive hepatitis, and this small group may explain the rare older males described with chronic active hepatitis who are Australia antigen positive (G8). It does seem that the combination of clinical progress, biochemistry, biopsy, immunoglobulin patterns, and serology advocated in 1967 (H23) will finally help to sort out some liver diseases.

(vi) The nonspecific pattern (Fig. 10:7) is seen with other liver diseases such as secondary biliary cirrhosis, sarcoidosis, tuberculosis, schistosomiasis, amebiasis, and with late mixed cirrhoses.

(vii) Normal levels are the rule with drug-induced jaundice or with simple nonfebrile extrahepatic obstruction. Because normal levels are uncommon in categories (i–vi), this finding may be clinically useful; indeed a raised IgM level could be a contraindication to surgery (B13).

Overall, I am of the opinion that serum immunoglobulins are very helpful in the differential diagnosis of liver diseases, provided diseases elsewhere can be excluded; pattern 4 is over 95% reliable, and patterns 3, 5, 6 are about 90% reliable, allowing for their different clinical pictures.

6.6. Febrile Heart Diseases

During active rheumatic fever elevation mainly of IgA and IgG (similar to Fig. 10:5) occurs in over 95% of subjects (H49, S5). In our experience of subacute bacterial endocarditis, all untreated patients have shown about equal elevation of IgG, IgA, and IgM, the nonspecific pattern of infection (see Fig. 10:7). In contrast, rickettsial (Q fever) endocarditis often shows a dominant IgM (see Fig. 10:3) (H46) which falls if treatment is successful, and two patients with fungal endocarditis have had all levels within normal limits. These unusual patterns can thus arouse suspicion in cases with negative routine blood cultures. The postcardiotomy syndrome is ill understood, but suggests an aberrant immune reaction to endogenous antigens possibly altered by the surgical procedures: in this light the marked isolated increase in IgG (Fig. 10:1) observed now in 6 such patients is distinct from the above patterns. Severe myocardial infarction may have a similar effect, but the IgG level rarely exceeds the 2 SD upper limit. In the given clinical context of isolated febrile heart disease, serum immunoglobulins can be helpful, e.g., following open-heart surgery (H27).

6.7. Respiratory Diseases

Since tuberculosis, bronchiectasis, emphysema, fibrocystic disease, intrinsic asthma, and hilar sarcoidosis are often associated with a dominant elevation of IgA (see Fig. 10:2), this has no specific value. Pneumocystis pneumonia in infants can elevate IgM (K7) (Fig. 10:6). Of course recurrent respiratory infection is a common mode of presentation of frank immune deficiencies (H32), and we find these in some 4% of patients with such histories. Reaginic states have already been considered under IgE globulin, and immunoglobulin deficiencies among atopic children support the hypothesis that atopic subjects may have a poor immunological dictionary (K2). Direct IgE assay of fluid from nasal polyps can also point to an allergic origin (D6). *Parotid saliva* normally contains only detectable IgA (see Table 1) (mixed saliva

contains detectable IgG and often IgM, is much more variable, and is not recommended). Since IgA matures to adult levels within 6 weeks of birth (S12), testing offers a simple early warning of IgA deficiency or agammaglobulemia. It is possible that inadequate secretory IgA can occur despite normal serum levels of IgA, and such inadequacy may be a contributory factor to dental caries (L4).

6.8. Gut Diseases

The raised IgA (Fig. 10:2) associated with malabsorption states—60% of children, (I1); 20% of adults, (H31) active Crohn's disease (H39, P10) and ulcerative colitis, etc.—similarly has no specific value. It seems fair to say persistence or relapse of a high IgA level indicates unmodified disease; therefore reassessment of the measures taken, e.g., milk sensitivity, may have been missed (I1) or a lymphoma may be developing (A10). The various immune deficiencies aggravating or resulting from malabsorption can be readily distinguished by their immunoglobulin patterns (H31), e.g., protein-losing enteropathy (see Fig. 10:8), celiac disease (see Fig. 10:9).

Jejunal juice. IgG-globulin is readily digested; so that samples require immediate inhibition of proteases (1 drop of 10% EACA and Trasylol) if IgG is to be assessed. IgM seems less readily digested and secretory IgA is unaffected. It is too early to fully evaluate jejunal juice estimations, but it can be said that excessive content of IgM is nearly always found in untreated celiac disease (D7). High immunoglobulin, albumin, and transferrin levels are easily demonstrated in protein-losing states (offer a simple alternative to ^{131}I-PVP), and with samples taken at different levels can delineate the area affected. Since juice can be sampled at the same time as biopsies, I believe jejunal juice estimations will have a useful future.

6.9. Renal Diseases

Interpretations of serum immunoglobulins in renal disease have two complications. Proteinuria itself can result in catabolic hypo-IgG-globulinemia (A4) yielding pattern 8 in Fig. 10 (H25). Renal failure can effect a toxic inhibition of synthesis affecting primarily IgM, then IgA, and last IgG, yielding pattern 9 (H19). Perhaps because these two factors hold down IgG and IgM, we commonly find a raised IgA in glomerulonephritis and pyelonephritis (see Fig. 10:2). It is worth mentioning here that a reduced serum level of complement components (β_1c/β_1a is most easily measured) is of diagnostic value in membranoproliferative glomerulonephritis (O2). In most other renal diseases serum immunoglobulin measurements alone are unhelpful. Extending the measurements to include other proteins and simultaneous urine samples enables

the invaluable clearance concepts of Hardwicke and Squire (H8) to be applied.

Proteinuria. The simplified determination of the IgG-globulin:transferrin clearance (C1) is the first investigation of choice in nephrotic children, whereby renal biopsy may often not be needed. In adults renal biopsy is preferable, and we tend to undertake such clearance studies only when the kidneys are small, single, or difficult of access. In pregnancy, preeclamptic proteinuria shows IgG:transferrin ratios of 0.20–0.30, so that values above 0.30 can point to some other renal pathology. In distinguishing between glomerulonephritis and pyelonephritis (e.g., in the hypertensive clinic), the addition of the IgM clearance can be very useful: infection evokes a much greater "clearance" of IgM than would be expected for the found IgG clearance (G12). Finally, the simplest way of proving a tubular proteinuria is by measuring β_2-microglobulin and albumin (P7) or transferrin, which is as good as the latter. By just adding transferrin and β_2-microglobulin to immunoglobulin assays an invaluable service can be instituted.

6.10. Central Nervous Diseases

Serum immunoglobulin measurements have been valueless except with dystrophia myotonica where inborn hyper-IgG-catabolism often results in pattern 8 of Fig. 10 (W12).

Cerebrospinal fluid. The blood-brain barrier is such that most elevations of immunoglobulins in the CSF probably largely result from local synthesis: subsequent retention against a normal background of very low levels (H10; see 6.2) makes the CSF a much more sensitive indicator of local immune reactions than is the serum. Elevations occur with infectious diseases of the CNS, and these typically involve IgG, IgA, and IgM (G17) disproportionately increased relative to the associated rises in albumin, etc. Hemorrhage and aseptic inflammations will result in raised levels of all these, but with clearances into the CSF relative to serum proportions. Multiple sclerosis, panencephalitis (probably cell damage due to antibodies catching measles at the membrane), and rare polyneurites (possible autoimmune) are usually associated with selective increases in IgG mainly, without concomitant albumin, and this has diagnostic value. There is good evidence for selective synthesis of IgG within the central nervous system in multiple sclerosis (C9, T12). The whole subject is more fully reviewed elsewhere (H15).

6.11. Skin Diseases

It is of interest but of no specific value that most skin diseases evoke some elevation of IgA (Fig. 10:2) (F9), and on the whole the more

extensive the lesions, the higher the level (Marks and Hobbs, unpublished observations). Together with the finding of secretory IgA in sweat (H3), these results suggest IgA plays a role in the defense of the skin. Dermatomyositis usually shows marked IgA elevation and erythema nodosum levels around 200% MNA (N3). Psoriasis and dermatitis herpetiformis often are associated with low IgM levels. Atopic eczema is associated with high IgE levels (J3) so these are also found in the Wiskott-Aldrich syndrome.

6.12. Aberrant Immunity

In those diseases where the patient's own immune reactions are thought to damage the patient's tissues, the final common mechanism is that, in contrast to the normal situation, those tissues behave as antigens, hence the term autoimmunity. In several instances, however, the antibodies are primarily directed against exogenous antigens, which unfortunately overlap with the patient's tissues (better called isoimmune disease). In other conditions there appears to be an imbalance in the patient's immune reactions (H24). In all types it could be said the patient's immune reactions have gone astray, and here the term aberrant immunity can cover the lot. Where aberrant immunity involves humoral responses, these occur mainly in the IgG class (although autoantibodies can be found in all classes). This can result in massive elevation of the total serum IgG (H21). Curiously, this is more marked and more frequent in those diseases (in capitals under Fig. 10:1) where the evidence for aberrant immune reactions is weaker than in the well-substantiated Hashimoto's thyroiditis or idiopathic Addison's. This may be because the IgG antibodies themselves are weaker, hence their activity is difficult to demonstrate. Good, easily detected IgG antibodies have high affinity for their antigens, therefore, they tend to complete their reactions more efficiently and less will be needed; e.g., a raised IgG level is unusual in thyrotoxicosis and LATS is highly effective: severe IgG-mediated hemolytic anemia can occur with hypogammaglobulinemia, and indeed pattern 9 of Fig. 10 occurs in 30% of warm autoimmune hemolysis (B15). It is sufficient to say that a large isolated increase in IgG should make one think of an aberrant immune situation. Exceptions to this rule are the mixed elevations of IgG and IgA common in fibrosing alveolitis (H47), though the excess of IgA may be due to concomitant liver disease (T14), Sjögren's disease (G24), and rheumatic fever. IgM elevation alone occurs in primary biliary cirrhosis. Later on, in some patients IgM elevation joins a preexisting raised IgG (Fig. 10:4).

Rheumatoid arthritis remains an enigma and is most often associated with a dominant IgA elevation similar to Fig. 10:2 (C6).

6.13. Mixed Cryoglobulins, Immune Complex Diseases

These are distinguished from other cold aggregates (7.5.4) by their content of usually more than one class of heavy chain and both types of light chain. Serum kept at 37°C (with sodium azide to discourage bacterial decomposition) can be compared with the 4°C supernatant. By measuring immunoglobulin in triplicate, significant differences in the mean levels can indicate how much IgM, IgG, or IgA has gone down in the precipitate. This is a better measure than the cryocrit, being superior also to immunoelectrophoresis of the cryoprecipitate, which is always contaminated by trapped plasma proteins. The best assessments are based on the void volume through Sephadex G-200, reconcentrated to the original serum volume applied and its contents then compared to the original serum (B6). Mixed cryoglobulins (M13) have so far mostly represented circulating immune complexes of IgM rheumatoid factors; or can also be IgA or IgG_3 (G20) (against IgG_1). Mostly the rheumatoid factors are polyclonal (although with a predominance of K light chains), but monoclonal rheumatoid factors are now well recognized (B18, G20, K10). Precipitation, slowly, in the cold offers a useful screening procedure for all binds of immune complexes (B6), and these cannot all be accounted for as rheumatoid factor formation against an initial complex of IgG with some other circulating antigen. Detection of DNA in such complexes, in some cases cytomegalovirus DNA (B6), affords evidence of an initial virus infection producing the first complex. This complex alone may be enough to cause disease as seems likely in Aleutian disease of mink and systemic lupus erythematosus, and the secondary formation of rheumatoid factors might aggravate the situation. The immune complexes are held up during transit through blood vessel walls, whereupon complement factors may become fixed to cascade to activation and subsequent local damage. The pressure gradient across the renal glomeruli offers a particular site prone to damage (D5). Vasculitis is the rule and inflammation may occur in many other sites, synovitis (polyarthritis), serositis etc. The skin lesions are typically raised and indurated, may be purpuric and biopsy reveals vasculitis with immunoglobulin fixed in the vessel walls. However complement is not always found, although typically the serum level falls during active immune complex disease. Raynaud's phenomenon is not uncommon. In many other patients there is no clear relationship between exposure to cold and their symptoms. Certain kinds of immune complexes can produce granulomata (G3).

It is therefore understandable why mixed cryoglobulins are reported in such a wide variety of diseases, such as those mentioned under aberrant immunity (6.12), e.g., S.L.E., rheumatoid arthritis, ankylosing

spondylitis, Sjögren's, sarcoidosis, syphilis, leprosy, glomerulonephritis (poststreptococcal especially), subacute bacterial endocarditis, various arteritides, hepatitis and cirrhosis, hemolytic anemia, infectious mononucleosis. That Aleutian disease of mink occasionally shows emergence of a monoclonal immunoglobulin (P11) suggests how a monoclonal rheumatoid factor can be found in the mixed cryoglobulin of aberrant immune disorders, which are usually polyclonal.

6.14. Antibody Measurements according to Immunoglobulin Class

The measurement of total immunoglobulins is a crude assessment. While total antibody measurements to a relevant antigen may be much more specific to a particular diagnosis, it may be poor evidence of disease activity. We are now entering an era where it is possible to evaluate the amount of antibody within each immunoglobulin class (T11), and this approach may much improve the clinical value of the information. The importance of identifying IgE and specific reagins has already been mentioned. Other precedents are the proofs that when brucella antibodies are mainly IgG the disease is active (R3), and similarly for listeria antibodies (O5). In the newborn the proof that antibodies against toxoplasma (R4) or treponema (S6) are in the IgM class indicates that the baby has been infected, not just given mother's IgG antibodies.

7. Paraproteins

Since a plasmacytoma has a specific histopathological identity, it seems a good idea to use the term of Heremans, immunocytoma, which can cover all the various patterns that can be taken by tumors capable of producing immunoglobulins. In considering these, three fundamental concepts have evolved over the last decade: the monoclonal concept (Section 7.1); the paraprotein level usually reflects the amount of immunocytoma (Section 7.2); biochemical dedifferentiation parallels malignant dedifferentiation (Section 7.3).

7.1. The Monoclonal Concept

By Burnet's theory, one plasma cell produces one antibody, that is, a single immunoglobulin; this is generally the case (M4), although some 2% of plasma cells seem capable of producing two immunoglobulins simultaneously, a fact confirmed in tissue culture (S3, T1).

Thanks to Waldenström (W1), we believe that if a single plasma cell precursor continues dividing to form a clone of cells, all the daughter cells will eventually try to produce the same single immunoglobulin. If all the molecules have exactly the same structure, they will share an identical electrophoretic mobility and will run as a single narrow band

(see Fig. 12). On testing, this band will contain immunoglobulin determinants of a single subclass of heavy chain and/or a single subclass of light chain; i.e., it can be proved to be monoclonal. This is not the way normal antibody responses usually appear. In contrast, even a single hapten will usually be antigenic to more than one clone of plasma cells in a given animal, and will therefore elicit several antibodies. A single protein will usually contain several haptens and will elicit a spectrum of antibodies. A natural challenge, e.g., diphtheria bacilli, more often presents many proteins so that a broad spectrum of antibodies is elicited, usually containing most of the classes of heavy chains and light chains. On electrophoresis this spectrum will show a diffuse range of electrophoretic mobilities (from α_2–γ_4, see Fig. 12) and can be recognized as polyclonal by its mixed content of immunoglobulin determinants.

Thus when we find narrow bands on electrophoresis, and can identify them as being due to a single type of immunoglobulin, we call them paraproteins. We believe a paraprotein is evidence that a *monoclone* of cells is growing in the subject, i.e., the subject has an immunocytoma. This cannot really be considered as normal, and in medicine, our concern then becomes whether or not such an immunocytoma is going to be harmful to its host. Monitoring the growth of such clones by measuring

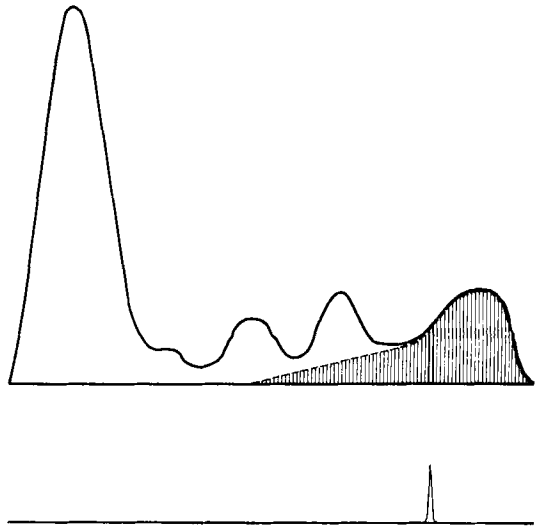

FIG. 12. The monoclonal concept. A paraprotein has a narrow electrophoretic mobility and contains heavy and/or light chains of a single subclass only. Polyclonal increases are broad and heterogeneous. Reproduced by permission of Athlone Press, from the *Scientific Basis of Medicine* (H19).

paraprotein levels and looking for evidence of dedifferentiation are important guides to prognosis.

7.2. The Paraprotein Level Reflects the Amount of Immunocytoma

With Potter's development of experimental plasmacytoma in mice, it was established that the turnover of paraprotein (N2), or more simply the serum level (O8), was directly related to the weight of solid soft-tissue plasmacytoma. In our laboratory an ascitic form of plasmacytoma has been studied, and using isotope dilution it has been possible to estimate the actual total number of plasmacytoma cells in a mouse. At the same time the serum level of paraprotein was measured and a simple correlation was shown (F2). To the best of my knowledge, this was the first time that the serum level of a tumor product had been directly related to the actually counted number of tumor cells. Incidentally it was noted that the paraprotein could be first detected in the serum, when a 23 g mouse had 3 million tumor cells.

It was further shown that tumor growth was exponential in the mouse.

While screening some 1000 human patients, chiefly for the Myeloma Trials of the Medical Research Council, we have now had the opportunity to follow 94 in whom a diagnosis was not initially certain, but who developed clear evidence of myelomatosis up to 8 years later. We found that the rise of serum IgG or IgA paraprotein levels, or indeed the 24-hour urinary output of Bence Jones protein was exponential, just as in the mouse. The Median doubling time for eventual IgA myelomatosis (our most reliable estimate, see H29) was 6.3 months. This is much slower than for normal antibody responses, which can double in 1 day. Among all our cases of proven IgA myelomatosis, the average serum level at clinical presentation was 2.8 g/100 ml. To maintain such a level in an average 70-kg patient would require the daily production of about 15 g of IgA paraprotein. Mammalian plasma cells on average produce 14 mg of immunoglobulin per gram of cells per day, so that our average IgA patient should have about 1 kg of immunocytoma at clinical presentation. Now this figure is based on estimates of paraprotein production, etc. With the help of Professor Hayhoe, we derived another means of estimating the tumor mass. From all the marrow biopsies in all our patients, he produced an estimate that 33% of the bone marrow cells were myeloma cells on average clinical presentation. A 70-kg patient could be expected to have 3.2 kg of bone marrow (M10), and so we had an independent parameter confirming the previous estimate of about 1 kg immunocytoma. This would be equivalent to 4.6×10^{11} tumor cells (see Fig. 13). With a little more than one further doubling, this would

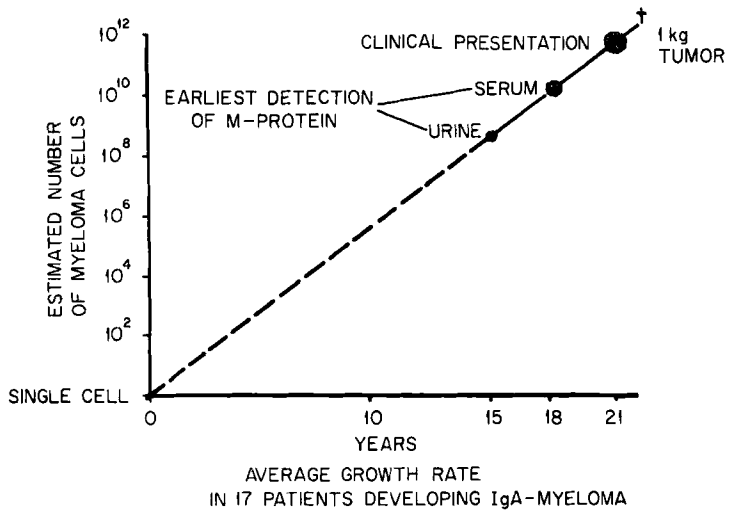

FIG. 13. The natural history of IgA myelomatosis. The log of the estimated number of tumor cells against time showed exponential growth for the solid part of the line. Serum IgA paraproteins are detected later than IgG, and so on average would only be found 2.6 years before clinical presentation at 4.6×10^{11} tumor cells. Bence Jones proteinuria can be detected before this. The broken line is extrapolation back to a single cell, assuming a monoclonal origin and a constant growth rate. Reproduced by courtesy of the *British Medical Journal* (H35).

become 10^{12} cells (identical to the estimate of the number of cells in acute leukemia, F10), and death follows. This is in accord with observations that less than half the patients survived one year from presentation (F3, I2; before melphalan). From the median doubling times, etc., it can be calculated that at the first chance that an IgG paraprotein can be detected in human serum there could be only some 20 g of immunocytoma (difficult to find, unless it is all in one vertebra, for example) or some 9000 million cells in our 70-kg patient. It is interesting to note that on a weight ratio (the mouse is 1/3000th of man) this is like that actually found in the mouse. From such chance detection, it would on average be 5 years before clinical evidence of myelomatosis emerged, and we have now indeed encountered such actually observed patients. By concentrating the urine some 300 times, it is possible to confidently detect Bence Jones protein at a level of 1 mg/100 ml original urine, or some 14 mg/day. Of 3 such patients, two have had their malignant immunocytoma verified by biopsy and clinical progress 6 and 8 years later, and the third has developed 3 discrete osteolytic lesions in her clavicles at 5 years from the initial observation. The solid portion of the line in Fig. 13 is therefore observed fact. The broken line is a back

extrapolation, assuming a constant doubling time (overall growth rate) as observed in the mouse. This suggests some 21 years for a single plasma cell precursor to go malignant and result in clinical IgA-myelomatosis. Our brief clinical view of the disease before death is only the tip of this chronological iceburg, which has important implications in treatment. The first estimate for IgG-myelomatosis was 39 years (H21), later modified to 33 years (H29) when more data became available. Normal polyclonal IgG exhibits an increased catabolic rate with rising serum level. Idiotypic IgG paraprotein might behave similarly so that doubling times would appear longer and the above estimates could be too long. Exponential increases of serum level would not be expected and curving should be seen in plots of log serum level against time. However, Drivsholm (D8) did not find changing rates of catabolism of autologous IgG idiotypic paraproteins, and in general we have seen only exponential increases in serum IgG levels. It is comforting that we have encountered only one patient with IgG-myelomatosis under the age of 33 years (he was 29, and his tumor had a fast growth rate). Salmon and Smith (S2) have recently studied IgG metabolism in 10 patients and have produced estimates for tumor cell masses of 0.5 to 3.1×10^{12} in IgG myelomatosis. They confirm this is compatible with a natural history of 20 years. For myelomatosis producing Bence Jones protein only, faster growth rates have been estimated (H29); these were further confirmed by finding such patients under the age of 33 years (one under 20). Doubling times of one month have been observed and would allow Bence Jones mutations to emerge within 3 years of treatment (v.i.).

Now not all immunocytomata become clinical myelomatosis, some remain benign, and studies can help in assessing prognosis.

7.3. Biochemical Dedifferentiation Parallels Malignant Dedifferentiation

First, we should consider synthesis of whole immunoglobulin. The heavy chain is synthesized on a large polyribosome and pulse labeling indicates that this takes 2.5 minutes. Light chain is synthesized on a smaller polyribosome, taking 1 minute (A9). Assembly follows, the Golgi apparatus adds carbohydrate, and intact molecules are secreted only some 30 minutes later. Free light chains cannot be detected outside such plasma cells, and there is only a small intracellular pool of free light chains (presumably from the initial 1.5 minutes before heavy chains are available). Light and heavy chain synthesis is presumably beautifully balanced in the well-differentiated cell.

It had been thought that Bence Jones proteins were breakdown products of myeloma proteins. This was disproved in 1958 when gluta-

mate-^{14}C was injected into a patient with myelomatosis and the Bence Jones protein had a much higher specific activity than the myeloma protein (P14). We now had a concept of de novo synthesis, and this I have taken further to a concept of biochemical dedifferentiation. The malignant myeloma cell has acquired an imbalance in heavy and light chain synthesis, presumably by dedifferentiation, and frequently produces too many light chains. These are secreted, and such free monoclonal light chains are indeed Bence Jones proteins. In some 20% of myelomatosis the process of dedifferentiation goes further and only light chains are synthesized and released. Such Bence Jones myelomata grow faster, can present at a younger age, seem more invasive in that more extensive bone and soft tissue lesions are found, and carry a worse prognosis (H28); in short they show that more biochemical dedifferentiation parallels more malignant dedifferentiation. Taking this further, a few myelomata produce half-light chains and/or small amounts of Bence Jones proteins relative to the tumor mass (e.g., down to one-fiftieth of the average 24-hour daily output of some 6 g at clinical presentation). Fluorescent study of such bone marrow reveals myeloma cells with a light chain content well below that of a normal plasma cell. Others even fail to produce any recognizable heavy or light chain at all; the so-called nonparaprotein myelomata. These latter groups clinically appear even more vicious than Bence-Jones myelomata, and 6 of our 8 such patients died within 6 months (4 within 3 months) of diagnosis, despite our best treatment. Rarer tumors can fail to produce light chains, and only heavy chains are found (Section 7.6.5). Still others produce only half-molecules (H42), found with soft-tissue plasmacytoma in 3 patients to date. IgM is normally secreted as a 19 S molecule, assembled intracellularly from five 7 S IgM units. After initial reports (B30, H5, S16, S29), a broader survey suggests that excess secretion of 7 S IgM in the adult is probably evidence of malignant dedifferentiation (C2). Further fragments of immunoglobulin synthesis and combinations of these continue to be reported, but almost throughout, such dedifferentiated immunoglobulin synthesis has been found only with malignant immunocytomata.

Our best evidence of this is a 3-year follow-up of 402 patients in whom Bence Jones protein had been detected in our laboratory. Dr. Corbett obtained biopsy evidence of malignant immunocytoma in 400. The various forms such malignant immunocytomata can take are listed in Table 7, which also includes the benign varieties in which I personally have not yet found immunoglobulin fragments.

Together with this tendency for their immunoglobulin synthesis to dedifferentiate, malignant immunocytomata also seem capable of sup-

pressing the synthesis of normal immunoglobulins, so that with IgG-myelomatosis the paraprotein band seen after electrophoresis on cellulose acetate usually sits on a white background because so little normal IgG remains (see Fig. 14). Levels of IgA and IgM are usually markedly subnormal. The serum level of paraprotein from malignant immunocytomata also shows a continued aggressive rise with time, so it is usually above 1 g/100 ml when first found. In benign immunocytomata, the tumor seems to have already reached equilibrium with its neighbors and the serum level (often less than 1 g/100 ml when first detected)

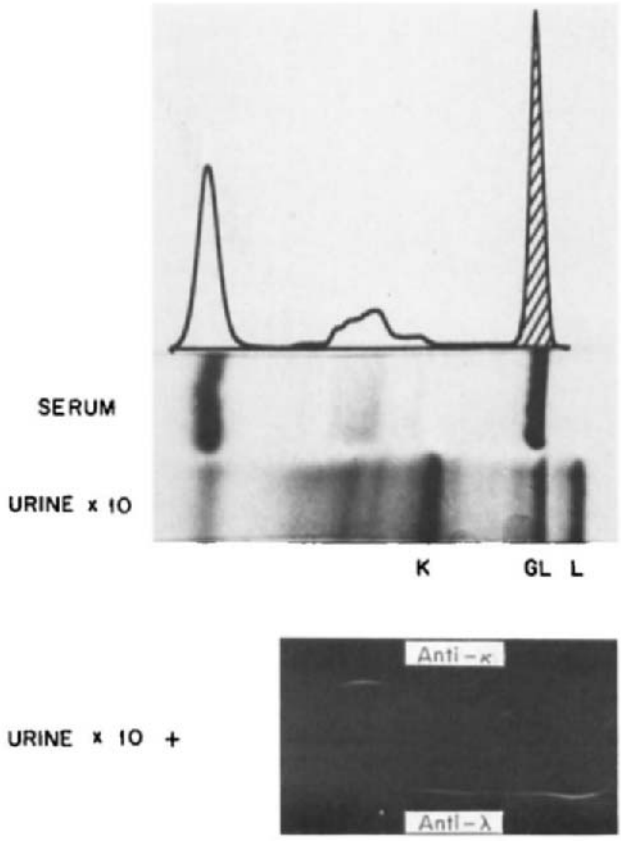

FIG. 14. Malignant paraproteinemia. Electrophoresis on cellulose acetate of serum (above) and concentrated urine (below) reveals: (i) Bence Jones proteinuria. In this case monoclonal bands are seen of each type. For L the relevant concentration to albumin indicates which are IgGL, L dimer, and L monomer; (ii) loss of normal γ-globulin; (iii) a high serum level of paraprotein. Immunoelectrophoresis of urine reveals paraprotein bows of κ, and λ, $\gamma_2\lambda_2$ and λ_2.

usually remains constant (see Fig. 15). The four features that are very useful in predicting a benign or a malignant immunocytoma are shown in Table 6, in order of importance, and even when apparently benign, follow-up of the patient at yearly intervals (watching for feature 4) is recommended in all cases, as a few can emerge as malignant.

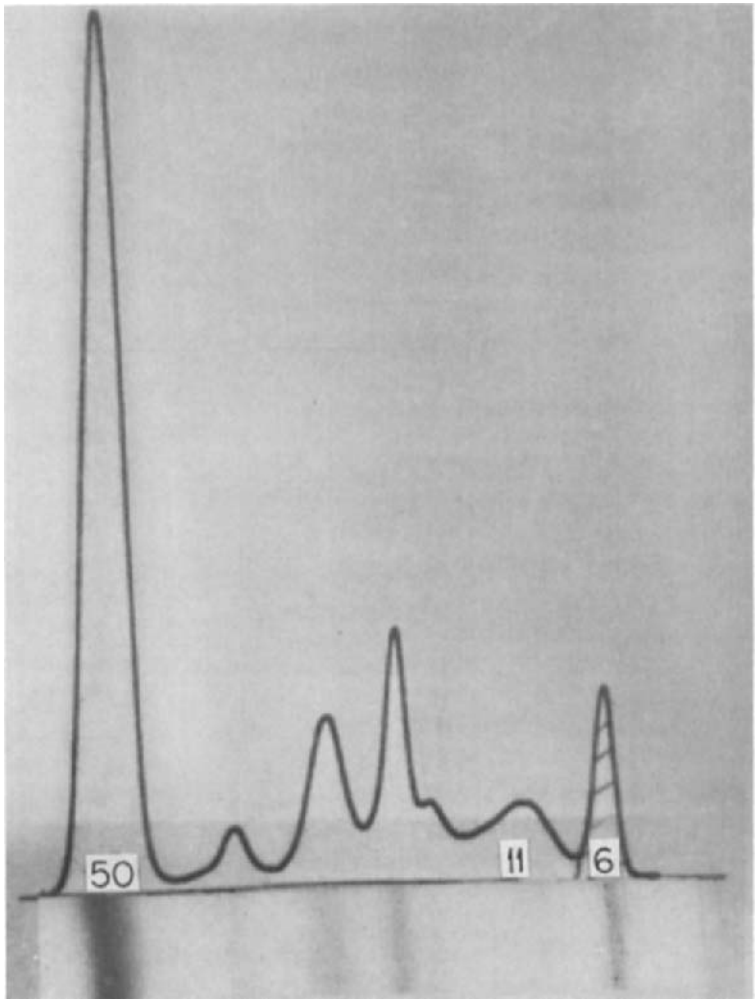

FIG. 15. Benign paraproteinemia. Electrophoresis on cellulose acetate of serum and concentrated urine reveals: (i) no Bence Jones proteinuria; (ii) no loss of normal γ-globulin; (iii) a low level of serum paraprotein. In this case the paraprotein is of post-γ-mobility, is type GL, and is typically (but not exclusively) found in lichen myxedematosus. Reproduced by courtesy of the *Proceedings of the Royal Society of Medicine* (H30).

TABLE 6
BIOCHEMICAL FEATURES OF VALUE IN THE PROGNOSIS OF IMMUNOCYTOMATA

Feature	Patients with immunocytomata	
	517 Malignant, biopsy proven	112 Benign, followed for 5 years
1. Immunoglobulin fragments	84%	0%
2. Suppression of normal immunoglobulins	98%	10%
3. In those with serum paraprotein, level > 1 g/100 ml	92%	15%
4. Of those followed up untreated, progressive rise in paraprotein level	99%	1%

7.4. INVESTIGATION OF SUSPECTED PARAPROTEINS

From Section 7.1. it follows that *paraproteins are immunoglobulins or fragments thereof with narrow electrophoretic mobilities due to heavy and/or light chains of a single subclass.* For several years, I followed fashion and used the term "M" protein, etc., but this has so often been misinterpreted as IgM that I now use only the term paraprotein. The first and the screening test is simple electrophoresis (Kohn's cellulose acetate is better than paper, see H19), and only narrow bands should be investigated further, unless the collateral evidence is very strong. Occasionally a spontaneously denatured IgG_3 paraprotein, a cryoglobulin

TABLE 7
DIAGNOSES ACHIEVED IN 691 PATIENTS WITH PARAPROTEINS

A. *Malignant immunocytomata*		74%
Myelomatosis (including 5 plasma cell leukemia)	420	
Waldenström's macroglobulinemia	32	
Soft-tissue plasmacytoma	20	
Lymphosarcoma	26	
Reticulosarcoma (including 5 atypical Hodgkin's)	6	
Chronic lymphatic leukemia	5	
Atypical myelosclerosis	2	
Giant follicular lymphoma	1	
Arabian lymphoma of gut (α-chain)	3	
B. *Benign immunocytoma*		23%
(i) Followed up for at least 5 years	112	
(ii) Monoclonal antibody		
Primary cold agglutinins (8 others had lymphoma)	37	
Lichen myxedematosis	4	
Transient paraproteins	5	
C. *Uncertain*	18	3%

run at room temperature or rare polymerizing paraproteins (e.g., α-chain) will show diffuse electrophoretic mobilities. Even in these situations, reduction of other immunoglobulins and some tendency to banding will alert an observer, and I have seen only 3 paraproteins (all α-chain) in over 1000 where the paraproteins had not been seen on simple electrophoresis.

Serum should be separated at 37°C to avoid underestimating cryoproteins. Urine should always be examined (Section 2.6) and electrophoresed alongside its donor serum, except in anuria when Bence Jones can be checked in the serum. The amount of any paraprotein can be estimated as a percentage of total dye binding on the electrophoretic strip. Using reliable methods (H18) and ensuring that the paraprotein band is not overloaded (H21), this affords the best available way of monitoring paraproteins. With all but IgM, a biuret total protein is adequate on either serum or urine: the latter should not be done using sulfosalicylic acid, which can fail to precipitate 18% of Bence Jones proteins (H20); 10% trichloroacetic acid is better (but not foolproof, see H20), the precipitate being redissolved in sodium hydroxide (B9). Since IgM contains 12% carbohydrate, macroglobulinemic sera are better estimated by specific gravity (L11) or refractive index.

The paraprotein is typed using immunoelectrophoresis. The three features of value in recognizing a paraprotein "bow" are (i) a change in density of the precipitate (e.g., broad anti-γ will be relatively weaker for a γ-idiotype); (ii) a sharp change in the angle with which the "bow" meets any normal residual immunoglobulin (again due to localized idiotype; e.g., excess polyclonal γ would blend into the rest); (iii) localized reduplication of precipitin lines (full length reduplication suggests denatured immunoglobulin). The most valuable antisera are the anti-κ and anti-λ, which will clearly show paraprotein bows against one only, with no reaction opposite (see Fig. 14). Antisera to D or E are reserved for those narrow bands that do not react with A, G, or M.

Serum immunoglobulins other than the paraprotein class are then measured (1.2). Most of the data required in Table 6 will then be available. Where desired, the molecular size of the paraprotein can be checked (Section 1.3), e.g., to provide evidence of 7 S IgM (see Table 8). The clinical chemist will then be able to advise on a sound basis. Where further desired (e.g., nonparaprotein myeloma, Section 7.6.1) bone marrow can be examined by (a) fluorescent antibodies, (b) direct insertion into agarose to electrophorese out and identify contained paraprotein, (c) tissue culture, etc.

Abnormal narrow bands on electrophoresis which might falsely be called paraproteins are in serum: (i) fibrinogen—a subsequent clot or

antifibrinogen soon clarify the situation; (ii) zoning—with old or uremic sera (H19) spontaneous denaturation can occur, IgG dimerizes, the Fab falls off and its Fc with largely the same mobility therefore shows as one or two narrow bands; anti-κ and anti-λ show no "bows," and the incautious might then claim γ-chain disease; however, it cannot be found in fresh serum or with improvement of the azotemia, (iii) excess lipoprotein in the α_2-region; (iv) free hemoglobin, readily seen from the color of the sample. In concentrated urine beginners often mistake (i) transferrin; its relative concentration to albumin avoids such errors. Other sources of confusion are (ii) free hemoglobin, or rarely myoglobin, seen as a band in the γ_1 position, but the sample will be colored; (iii) lysozyme, seen as a post-γ band (O7); (iv) excess α_1-glycoproteins, seen with cancers; (v) rarely other mucoproteins (α-γ) from pseudomucinous cystadenocarcinoma, etc. None of these react with reliable antisera (Section 1.5.).

In some samples more than one paraprotein band will be found (see Fig. 14). This may be due to many causes; (1) postsecretory alterations, either by (a) e.g., deamidation (K6), (b) polymerization (IgA frequently shows a double band, 7 S + 11 S), (c) complexing to other proteins (e.g., IgA + albumin), (d) degradation (IgG_3 frequently splits, with parent + its Fc); (2) presence of parent immunoglobulin together with its fragments (see Fig. 14, IgGL + L); (3) a trible of clones, i.e., closely related paraproteins such as IgGL + IgML yet with the same V genes (W3) or two Bence Jones with a single amino-acid difference (W7); (4) a mosaic of clones, i.e. two unrelated immunocytomata in the same patient (IgGL + K, see Fig. 14). Where there is truly more than one clone, the mosaic seems much commoner (2% of all paraproteinemia, and 16 out of 18 personal diclonal cases), than the tribe. Finally, it does seem that occasionally two paraproteins with the same light chain can be produced in one cell (S3).

7.5. Effects of Paraproteins

Some of the symptoms or findings in a patient are actually due to the paraprotein itself, though in the majority of patients it is the underlying tumor that kills.

7.5.1. *Amyloidosis*

It has long been recognized that amyloidosis could occur in association with paraproteins, and in 35 personal cases it was the amyloidosis that brought the patient to the doctor. The commonest features were nephrosis, bilateral carpal tunnel syndrome (and often tarsal), heart failure, thrombosis, malabsorption, and macroglossia. Eventually in 33 of these

patients, who might otherwise have been called idiopathic or primary amyloidosis, biopsy evidence, abnormal or invasive immunocytes, was obtained to establish a diagnosis of malignant immunocytoma. Among our patients presenting with undoubted myelomatosis, some 8% have had amyloidosis proved during life. It is thus clear that amyloidosis can develop during the growth of an immunocytoma, and either it or the tumor may be the presenting feature.

Apitz (A6) noted the association with Bence Jones proteinuria, to be emphasized by Osserman's fluorescent studies (O9), but others were unable to confirm that significant amounts of κ or λ determinants could be detected in the amyloid using reliable fluorescent antisera. The association remains, however, and our highest detection rate of amyloidosis (12%) is among myelomata producing only Bence Jones proteins. This enigma was resolved when it was shown it was the V portions of the light chains (see Section 1) which were involved in the amyloid (F8, G11), and these would not have κ or λ determinants, which are in the C portions. Amyloidosis presumably reflects the production of excess free V portions (more probable from dedifferentiated plasma cells) with affinity for the tissues. I remain unconvinced that amyloid can be made to regress in man, as judged by serial biopsies, but it is possible using cytotoxic treatment to depress paraprotein (and V) production, and presumably arrest further deposition. If this is done while the patient has a full-blown nephrotic syndrome, the patient notices no benefit. On the contrary, where the amyloid progresses, less filtrable surface results and the proteinuria lessens (hence spurious claims of regression) although the patient will then become azotemic. It would seem that at about this point in time, it would be apt to use cytotoxics in the effort to prolong comfortable life.

Where heart failure due to amyloidosis has already set in, we have had no success. In one patient macroglossia became less edematous, and caliper measurements confirmed that a previous width increase (of 1 cm each 4 months for 8 months) was arrested for 2 more comfortable years by the use of melphalan, before the reappearance of Bence Jones proteinuria and further width increase heralded relapse and death.

7.5.2. *Renal Damage*

The kidneys can be damaged by paraproteins in three known and two unknown ways. (i) Myeloma kidney is a term used to describe blockage of the distal tubules by casts containing paraproteins (Bence Jones and IgA have been identified), with giant cell formation. This is found in only 10% of myelomatosis and seems largely irreversible. Why it develops in some patients with only minimal proteinuria, and not in

others with up to 72 g daily, is unknown. In two of our patients, intravenous pyelography (IVP) precipitated anuria, which was fatal. In one, adding the expected concentration of the Hypaque used to the preexisting urine produced a massive precipitate. Although the risk of IVP has been minimized by some authors (C21, M20), others collected 17 such cases (G21), and I support the latter. (ii) Amyloidosis (see Section 7.5.1) can result in nephrosis and azotemia, and also predisposes to renal vein thrombosis. (iii) An acquired Fanconi syndrome is well though rarely recorded in association with myelomata producing Bence Jones protein, and can reasonably be attributed to it (E1, H2, H9, R6, S5). (iv) IgA-myelomatosis without Bence Jones proteinuria has in 3 patients been associated with azotemia. Renal biopsy has revealed no abnormality; hyperuricemia, hypercalcemia, and hypercalciuria have been excluded. Reduction in the high serum levels of IgA paraprotein (in 2 by cytotoxics, in 1 by plasmaphoresis) has been followed by marked falls in blood urea (e.g., 350 to 80 mg/100 ml). During relapse, the serum IgA rose and so did the blood urea. After death from renal failure, thorough study of the kidney has revealed no known mechanisms. (v) IgG myelomatosis without Bence Jones proteinuria has been associated with nephrosis in 3 patients. Biopsy and electron microscopy revealed no amyloid and only a "minimal lesion." Treatment with melphalan in one, together with prednisone in another, reduced the IgG paraprotein level, and remission of the nephrosis occurred. With relapse of the paraprotein level, the nephrosis relapsed and at postmortem, no lesion of the glomeruli could be proved. In that an identical syndrome in relation to the growth of 2 lymphomata has been described (G4), the lesion may be mediated by some other product of lymphoid neoplasia.

Renal damage with immunocytomata can also result from other known causes: (a) Hypercalcemia, found in 45% of myelomatosis (H28), is aggravated by putting the patient to bed or allowing a fluid intake below 3 liters daily. Furthermore, not all myeloma patients show cortisone suppression, so paraproteins should be excluded before rushing to parathyroidectomy. (b) Pyelonephritis is found postmortem in some 30% of patients with myelomatosis, presumably predisposed to by their immune paresis. (c) Hyperuricemia was not found to be a cause of renal failure or death in over 300 patients given intensive cytotoxic treatment. The highest level of serum uric acid detected was only 17 mg/100 ml, presumably because of the generally slow growth rates (Section 7.2.). (d) Invasion of the kidney by tumor is seen in about 10% of patients, especially those with Bence Jones only, or IgD myelomatosis (H36).

Finally in the viscosity syndrome (7.5.6) or with cold aggregates

(7.5.4) the loss of water between the afferent and efferent circulations through the glomeruli increases the protein concentrations and aggravates the situation. Renal failure is common in these situations.

7.5.3. *Apparent Hyponatremia*

A low plasma sodium (e.g., 120 mEq/l), as found in about 8% of IgG myelomatosis (H22), may mislead one into the giving of sodium supplements, with disastrous results. The sodium in the whole plasma aliquot is apparently low for two reasons: (i) occupation of normal plasma water space by large amounts of paraprotein (>8 g/100 ml); (ii) a high isoelectric point of the paraprotein, allowing it to act as a base at normal blood pH (F11, T3), and in my experience all have been IgG_1. Simple dialysis of the plasma against sodium chloride 145 mEq/l will fail to normalize the apparent plasma sodium, and contraindicates foolhardy electrolyte treatment.

7.5.4. *Cold Aggregates*

If these are to be studied, separation of oxalated plasma (heparin can precipitate cryofibrinogen) and serum held at 37°C is essential, using a proper 37°C centrifuge. Should an aggregate become visible at 4°C within the next 24 hours, its return to solution at 37°C should be verified (although 100% of a given precipitate may not always redissolve) and then the temperature at which it first reappears should be ascertained. If this is not above 21°C (the lowest skin temperature naturally encountered) exposure to cold is unlikely to cause symptoms in the patient. Most symptomatic cryoproteins appear above 28°C, with the exception where the cold aggregate is a mixed cryoglobulin evoking symptoms as an immune complex, rather than by simple gelification. Although it has been stated (M12) that the majority of monoclonal cryoproteins are asymptomatic, in my own experience, in some 1000 consecutive paraproteins, only a minority precipitate between 4° and 20°C, 19 of 23 achieving precipitation above 21°C and 16 being symptomatic. For interest, cryoproteins can also be checked for pyroglobulin changes (Section 7.5.5).

Raynaud's phenomenon is a common symptom, and this may be the presenting feature of four kinds of cold aggregates: (i) cryofibrinogen well reviewed elsewhere (Z2) but readily recognized as cryoprecipitate only from the plasma and not from the serum (ii) cold agglutinins (Section 7.7.3), which greatly increase the erythrocyte sedimentation rate (ESR) on cooling; (iii) monoclonal cryoglobulins which typically decrease the ESR on cooling; (iv) mixed cryoglobulins (Section 6.13).

The skin lesions of (i) and (iii) can be identical as flat areas of necrosis, marginated by erythema, due to simple occlusion of peripheral vessels. Skin lesions do not occur in (ii). Lesions in (iv) are raised indurated nodules, usually tender, and biopsy reveals vasculitis (6.13). The severity of such lesions is related to the critical temperature of gelling on cooling, and this itself depends largely on the protein concentration (L5), therefore being more common in the dependent areas (shins, buttocks, elbows, etc.). Lowering the concentration of cryoprotein, initially by plasmaphoresis if severe and more slowly by cytotoxic treatment can much improve the patient. Thiols may be useful in preventing aggregation, e.g., a 7S IgG which polymerizes in the cold, but cannot be used in the amounts needed to dissociate preformed complexes, as in cryomacroglobulinemia (IgM).

Viscosity syndrome (Section 7.5.6) may coexist, and renal failure is common (Section 7.5.2).

Monoclonal cryoglobulinemia is reserved for those paraproteins which complex to themselves, so that the cryoprecipitate contains predominantly only the heavy and light chain classes of the paraprotein. It is realized that rheumatoid factors (e.g., anti-IgG) may themselves be monoclonal (Section 7.7.6), but their cryoprecipitates are mixed ones and largely behave as such (see Section 6.13). Monoclonal cryoglobulins may be IgG or IgM in class. A majority of the IgG cryoglobulins belong to subclasses IgG_3 and IgG_2 (V2), and the critical temperature varies widely for the same concentration (M12). They represent about 2% of all myelomatosis. In other patients the immunocytoma is not obvious, the cold symptoms causing the patient to present before the average presenting serum level of 4.3 g/100 ml for IgG myelomatosis (H45), but on follow-up this so-called essential cryoglobulinemia frequently declares an invasive immunocytoma. Others die of the cryoprotein before frankly invasive tumor can be found, or the immunocytoma itself could be noninvasive.

IgM cryoglobulins represent some 6% of all macroglobulinemia, and seem especially associated with lymphosarcoma—indeed benign IgM cryoglobulinemia is a rarity. One IgA cryoglobulin is recorded (A11), but it might have had anti-IgG activity (Section 6.13).

Six published and personal examples have been detected of Bence Jones protein precipitating in the cold, but all under 20°C, and causing no symptoms in the patients. They were mostly type L, and this class shows an excess among monoclonal cryoimmunoglobulins. Rarely, true crystals may form in the cold, and any such protein would be welcome to X-ray crystallographers.

7.5.5. *Pyroglobulins*

Although several examples of paraproteins gelling irreversibly between 45° and 56°C are recorded, I am not aware of these ever causing symptoms in a febrile patient. Such a finding merits no more than the attention that any paraprotein should receive, although it is fair to say most pyroglobulins have come from malignant conditions.

7.5.6. *Viscosity Syndrome*

Since the original description by Waldenström, it has become clear that this syndrome is not confined to macroglobulinemia, and so it is better called viscosity syndrome. It has been found in some 4% of IgG-myelomatosis (H22), occasionally with IgA paraprotein and even with Bence Jones proteinemia, usually due to polymerization [reviewed by Somer (S19)], and it is also recorded with IgE (O1). With regard to viscosity syndromes due to paraproteins the relevant abnormality is detected in viscosity measurements on either whole blood, plasma, or serum (S19); the latter is the most convenient. This is not the case in polycythemia, etc. (W5).

With a few rare exceptions (?7 S, 28 S, or cryomacroglobulins or IgM + IgG complexes, Section 6.13), the viscosity of IgM is simply related to increasing serum concentration, and the clinical syndrome presents at levels above 3 g/100 ml. The viscosity syndrome due to IgG, IgA, or Bence Jones proteins seems to depend on unusual forms of these proteins, or complexes such as IgA rheumatoid factor + IgGKL, or IgA, IgG, or IgM binding lipoproteins (Section 7.5.7), etc. Unusual polymerization is common with those that also behave as cryoproteins (Section 7.5.4), so that bizarre changes in viscosity occur with increasing serum level or temperature change (V2). Thus viscosity symptoms have occurred with IgG_3 at 1 g/100 ml and have been absent with IgG_1 at 13 g/100 ml. Among the IgG subclasses we could not find any special preponderance to viscosity (V2), so it seems that polymerization can occur with any class of immunoglobulin.

The following symptoms and signs can be directly attributed to the viscosity of such paraproteins, in that they all revert toward normal within hours of efficient plasmaphoresis (e.g., using the IBM cell separator): severe lassitude, impaired phagocytosis [infection as a result of blindfolding by the paraprotein (P4)], impaired platelet function [bleeding, petechiae, also due to coating (P1)], anemia [due to blood volume changes and also due to nonspecific enhancement of destruction by coating red cells (P1)], sausage-shaped distension of retinal veins, bilaterally and independent of arterial changes (leading to perivenous

haemorrhages and blindness), coma, renal failure (Section 7.5.2), and simulation of congestive heart failure. Cytotoxic treatment (M7) per se usually takes 2–12 weeks to effectively reduce symptoms; if symptoms are severe, plasmaphoresis (or even exchange transfusion if nothing else is readily available) will have to be maintained until the paraprotein is seen not to rise again to the symptomatic level. With IgM, 3-day intervals are the most frequent needed, but with IgG, e.g. (which are largely in the tissue fluids), the plasma often refills within 24 hours. Strict asepsis should be observed and prednisone be avoided during these procedures because of the grave risk of septicemia.

7.5.7. *Xanthomatosis*

Initially recorded with rare paraproteins (B23), mainly of the IgA class, this has also been associated with IgG and IgM. Usually the total serum cholesterol is raised, but in one case it has been within normal limits; curiously it is subnormal in most patients with IgA-myelomatosis without xanthoma (S7). The xanthomata are typically of the soft eruptive variety and contain complexes of the paraprotein and β-lipoprotein. Beaumont (B8) has collected evidence suggesting that the paraproteins are antibodies to the β-apoprotein. Occasionally excess complexes can result in viscosity syndrome (Section 7.5.6). If a lipid stain is used, the paraprotein band is positive. In such cases, regrettably, Potter has been unable to relate the antibody activity to phosphorylcholine (P13). Cytotoxic treatment can reduce the serum levels of lipid and paraprotein.

7.5.8. *Paraproteins with Antibody Activity*

The whole question of paraproteins with antibody activity is beyond the scope of the present review, and is well reviewed elsewhere (M15). In clinical practice it should be remembered such proteins offer splendid research material for sequencing, etc., but no particular syndromes, apart from those described in Sections 7.5.7, 7.7.3, and 7.8.2, can as yet be attributed to such activities.

7.6. Malignant Paraproteins

Our main concern with paraproteins in clinical chemistry is to find out whether the monoclones producing them will behave like malignant tumors or whether they will be benign, striking a balance with neighboring tissues. The biochemical criteria useful in this decision are listed in Table 6. The various diseases associated with paraproteins are listed in Table 7, which also gives the incidence to be expected in general hospital practice. While it is realized that the normal population may contain a

much higher proportion of benign paraproteins, in clinical medicine all paraproteins should be initially considered as potentially malignant and the diseases which follow should be excluded together with yearly follow-up for at least 5 years and really for life, before permitting a benign label. At the same time, however, no patient should be given cytotoxic treatment until malignancy is clearly established, as the treatment may itself induce mutations (H35). I personally have not yet witnessed a clearly benign paraprotein (with a static level for 5 years) suddenly change spontaneously to malignant (with a sudden rise in level, etc.) in over 12 years experience of over 1000 well-followed paraproteins, although others have. Mostly the malignancy which is later diagnosed has been there all along, and its emergence is steadily progressive. As far as I can judge no good evidence exists of paraproteins ever being produced by carcinomata, indeed all the tumors are either clearly related to plasma cells or closely similar lines, lymphoid, myeloid, monocytoid, or reticulum cell, hence justifying the use of Heremans' term, immunocytoma.

7.6.1. *Myelomatosis*

This diagnosis remains the privilege of the physician, requiring evidence from three sources, (i) radiological, discrete bone lesions in some 60%; 96% have a normal serum alkaline phosphatase (H28); not just osteoporosis, which is so common in the usual age group; (ii) paraprotein; (iii) biopsy (not just excess plasma cells, but cells which look abnormal to the experienced hematologist). In some 10% of patients only two are positive, but the diagnosis should not be made on just one. Myelomatosis is the commonest immunocytoma (see Table 7) and is also a common neoplasia being found in some 1% of our patients over 70 years of age. The different classes of paraprotein show statistically significant differences when compared in groups (H28), but any of the complications may occur in the individual patient, so that class data are required mainly to ensure proper trials, etc. Some 2% of patients show more than one class. The remainder can be considered as follows.

IgG (53% myelomatosis) patients present on average with a serum level of 4.3 g/100 ml (because of the longer $T_{½}$, see Section 2.1). They have the most severe immune paresis, so that infection requiring hospitalization is common (60% within three years); but less hypercalcemia (33%), renal failure (16%), Bence Jones proteinuria (60%), and amyloidosis. Apparent hyponatremia (8%, see Section 7.5.3), viscosity syndrome (4%, see Section 7.5.6), and cryoglobulinemia (4%, see Section 7.5.5) with myelomatosis relate to IgG paraproteins.

IgA (22% myelomatosis) patients present on average with a serum

level of 2.8 g/100 ml. Immune paresis is not so severe and serious infection is less common (33% in three years): but hypercalcemia (59%) Bence Jones proteinuria (80%), and amyloidosis are more frequent although renal failure is not (17%). Rarely (1%) xanthomatosis occurs (see Section 7.5.7).

Bence Jones only (20% myelomatosis) patients on average excrete 6 g daily (a reasonable proportion relative to molecular weights 44,000–150,000 and average turnover of whole immunoglobulin 15 g daily, see Section 7.2). Severe immune paresis and infection are not so common (20% in three years). Hypercalcemia (62%), osteolytic bone lesions (78%), renal failure (33%), and amyloidosis are common. Most of these tumors seem faster growing, more invasive of soft tissues, can occur in younger age groups, and have a worse prognosis (H28).

IgD (1.5% myelomatosis) paraproteins are harder to find and usually are associated with heavy Bence Jones (90%L) proteinuria. Severe immune paresis and infection are not so common, but hypercalcemia (47%), osteolytic lesions (77%), extraosseous tumor (63%), and renal failure (52%) are common, as is presentation under 50 years of age (H36).

IgM (0.5% myelomatosis) can truly be associated with typical bone lesions (see Section 7.7.5).

IgE (0.1% myelomatosis). The two recorded patients (O1) initially had no bone lesions (but Johansson's case did later), and both seemed to have a tendency to plasma cell leukemia and hyperviscosity, perhaps because many of our few normal IgE precursors may circulate in the blood stream.

Nonparaprotein (1% myelomatosis) patients are well recorded (H28). Osteolytic lesions and positive biopsy have been needed to make the diagnosis, and our 8 such patients all showed severe immune paresis. In the abnormal plasma cells of 5, we were unable to detect heavy or light chains using reliable fluorescent antisera, and electron microscopy (one case) showed endoplasmic reticulum but with empty sinuses. Other workers have claimed a "forme frustre," with plasma cells showing immunoglobulin retention (H51), and it is conceivable a defective Golgi apparatus could impair secretion. The prognosis seems bad, only 2/8 surviving 6 months, and in these recovery of normal immunoglobulins offered some guide to the success of treatment.

During the treatment of myelomatosis, careful paraprotein measurements have been invaluable. They have only belied the prognosis in 3% of patients, who showed *nonparaprotein escape* with the emergence of reticulosarcoma, or monocytic leukemia some 3 or more years later (H35). Response has been preceded by falls in paraprotein levels, and

the slow response has had a much better prognosis than the fast response (halving within 3 months) (see H29). In some 15% of patients paraprotein has become undetectable by electrophoresis. In those who responded, relapse has been heralded by rising paraprotein levels, and in about half faster growth rates, with disproportionate increases in Bence Jones excretion, *Bence Jones escape* or *mutation escape* has been seen (H29). It is therefore important to monitor both serum and urine. Despite this mutagenic hazard, a majority of patients enjoy longer pain-free lives, and treatment is well worthwhile.

7.6.2. *Sclerotic Myeloma, Myeloproliferative Syndromes*

Some 11 patients have been recorded since the first report (S13) of plasma cell neoplasia and paraprotein with the rare osteosclerosis. Because Paget's disease is so common in the same age group, coincidence of the two diseases (or others) is possible. In genuine cases the serum alkaline phosphatase has usually been within normal limits, which would be most unusual for Paget's, and the work of Evison and Evans (E2) clearly shows the myeloma cells directly related to the bone sclerosis. Accepting that myelomatosis can occasionally evoke osteosclerosis, it becomes likely that it could also evoke myelofibrosis, etc., and this is one explanation for the association of paraproteins with myeloproliferative syndromes (B24), although this is not much above the natural incidence as only 1 paraprotein was found in our 56 patients.

If neoplastic plasma cells can sometimes stimulate osteogenesis, which they more usually inhibit, they might also stimulate erythroid cells, which again it seems they most commonly inhibit. This could be one view of paraproteins associated with polycythemia and even erythroleukemia. Another view is that the general myeloproliferative state either per se or as a result of mutagenic treatment has evolved a monoclone of plasma cells. A third view (see Section 7.6.4) can explain some erythroleukemia as misinterpretation of light microscopy.

7.6.3. *Soft-Tissue Plasmacytoma*

As these are mostly diagnosed by excision biopsy, there is a paucity of data as to the protein status before excision. At times up to 20 years later, when dissemination has followed, apart from some predilection of metastases for unusual bony sites (W11), the disease can be indistinguishable from myelomatosis, and I have usually had no difficulty in finding paraproteins. Careful study of preoperative serum samples in 26 patients with biopsy proven soft-tissue plasmacytoma and no obvious metastases only detected paraproteins in 11 (γD1, γG9, γM1), and this may be because at least 20–50 g tumor is needed to render them visible.

However in concentrated urine, Bence Jones was found in 15 and half-molecules in 3 others, leaving only 4 with no detected paraprotein in either serum or urine. The heavy-chain diseases also seem to be associated with soft-tissue plasmacytoma (see Section 7.6.5). The impression gained is that dedifferentiated immunoglobulin production (more Bence Jones, etc.) is commoner with extraosseous immunocytomata, and with more careful preoperative examination of urines such paraproteins may be found in most cases.

7.6.4. *Lymphoma and Leukemia*

Rundles, Coonrad, and Arends (R13) surveyed 35 patients with leukemia and detected paraproteins in at least 4, and possibly in 2 others. Since then there have been numerous reports of paraproteinemia with all kinds of lymphoma or leukemia, although almost no further surveys until 1969 when 19 of 76 patients were found to have excess light-chain excretion (L6). Personal surveys have revealed serum paraproteins in 3 atypical cases out of 124 consecutive patients with Hodgkin's disease, 26 of 207 with lymphosarcoma (see Section 7.7.2), 1 of 45 with reticulosarcoma, 0 of 31 with giant follicular lymphoma, 3 of 84 with chronic lymphatic leukemia, 0 of 43 with chronic myeloid leukemia, 0 of 57 with acute leukemia, and 1 of 5 with chronic monocytic leukemia as established by high lysozyme levels. Urine has revealed Bence Jones proteins in many of those with serum paraproteins and in addition only Bence Jones in 1 atypical Hodgkin's, 2 lymphosarcoma, 1 giant follicular lymphoma, yet 8 with chronic lymphatic leukemia.

It is therefore clear that a minority of patients with lymphoma and leukemia can have associated paraproteins, especially lymphosarcoma, atypical Hodgkin's, and chronic lymphatic leukemia. A special case can also be made for monocytic leukemia (O7). It has been stressed that the Hodgkin's was atypical, and this is in accord with others (A13), because histology revealed a marked plasmacytosis or excess large pyroninophilic cells, with forms intermediate between reticulum cells, lymphocytes, and plasma cells. Fluorescent studies indicate that these are the cells producing the paraprotein. It is such transitional neoplasia that underlines the limitations of morphology and favors the use of the term immunocytoma. Neoplastic immunocytes can be called stem cell leukemia (R8), etc., but electron microscopy and other studies suggest the few so-called myeloid leukemia producing paraproteins are really primitive plasma cells (T5).

The 12% of lymphoma and 10% of chronic lymphatic leukemia with paraproteins may actually represent neoplasia of the B-lymphocyte line (bursa-equivalent), i.e., true plasma cell precursors. The prognosis

of these tumors when producing paraprotein seems to be worse (K9), (more like myelomatosis); tribes of clones (more than one paraprotein) and cryoglobulins seem commoner. Eventually it may be possible to distinguish these from tumors of the T-lymphocyte line (thymus dependent). Concepts of derepression are best reserved for embryologically closely related cell lines (e.g., oat-cell carcinoma bronchus-branchial pouch endocrine activity), and thus B-lymphocytes (plasma cell precursors), T-lymphocytes, and monocytes could be expected to overlap in their neoplasia.

Finally there is the probability that severe immune paresis from the primary lymphoid neoplasia predisposes to monoclone formation (H19).

7.6.5. *Heavy-Chain Diseases*

Since Franklin's original description of γ-chain disease, a total of 17 cases are now known (F7), and tumors producing α-chain (some 20 cases) and μ-chain (some 4 cases) have been found. All these tumors have been primarily in the soft tissues, either as malignant plasmacytoma or lymphoma, with little if any involvement of bone. In common with other malignant immunocytomata, immune paresis is the rule and infection is often a cause of death.

While edema and redness of the uvula and soft palate were initially emphasized with γ-chain disease, this sign has been found in other lymphoid neoplasia and is also not essential to γ-chain. The γ-chain has often been difficult to see on simple electrophoresis, and only a little enters the urine, in which Bence Jones protein has not been found. The γ-chain exists as dimer, and represents mostly Fc (see Fig. 2) with some 8 or so amino acids of the Fd in front of it. The Fd seems quite untypical (F7) as if nonsense coding had resulted in a failure of most of it, so that no site is available for binding to light chains. As light chains seem to be not synthesized at all, presumably their coding has become completely nonsense or deleted. The molecular weights of such γ-chains vary from 40,000 to 70,000.

Seligmann's team (S8) first recognized α-chain in a disease which had been well known in the Middle East for some years as Arabian lymphoma of the small gut. This seems to have a genetic basis, the two Pakistanis I have seen possibly reflecting the travels of Alexander the Great. It often presents in the late teens as progressive, then intractable, malabsorption. Radiology reveals a stove-pipe small gut, whose whole wall becomes involved with plasmacytoma. Initially the tumor seems to be confined to the small gut, with normal secretory IgA in the rectum and in saliva, but later may spread locally, to tonsils, etc., and to bone marrow. The serum alkaline phosphatase develops an excess of intestinal

isoenzyme. The α-chain imparts a diffuse hump of $\alpha_2 - \gamma_2$ mobility, which characteristically reacts with anti-α far forward into the α-mobility region, where no reaction can be found with anti-κ or anti-λ and indeed Bence Jones protein is not found. In one tissue culture of jejunal biopsy we did find synthesis of a narrow monoclonal band of α-chain of β_2-mobility. It therefore presumably becomes polymerized post-secretion to yield the diffuse mobility seen in serum, with very little in the urine or intestinal secretions, and unassociated with secretory piece (which retains its usual mobility when free as with IgA deficiency). The regional nature of this disease implies amazing IgA phylogeny, and this is strengthened by the recent discovery (Dr. R. Ballieux, to be published) of α-chain disease confined to the lungs in a child of Dutch descent.

Synthesis of μ-chain only was first found in tissue culture lines grown from human lymphoma cells (T1). The patient later described (B3) is just like a typical case of Bence Jones only myelomatosis, with amyloidosis, except that a fast-migrating component reacting only with anti-μ was also found. This was not visible on serum electrophoresis. It might just represent a ghost of memory of the IgM type of immunoglobulin the original plasma cell precursor should have made.

7.7. IgM Paraproteins

After the discovery of 19 S IgM and the description of macroglobulinemia by Waldenström, it has become apparent that monoclonal IgM does not indicate one type of disease, but rather a spectrum of diseases ranging from frankly invasive reticulosarcoma to a benign condition. Because of this wide range of prognosis, IgM paraproteinemia is here considered under 8 subheadings (as shown in Table 8), which it is possible to delineate within the spectrum, although realizing that overlap can occur.

In general measurements of IgM paraproteins are difficult because of trailing on electrophoresis and occurrence of differing molecular sizes such as μ-chain (see Section 7.6.5), 7 S, 19 S, 24 S, 28 S, etc., as well as complexes with IgG (Section 7.7.6) or lipoprotein (Section 7.5.7). I tend to rely on electrophoretic dye-binding but am hopeful of Laurell's technique (H5). Among IgM paraproteins there is an excess of κ light chains (possibly due to the conditions described in Sections 7.7.3 and 7.7.6). Serum levels of IgM can also be misleading because of amazing expansions of plasma volume that occur (M2), and these can swing widely during plasmaphoresis. Apparent doubling times have often been "impossible" (C14) and of much less value than with other paraproteinemia.

Treatment, once started, should be continuous (M7). Because of the

TABLE 8
IgM PARAPROTEINEMIA[a]

Disease	Patients (%)	Serum IgM level (g/100 ml)	% Patients			
			7 S IgM subunits	Bence Jones	Subnormal IgG	Subnormal IgA
Myelomatosis	2	0.4–3.6[b]	66	100	100	100
Lymphoma	25	0.2–5.4[b]	45	100	40	60
Waldenström's	50	3–10	20	95	5	60
Cold agglutinin	3	0.4–2.4	9	9	12	32
Chronic lymphatic leukemia	2	0.2–4.5[b]	?	10	60	75
Rheumatoid factor	3	0.4–5.8[b]	0	0	0	5
Benign	10	0.2–2.0	0	0	0	0

[a] Varieties of disease (in order of average survival) with immunoglobulin findings and estimated incidence (after C2). A further 5% are associated with cancer.

[b] The few patients with high levels overlap with viscosity syndrome like Waldenström's.

risks, it is prudent to try and select one of the following diagnoses, and in this the assessment of 7 S subunits (see Section 1.3), Bence Jones proteinuria, and immune paresis seem to be valuable (see Table 8) in selecting lymphoma and myeloma for immediate treatment. Waldenström's disease has a long course if viscosity can be controlled (the first choice today may be plasmaphoresis alone). The others should be given cytotoxic drugs only when progressive tumor growth can be shown or life is threatened.

7.7.1. *Waldenström's Macroglobulinemia*

Unless discovered accidentally, the major presenting symptom is lassitude (out of proportion to the anemia which is usually present). There may be a history of susceptibility to virus infections, herpes zoster, or second attack of mumps, etc., although IgG is usually normal or raised (see Section 7.5.6). The onset is usually insidious and slow and may progress to semicoma before the patient is admitted to hospital. There is often purpura, or bleeding from gums or gut. The fundi by then usually show, bilaterally, distended tortuous veins with or without perivenous hemorrhages, and the viscosity syndrome (see Section 7.5.6) can be diagnosed. The spleen is usually palpable but is rarely more than grade 1 (grade 2 reaches the umbilicus). Lymph nodes, if palpable, are similarly not prominent.

With viscosity symptoms, the monoclonal IgM level is nearly always over 3 g/100 ml of serum. The plasma volume is expanded to some 1–2 liters more than expected, accentuating the anemia due also to a

decrease of the total red cell mass (M2). The bone marrow will show involvement with a lymphoid and/or plasmacytoid picture, but radiologically the bones are normal for age. Lymph node biopsy has a classical picture. The architecture is well preserved but diffusely infiltrated with round cells, which frequently involve the surrounding tissue. There is often an excess of mast cells, and the round cell nuclei frequently contain inclusions. Fluorescent antibodies reveal clusters of IgM-containing cells usually of a single light-chain type whereas in normal or reactive lymph nodes both K and L are present.

In summary Waldenström's macroglobulinemia seems to be a slow-growing infiltrating, widespread "lymphoma" whose protein production is thereby allowed to gain viscosity syndrome levels before the tumor does any direct harm. With adequate control, the prognosis is good, up to 10 years.

7.7.2. *Malignant Lymphoma*

That malignant lymphoma occurred with IgM paraproteinemia was clearly emphasized by Mackay, Taft, and Woods (M1). Here it is the tumor that brings the patient to the doctor. There is usually massive regional enlargement of a group or more of lymph nodes, and/or the spleen, and/or the liver. The history is shorter and more rapid in onset; usually malaise is more obvious. It is unusual to have achieved an IgM level giving rise to symptoms or signs of viscosity, though sometimes symptomatic cryomacroglobulinemia is seen (see Section 7.5.4). This occurs in about 10% lymphosarcoma with IgM paraproteins, and pyroglobulins and more than one paraprotein also seems commoner than with any other disease associated with paraproteins.

The serum IgM level is usually around 1 g/100 ml. Biopsy of the affected lymphoid tissue reveals frank invasion and destruction of normal architecture, and sometimes only a minority of the cells stain with fluorescent anti-μ (H21). The bone marrow biopsied can be free of involvement. Overall there is little doubt of a usually malignant lymphosarcoma, but this may be classified as atypical Hodgkin's disease or reticulosarcoma etc. The prognosis is correspondingly bad, and few survive the first year.

7.7.3. *Primary Cold Agglutinin Disease*

This disease is a rare form of chronic hemolytic anemia with Raynaud's phenomenon and occasional hemoglobinuria, which can be attributed to a high-titered 19 S IgM antibody which reacts at reduced temperatures with the red cell I antigen.

Primary cold agglutinins are distinguished from those secondary to

infections with *Mycoplasma pneumoniae, Listeria monocytogenes,* infectious mononucleosis, rickettsial endocarditis, etc., by being monoclonal, with one particular subclass of IgM (C13) and nearly always with K-type light chains (H7), whereas the others are polyclonal showing both K and L light chains (C17).

A monoclonal band can be found in most cases, but serum must be separated at 37°C. The ESR in the cold is much higher than at 37°C, due to massive agglutination in the cold. The monoclonal IgM is readily measured (C14), averaging 0.4 g/100 ml (range 0.1–2.4). The residual IgM and IgA and IgG are usually normal. In some 30% patients, mostly those with high IgM levels, a subnormal level of IgA is found. The cold agglutinin IgM is mostly well produced, with no Bence Jones proteinuria or 7 S IgM, and, on a molecular basis, specific lytic activity against enzyme-treated red cells was very similar.

Where Bence Jones proteinuria, 7 S IgM, subnormal IgG, or atypical cold agglutinin (anti-i, type L, etc.) are found, the probability of lymphosarcoma looms large (C2, C14). Some 10% of IgM lymphomata present with atypical cold agglutinins, and conversely disease terminates as lymphosarcoma. Apart from these, the prognosis is fair, for this would be a benign paraprotein in most cases, were it not for its hemolytic potential.

7.7.4. *Chronic Lymphatic Leukemia*

Some 1–5% of patients with typical chronic lymphatic leukemia may show small paraproteins in their sera, which are often IgM at levels from 0.2 to 2.5 g/100 ml, though occasionally higher (F1). Insufficient data are available to appreciate the significance of such findings (see Sections 7.6.4, 7.7.7, v). The emergence of such paraproteins together with Bence Jones proteinuria has been associated with Richter's syndrome—an emergence of a rapidly malignant lymphomatous termination of preexisting slowly progressive chronic lymphatic leukemia. They can thus be warning signs, and the overall prognosis is usually worse than for Waldenström's (Section 7.7.1).

7.7.5. *IgM Myelomatosis*

With IgM as the paraprotein, 25 known examples of radiological and clinical myelomatosis have been collected (H28). There is thus no doubt this is an entity accounting for 0.5% of all myelomatosis and some 2% of all IgM paraproteinaemia. Typically this disease differs from 7.7.1 in that the IgM level has been on the low side (average 1.9 g/100 ml), with heavy Bence Jones proteinuria and marked immune paresis. It has been the myelomatosis that has brought the patient to the doctor. At post-

mortem the tumor has been largely confined to bone marrow with little involvement of lymph nodes or spleen. Occasionally enough IgM has been present to result in a viscosity syndrome and overlap with Waldenström's macroglobulinemia (Section 7.7.1).

The prognosis is worse than for Waldenström's, few surviving for 3 years.

7.7.6. *Rheumatoid Factor Paraproteins*

Recognized in 1961 (K10), these are now well described (B18) and mimic the syndrome of mixed cryoglobulinemia (see 6.13). Skin lesions in this condition are raised, painful, and edematous with or without necrosis. Biopsy always reveals arteritis with a mononuclear and neutrophile infiltrate. There is in most cases a preceding history of rheumatoid arthritis, Sjögren's syndrome, syphilis, sarcoidosis or other "hyperimmune" states, and this will dominate the clinical findings. Rarely the protein interactions build up to a level presenting as a viscosity syndrome so that this group can overlap with 7.7.1 unless the serum is carefully examined.

If serum is separated at 37°C, a cryoprecipitate will usually form within 3 days at 4°C. Often the precipitate does not form at ranges that can exist in the skin (21°–40°C). The complexes usually are of 23 S size or above, and if dissociated at acid pH can be shown to contain polyclonal IgG KL as the antigen with a monoclonal IgM (usually K) as the rheumatoid factor antibody. The other findings and the prognosis depend on the associated diseases, but the bone marrow is usually nonspecific, as are the lymphoid tissues (unless studied by fluorescence which reveals abnormal clusters of IgMK-producing cells). In a few rare cases, frank lymphoma is seen and this syndrome then overlaps with that of malignant lymphoma (Section 7.7.2). It seems that there is preexisting lymphoid preoccupation by the "hyperimmune" process. Altered IgG-globulin arises, and in reactors this can stimulate IgM rheumatoid factor formation. Because of the preoccupation, there may be only a limited number of IgM clones left that are capable of responding. One of these may gain ascendancy and, provoked by the antigen, may go wild. Occasionally the monoclonal rheumatoid factor is IgA or an IgG subclass.

7.7.7. *Paraproteins and Cancers*

Because IgM are the commonest paraproteins associated with carcinomata and other nonlymphoid tumors, this discussion has been reserved until here. The same remarks, however, apply to other paraproteins, IgG, etc., found with cancers. Five explanations are possible:

(i) simple coincidence of two common diseases in the age group under study; (ii) the paraprotein is made by the carcinoma; (iii) the carcinoma has evoked a monoclonal immune reaction; (iv) the carcinoma and the immunocytoma have both been initiated by the same process, e.g., virus induced; (v) immune paresis due to the carcinoma has (a) reduced immunosurveillance, (b) enabled only a few clones of immunocytes to compete, so that a monoclone has emerged. Let us consider these possibilities in the light of available evidence.

(i) In the age group 50–60 years we can expect 2% population to have paraproteins (A12). Among some 3000 collected patients with cancers the incidence of paraproteinemia was 2%, as expected (H30). Coincidence is therefore presumably the commonest association, and indeed in many patients independent diagnoses of myelomatosis and carcinoma were clearly defined (H21, H50): However, Osserman (O6) has made the point that certain kinds of rare cancers, e.g., cholangiocarcinoma, bronchial adenocarcinoma, are associated with paraproteins, and he has the impression the frequency is greater than would be expected. With (iii) in mind, I have very carefully studied two patients who had cholangiocarcinoma, and both had undoubted independent myelomatosis.

(ii) I can find no good evidence that any histologically defined carcinoma has ever produced paraprotein. I found no positive immunofluorescence of the carcinoma cells for the class of paraprotein, no disappearance of paraprotein following excision of all the carcinoma [the day claimed by some authors (C8) is impossible for the known $T_{½}$ of paraproteins], and no production of any immunoglobulin in successful tissue cultures of 5 different cancers from patients with paraproteins.

(iii) Following Waldenström's suggestion, we isolated the paraprotein, added a fluorescent label, and tried to see whether it had any affinity for its associated carcinoma, with negative results in all the 8 cases tested. Others (W10) raised idiotype-specific fluorescent antisera and claimed staining of plasma cells within the carcinoma. This elegant method supported the idea of a monoclonal reaction to the carcinoma. In 5 of our 8 such cases, we could not confirm this; indeed ordinary anti-γ, etc., completely failed to show any plasma cell infiltration. Since carcinoma cells can imbibe plasma proteins in a completely nonspecific manner (C10), it may be that the idiotype-antisera just detected the most greedy, who had saturated their catalytic sites for that paraprotein.

(iv) The possibility of common tumorogenesis remains open, but fails in that gut cancers do not show the excess of IgA paraproteins that could be expected.

(v) Immune paresis is not a common feature of carcinomata (see

Section 4.0), although late and extensive metastases may have such a result. In that immunocytomata generally have very slow growth rates, they could hardly emerge before death from the extensive carcinoma. However, with slow-growing lymphoid neoplasia, it is quite conceivable that some of the paraproteins we find do arise in such ways: (a) loss of immunosurveillance, (b) loss of high-affinity competitors, allowing a low-affinity monoclone to emerge, with corresponding low-affinity feedback and thus continued multiplication. The occurrence of transient paraproteinemia in chronic lymphatic leukemia indicates an intermediate behavior (H19).

7.7.8. *Benign IgM Paraproteins*

These are uncommon and cannot be diagnosed with complete assurance until some 10 years of observation have passed. In general in Britain such patients are symptomless and have normal lymph nodes, spleen, and bone marrow. (In Africans the condition may be associated with parasitic infections, see M17.) The serum level is mostly under 1.0 g/100 ml, and hitherto always under 2.5 g/100 ml. It shows no tendency to rise with many years of follow-up; indeed it may disappear spontaneously. In our experience there is no Bence Jones proteinuria, and IgA and IgG levels are usually normal. Such apparently benign IgM paraproteins can be found in relatives of patients with malignant IgM paraproteins (S9), emphasizing the need for careful follow-up.

7.8. Benign Paraproteins

After excluding those paraproteins considered in Sections 7.6–7.7.7, the remainder can be followed up at yearly intervals. After 5 years with no emergence of malignancy, they can reasonably be called benign. However, continued yearly follow-up is recommended, although I have not seen an unprovoked benign immunocytoma change its spots and suddenly become malignant. Waldenström, with longer experience, has witnessed a sudden rapid increase in a paraprotein level which had been steady for many years, but this does appear to be a rare event.

Much has been made of the 63 benign paraproteins found in a survey of an adult population of 6995 (A12), but the 5-year follow-up is not yet available. As clinical chemists, we are largely concerned with the patients who attend doctors. Among these, with sound criteria, I find (H19) the same incidence as Axelsson *et al.* (A12), yet achieve a diagnosis of malignancy in 74%, only 26% at the most being truly benign after 5 years (see Table 7). This is also in accord with others who study patient populations (C12). A paraprotein should therefore be considered as potentially malignant until proved otherwise.

There has also been a tendency to ascribe a genuine association between paraproteins and liver diseases, gut diseases, and certain infections (reviewed by M17). However, among our collection of 368 patients with liver diseases, repeated serum electrophoreses have revealed only 3 with paraproteins, and among 326 with gut disease only 1; allowing for their ages this almost seems less than the expected natural incidence. Furthermore one would expect IgA paraproteins to predominate, and they do not. The incidence of the various classes of paraproteins agrees closely with the proportions of IgG, IgA, IgM, IgD, IgE (K:L) catabolized daily, and since this reflects the actual numbers of plasma cells of each class, the risk of a monoclone developing seems to be a random event (H26). With increased immunoglobulin levels, there must be more plasma cells at risk, and the available evidence suggests that paraproteinemia is commoner in populations native to areas with high endemic rates of infection (M17). Aleutian disease of mink provides just such a model (P11).

Curiously paraproteinemia also seems commoner with immunoglobulin levels at the other extreme, in immune deficiency states (H6, H19, M17, R1, Y2), and Heremans has suggested that this is due to less competition (see 7.7.7, v). These paraproteins may well be antibodies with such weak affinity for antigen that they do not switch themselves off.

7.8.1. *Transient Paraproteins*

The whole subject of transient paraproteins has been well and concisely reviewed (Y2), reserving this term for proven paraproteins, suddenly discovered, rising rapidly to a peak value, and disappearing spontaneously within weeks or months. In 4 patients the actual emergence of such paraproteins onto preexisting negative electrophoreses was witnessed (Y2). The paraprotein levels doubled in 8–12 days, very much faster than myeloma levels (28–700 days), and halved in 7–14 days. This is still slightly slower than for normal antibody responses (1 day), and this supports the idea that perhaps such transient paraproteins represent a weak recognition of antigen, but eventually with enough affinity for combination to occur and switch off the response. There has been no secondary immune paresis or Bence Jones production. Three patients had had multiple blood transfusion, and after cross-matching has eliminated likely strong reactions, it is plausible to see the transient paraprotein as a single clone in the patient, finding a small residual difference between donor and host (Y2). In this light, the transient appearance of paraprotein in an immune deficient child transplanted with closely matched marrow (H6) also becomes understandable.

7.8.2. *Lichen Myxedematosus*

This is a rare chronic skin disease in which an unusual paraprotein has been consistently found (J1) since being first described (P5). It is cathodal to the normal γ-globulin (which is well preserved, see Fig. 15). It is always IgGL, and in 5 personal cases IgG_1L. No Bence Jones proteinuria has been detected, and the serum paraprotein level seems largely stationary, so that this would qualify as a benign paraprotein. Immunofluorescence showed IgG localized in the skin lesions (J1) and in confirming this we also found it was type L. This suggests the IgGL is a monoclonal antibody related to the skin disease, just as IgMK so consistently relates to primary cold agglutinin disease (see Section 7.7.3).

8. Summary of Clinically Useful Immunoglobulin Studies

I. In certain clinical contexts and with only one disease affecting the results, the crude but simple overall measurement of immunoglobulins can be useful.
   A. Valuable areas are:
      Immune deficiency, recurrent respiratory infections (parotid saliva also), malabsorption (jejunal juice also), idiopathic splenomegaly, etc.
      Lymphoid neoplasia
      Neonatal infections
      Liver diseases
      Multiple sclerosis, etc. (CSF only).
      Proteinuria in childhood, with hypertension, etc., or tubular lesions (clearance studies are invaluable).
   B. It can often be rewarding in monitoring:
      Crohn's and celiac diseases
      Endocarditis
      Aberrant immunity
   C. When IgE reagents become more readily available these will have obvious application to the clinical evaluation of atopy.

II. Establishing the level and class of a paraprotein enables a better appreciation of that level, and possible associations. Immunoglobulin fragments (urine should be properly examined for Bence Jones protein: IgM for 7 S, etc.) and reductions of other immunoglobulins imply a serious prognosis. Follow-up is essential and can indicate the natural history or response and escape on treatment.

III. In special areas (cryoproteins, cold agglutinins, rheumatoid factors, lichen myxedematosus, etc.) special studies will be needed.

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THE BIOCHEMISTRY OF SKIN DISEASE: PSORIASIS

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1. Introduction

We were uncertain as to the kind of review about the biochemistry of skin disease which would be of interest to a clinical chemist. On the other

hand, dermatologists would presumably also be interested in an article on this subject. What do they have in common? Obviously information about those diseases of the skin which are of general medical and biochemical interest, such as lupus erythematosus or the dyslipoproteinemias or porphyria, could just as easily be written by specialists from a field other than dermatology and with more authority. For some time we pondered the possibility of discussing those laboratory tests which were of special interest in the diagnosis and treatment of disorders treated by dermatologists; however, this was even less appealing since we would be discussing tests more familiar to the reader of the article than to the authors. We were then left with discussing a subject of biochemical interest, of dermatological interest, which fell within the special province of the dermatologist. There is only one such disease—psoriasis. This is the only skin disease which has been extensively studied and whose clinical, biochemical, and physiological aspects are unfamiliar to most scientists outside dermatology.

We do not apologize for the choice, since, although there are no biochemical tests which can yet be performed in the laboratory to help confirm the diagnosis or to aid in the prognosis or choice of therapy, the biochemical enigmas encountered in the study of this disease are or should be of interest to all biologists. Psoriasis is common; it has a definite genetic background; it has uncontrolled yet not malignant growth as its outstanding feature; it has disordered maturation and differentiation; it has an experimental model in the Koebner reaction; it has intense metabolic and mitotic activity; it has material readily available for study; and it offers the possibility of helping millions of people who suffer but usually do not die.

2. General Information

2.1. History

There is reason to believe that some of the "lepers" referred to in Leviticus might actually have been suffering from psoriasis (G7); however, Robert Willan (W17) in 1808 is usually credited with the first clear separation of this disease from the numerous other scaling dermatoses. During the past 150 years, therefore, this disease has been well recognized, intensively studied, and treated by every imaginable means both internal and external. Some recent reviews include those of Tickner (T9), Baer and Whitten (B4), Farber *et al.* (F1, F2, F3, F4), Shelley and Arthur (S9), and Braun-Falco (B29). In addition, a fascicle of the *Annals of the New York Academy of Science* (A4a) is devoted to psoriasis, as are books by Sidi *et al.* (S12) and Jarrett and Spearman (J3). The latter is limited to histochemistry.

TABLE 1
PREVALENCE OF PSORIASIS

Author	No. in population studied	Prevalence (%)	Reference
Bereston and Cecclini	20,000	0.27	(B9)
Farber and Peterson	1,000	3-4	(F3)
Forssman	693	1.4	(F16)
Gahan	Estimate	1.0	(G1)
Hellgren	39,571	3.0	(H14)
Lane and Crawford	231	0.51	(L1)
Lomholt	10,984	2.8	(L10)
Sutton	4,732	0.2	(S29)

2.2. PREVALENCE

Psoriasis affects men and women equally often and is found in all races examined except perhaps some South American Indians (C9). It is infrequent in American Indians and in Negroes compared to Caucasians (F5).

How common is psoriasis? Estimates of the prevalence of psoriasis in the population at large vary from 0.2 to 4% (Table 1). Bereston and Cecclini (B9) and Sutton (S29) based their data on a study of Army personnel; therefore, the mean age of the population studied was low and all were men. This no doubt explains the low frequencies since most studies indicate that the mean age of onset of psoriasis is in the twenties and about four years later in men than in women (Table 2). Lane and Crawford (L1) actually reported an incidence rather than a prevalence figure for psoriasis since they were reporting the percentage of new cases of psoriasis admitted to the Massachusetts General Hospital per year per total hospital admissions. The prevalence of the disease would be expected to be much higher than the incidence, since once it appears it rarely goes away and it does not decrease the life expectancy of the

TABLE 2
SEX DISTRIBUTION AND AGE OF ONSET OF PSORIASIS

Author	Male/Female	Mean age of onset	
		Males	Females
Hellgren (H14)	20,569/19,002	30.0	26.1
Farber *et al.* (F4)	968/1176	28.2	26.2
Romanus (R12)	461/307	18.4	13.8
Steinberg *et al.* (S24)	251/213	32.0	28.0

affected population. The remaining figures in the table are all greater than 1%, and those from the largest and most adequately performed studies (i.e., Lomholt and Hellgren) indicate that about 3% of the population at large have psoriasis.

Because of the periodic character of psoriasis, the poverty of lesions in some cases, and a distribution on sites hidden by clothing, most surveys would tend to underestimate the prevalence. There are many psoriatics who never go to a dermatologist for care and some who never seek care from any physician; however, psoriasis represents about 6% of all new patients seen by dermatologists (B20, L1), and an even greater proportion of his patient visits since this is a chronic disease.

2.3. Inheritance

Since the first clear description of psoriasis in 1808 by Willan, its familial occurrence has been recognized, but the exact genetic nature remains unresolved. The rather irregular distribution of cases in most pedigrees, the wide age range of first appearance, and the many environmental factors which seem to precipitate recurrences suggest that the effect of the genotype is highly variable. Table 3 presents the frequency of familial occurrence found in the studies of some large kindreds which have appeared in the literature.

Most authors have concluded that psoriasis is inherited as an autosomal dominant with incomplete penetrance (A1, A5, G11, H14, L5, R12, S5, W2); Steinberg *et al.* (S24) suggested, however, that affected individuals were homozygous for two independent recessive genes. There are valid arguments against both of these suggested modes of inheritance. The rise in consanguinity in parents which would be expected if the disease is inherited as a double recessive trait was not present in Steinberg's kindred. On the other hand, if the disease is inherited as a single dominant, then the proportion of offspring affected should be the same whether one parent or neither parent has the disease. Table 4 illustrates

TABLE 3
Familial Prevalence of Psoriasis

Author	No. in population studied	Familial occurrence (%)	References
Farber *et al.*	2,144	36	(F4)
Grayson and Shair	248	18	(G11)
Hellgren	39,571	35	(H14)
Hoede	1,437	39	(H23)
Lerner	172	42	(L5)
Romanus	1,417	30	(R12)
Schamberg	592	13	(S5)

the frequency of affected sibs as related to parental involvement. This indicates that in a family with a psoriatic child there is a two to four times greater probability of having a second psoriatic child when one parent has psoriasis compared to a family with a psoriatic child where neither parent has the disease. This is not compatible with a single dominant mode of inheritance, but is more suggestive of autosomal recessive inheritance.

This controversy is unresolvable at the present time. When the frequency of a trait in the population is low and the concentration of cases in families is high then identification of the type of monofactorial inheritance does not usually present much difficulty. However, the higher the frequency of the trait in the population relative to that in close relatives of an index case, the greater the difficulty in discriminating between monofactorial and more complex genetic explanations (C1). Psoriasis occurs with sufficiently high frequency in the population at large that additional genetic markers will have to be discovered and studied before a definite mode of inheritance can be proved. The possibility also exists, of course, that psoriasis is not one disease, but several, and perhaps in different kindred different modes of inheritance are present. An excellent discussion of these problems as well as other more complex aspects of the genetics of psoriasis can be found in Stevenson and Wells (S26).

Chromosome studies (G6, H21, R21, V7) have failed to reveal any abnormalities in cells from psoriatic patients.

2.4. Clinical Notes and Therapy

Psoriasis can take many forms varying from a few mild red scaling patches on the knees, elbows, or scalp to universal involvement of the skin with marked signs of systemic illness and debility. These clinical features of psoriasis as well as the associated arthritis are treated in the reviews cited as well as in standard texts on dermatology. In addition the response to therapy, while of great interest to clinicians, is not considered pertinent here and will not be discussed except to note that any

TABLE 4
Effect of Psoriasis in Parent on the Chances of a Second Child Having Psoriasis

Author	Percent affected when neither parent has psoriasis	Percent affected when one parent has psoriasis	Ratio of sibship frequency
Hoede (H23)	4.75	12.5	2.5
Lomholt (L10)	17	31	1.8
Steinberg *et al.* (S24)	2.5	9	3.7

cellular poison or mitotic inhibitor (ultraviolet light, ammoniated mercury, steroids, or cytotoxic agents) is helpful in treatment.

2.5. The Koebner Phenomenon

This reaction is named after Heinrich Koebner (K6), who described this striking and characteristic occurrence of lesions of psoriasis in areas of skin injury. The lesions usually take 10–15 days to appear, are sharply limited to the injured area, and can be provoked in up to 40% of patients. This provides good material for the study of the early events in the formation of a psoriatic lesion. Extensive investigations of the kind and extent of the trauma necessary to provoke these lesions have been carried out; the general conclusion is that there must be some kind of epidermal damage, not just dermal injury (F6, P3, R7, S18, S19).

3. The Lesion

3.1. Microscopy

For those unfamiliar with the histology of either normal or abnormal skin, Fig. 1 shows photomicrographs taken at identical magnification of normal skin on the left and the lesion of psoriasis on the right. Good descriptions of these lesions as seen with the light microscope have been written by Helwig (H15), Gordon and Johnson (G9), and Braun-Falco (B28). A detailed study of their electron microscopic appearance has been published by Brody (B35, B36, B37, B38, B39, B40, B41).

Most striking is the marked, regularly spaced thickening (acanthosis) of the epidermal layer. The normal 5–6 living cell layers are increased severalfold in the elongated epidermal ridges which reach down to interdigitate with and enfold their equally hypertrophic dermal papillae. Each dermal papilla contains an enlarged and straightened capillary which forms a simple loop. Polymorphonuclear leukocytes extend from the capillary into the epidermis in varying numbers. Where the dermal papillae reach toward the surface, the overlying epidermis is not thickened; after the scale is removed only slight abrasion of the lesion is needed to open these capillaries and cause regular pin point bleeding spots to appear ("auspitz sign").

The normal epidermis is less than 0.1 mm in thickness whereas the epidermis of the psoriatic lesion reaches to at least three to four times this depth. The material within the enlarged papillae must be rather soft or easily compressible since a dermatome set to cut at 0.1 mm (just below normal epidermis) will actually cut at a depth of about 0.3 mm when used to remove a superficial slice of skin from a lesion of psoriasis (H2, H16).

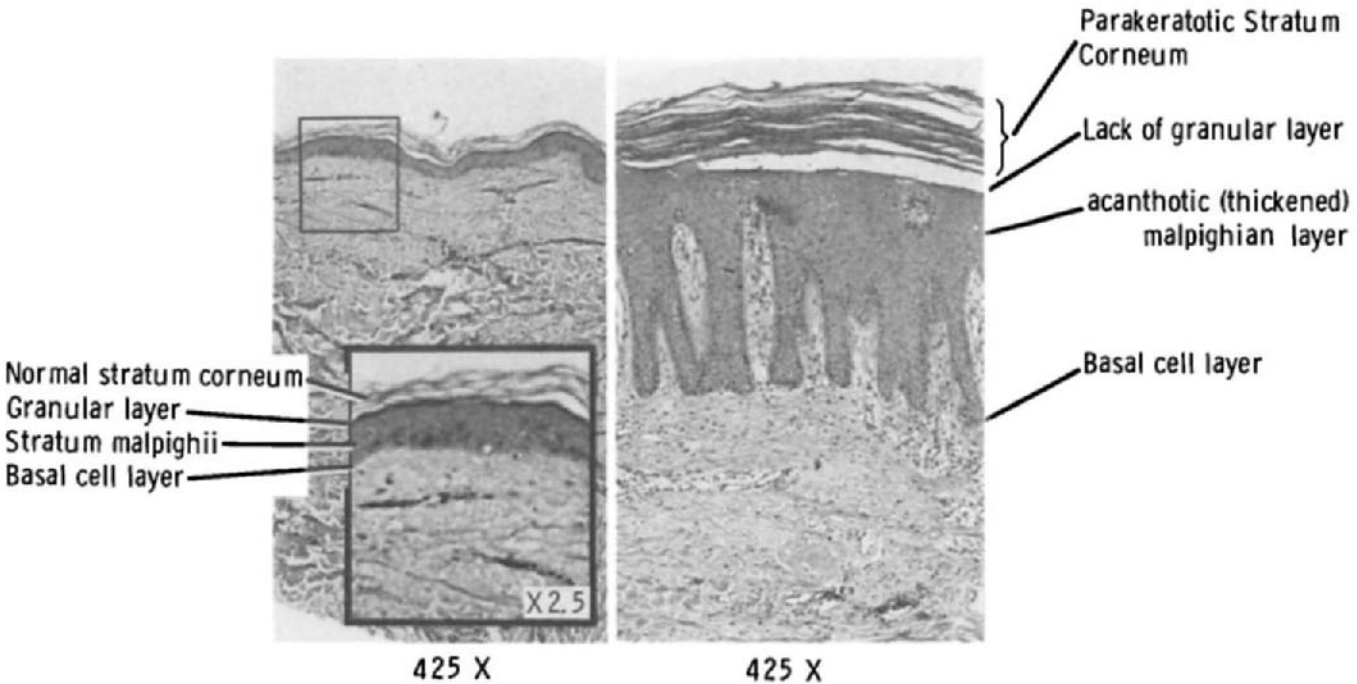


FIG. 1. Light microscopy of normal skin and a psoriasis lesion. The normal skin (on the left) and the psoriasis lesion (on the right) were photographed at the same magnification (×70 original).

Above the living cells, or malpighian layer, of the normal epidermis is the multilayered, dead, anuclear stratum corneum, which is instrumental in keeping water in and the environment out. It sheds so inconspicuously that the process is not normally noticed. Below the stratum corneum are the topmost layers of the living cells, the so called "granular" layer. It is called by this name because the cells contain prominent granules staining a deep blue with hematoxylin. Brody has suggested calling this layer the intermediate layer (B34) rather than the "granular" layer since, although the layer of cells immediately below the stratum corneum or "horny layer" is always intermediate between living and dead cells, in pathological situations it may not always contain "keratohyalin" granules. This layer is better illustrated by the higher power photomicrograph shown in Fig. 2.

By contrast the horny layer of the psoriasis lesion is greatly thickened (hyperkeratosis) and contains nuclear remnants (parakeratosis). Although this layer of horn is thick and forms the clinically visible scale which is the hallmark of the disease, the scale or horn is physiologically inefficient as a barrier.

Immediately below the thickened parakeratotic horny layer of the

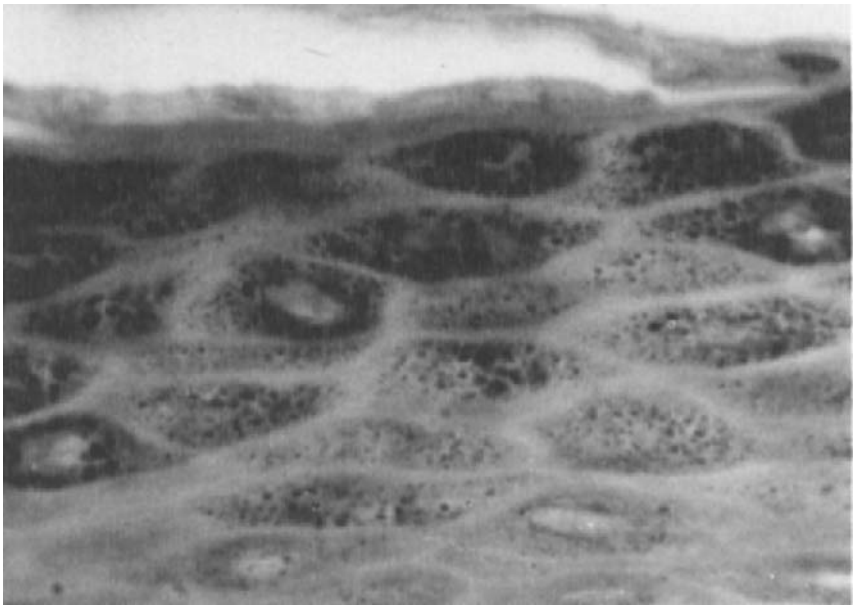


FIG. 2. Higher magnification of the normal stratum granulosum (granular or intermediate layer). The dense, dark-staining keratohyalin granules filling the cytoplasm of 3-4 cell layers can easily be seen. ×680.

psoriasis lesion is a layer of cells which are intermediate but contain no granules. Improvement of the lesion clinically is usually accompanied by the reappearance of granules within the intermediate layer and the formation of a more normal horny layer. Normal maturation of the epidermal cell has as its end product the "horn cell" or stratum corneum cell packed full of protein (keratin) fibrils in an α-helix configuration (by X-ray diffraction). These fibrils, which measure about 70 Å, are surrounded by a denser matrix material to form a characteristic lattice pattern (α-keratin pattern). Nuclear and cytoplasmic organelles are not seen since they are removed at the level of the intermediate or "granular" layer. Below the intermediate layer the epidermal cells contain fibrils measuring about 50 Å in diameter. The "granular" layers with their masses of keratohyalin represent a stage of cell maturation in which the change from a 50-Å fibril to a 70-Å fibril and the beginning of the packaging process are accomplished.

In psoriasis the 50-Å fibrils which start in the basal or most inferior cell of the stratum malpighii are never transformed into the 70-Å fibril found in the normal stratum corneum. They remain as 50-Å fibrils poorly integrated into the surrounding denser matrix and do not assume the nicely arrayed keratin packing found in normal horn cells. In addition keratohyalin granules are not found in the cells of the intermediate layer, and the cytoplasmic and nuclear contents of the cell are removed only to a very limited degree. An alternative picture involving lipid has been discussed by Swanbeck (S30, S31).

In addition to the epidermal abnormalities noted above, there is an increase in the number of dermal cells which is probably of secondary importance. However, it should be noted that Weddell (W7, W8) using special histochemical techniques has reported an increased production and breakdown of Schwann cells in the psoriatic lesion, and he believes that these changes are of primary importance in stimulating the epidermal lesion. Along similar lines is the suggestion by Pinkus and Mehregan (P7) that the polymorphonuclear leukocytes are of prime importance in stimulating the epidermis and that the dilated capillary in the papilla "squirts" these leukocytes into the epidermis as an initial event.

Other investigators have cited an abnormal capillary as being causative (H24, R13, T7), but although it is true that there is increased blood flow in the lesion (N2), most workers have not been able to confirm the findings of a capillary change in normal-appearing skin of the psoriatic patient or of an abnormal capillary response to injury (E1, I1) as being a primary event.

Recent studies by Christophers and Braun-Falco (C5) indicate that at the edge of lesions there is increased epidermal thickness and mitotic

activity prior to any change in the dermal papilla, and studies reported by Petzoldt *et al.* (P5) showed partial healing of lesions under occlusive therapy with no change in the number of polymorphonuclear cells as indicated by histochemical identification.

Although the question is far from settled, the weight of evidence is on the side of an epidermal rather than a dermal defect, and for the purposes of this review we shall assume that the primary event lies within the epidermis and that the various responses seen in the dermis are secondary.

3.2. Functional Aspects of Disordered Stratum Corneum Formation

Despite the thickness of the stratum corneum of the psoriatic lesion, it functions poorly. It is rapidly made, inadequately packaged, and easily disrupted. Normal people lose approximately 0.5 g per day of cornified material from the surface of the body (G8) by an exfoliative process which is so finely tuned as to be unnoticeable. These thin dead cells of normal stratum corneum are remarkably efficient as a water barrier since they allow the exit of less than 0.5 mg/cm^2/hour, or a total body water loss of less than 240 ml per day (K5).

On the other hand, psoriatic scale may be produced and sloughed off at a rate of up to 17 g per day (F18), and despite this overproduction it allows the escape of ten times more water than normal (G13). Grice and Bettley (G13) have calculated that the water loss, which can be over 2000 ml per day, represents a heat loss of 1000 kcal per day; this might explain the hypothermia and increased basal metabolic rate seen in patients with extensive disease. It might also contribute to some of their other systemic problems, such as high output cardiac failure reported by Fox *et al.* (F17). Another approach to the magnitude of the psoriatic defect is the study of Croft *et al.* (C12). They measured DNA in psoriatic scale and found that their patients lost up to 28 g of scale per day containing enough DNA to account for a loss of about 4×10^6 cells per minute. Since de Bersaques (B14) has shown that only about one-third of the DNA is still left in the cells of the parakeratotic stratum corneum, there is probably an actual cell loss of about 1.5×10^7 cells per minute, or a loss of approximately one billion cells per hour. This can be compared with the 16.7×10^7 cells lost per minute in the human gut (C12).

The whole subject of the functional biology of the stratum corneum has been most thoroughly and enthusiastically reviewed by Kligman (K5).

3.3. Cell Kinetics

The single characteristic which most adequately sums up the pathogenesis of psoriasis is the markedly increased proliferation of the epi-

dermis. This abnormally rapid epidermopoeisis leads to the histologic findings of acanthosis and increased numbers of mitotic cells. The hyperkeratotic and parakeratotic stratum corneum as well as the absence of a "granular" cell layer may also be due at least in part to the rapidity of new cell formation. According to Sutton and Sutton (S28), Crocker first suggested that the epidermis proliferates rapidly in psoriasis, but scientific study of this phenomenon is only 10 years old.

The first report which clearly substantiated an increased epidermal proliferation in psoriatic skin was published in 1961 by Rothberg, Crounse, and Lee (R18). These investigators injected ^{14}C-labeled glycine intravenously into two patients with terminal leukemia and fed the material to two patients with psoriasis. They serially collected scale from the psoriatic patients and scraped the stratum corneum from the backs of the patients with leukemia. The radioactive glycine was isolated chromatographically from the hydrolyzates of the scales, and its specific activity was determined. For the first 13 days they could detect no radioactivity in scrapings from the normal skin, the glycine-specific activity then increased gradually to reach a peak on the 26th and 28th days following administration. Psoriatic skin reached peak activities after only 3 days, indicating a cellular turnover rate 8–9 times greater than that found in normal skin. These authors postulated that the glycine-^{14}C was incorporated into proteins synthesized by all viable epidermal cells from the basal cell layer to the granular cell layer; therefore, the delay of 13 days in the normal skin before radioactivity could be detected represented the time required for a cell to travel from the bottom to the top of stratum corneum. They further postulated that the peak glycine specific activity represented the time necessary for cells to go from the area of maximum incorporation (which they presumed to be the basal cell layer) to the skin surface. Unfortunately for this latter measurement, there is no evidence that glycine is preferentially incorporated into the basal cell layer; in fact, it is either uniformly incorporated (W9) or accumulates preferentially in the "granular" layer (F27). Their figures of 28 days for transit time from the basal layer to the surface in normal epidermis and 3 days for psoriatic skin are, therefore, too low. Another difficulty inherent in this study is the fact that the backs of the normal patients were repeatedly scraped. This trauma was probably sufficient to speed up the rate of epidermal proliferation.

Van Scott and Ekel in 1963 (V4) studied epidermal cell kinetics by projecting histologic sections of lesions. They traced the architecture of the epidermis, counted the number of mitoses present, and determined the relationship of the proliferative cell population to the skin surface, to the viable epidermal cell population, and to the epidermis as a whole. This was repeated in from 6 to 24 sequential 8-μ-thick sections, and a

three-dimensional model was made in acrylic plastic. From these studies, they measured 27 times more cells in mitosis per unit surface area in psoriatic epidermis than in normal epidermis and calculated that psoriatic epidermal turnover time was 4 days. Inherent in this calculation was the assumption that epidermal turnover time as determined by Rothberg *et al.* (R18) was correct, and since this assumption is probably not correct their estimate must also be too low.

Most subsequent studies of epidermal cell kinetics have relied on autoradiography after the local injection of tritiated thymidine (E5, K2, W9, W10, W11, W12). Technical advances in applying a thin film of photographic emulsion over histologic slides prepared from tissue previously exposed to a radioactive isotope made localization of the site of incorporation of an isotope into macromolecules within the cell population possible. This technique was further improved when tritium-labeled isotopes became readily available. Nuclear emissions from tritium are much less penetrating than those from carbon, sulfur, or phosphorus, and silver granules are reduced only in the immediately adjacent emulsion and, therefore, give fine localization. Of the purine and pyrimidine bases which make up nucleic acids, only thymidine is incorporated exclusively into DNA and the availability of tritiated thymidine made possible the specific labeling and localization of those cells within a population which are actively synthesizing DNA in preparation for mitosis. These techniques have been of great value in our understanding of cell population and cell cycle kinetics and several good general reviews of the subject have appeared (B18, C7, S10, S27, W11, W23).

The first detailed study of normal human epidermal population and cellular kinetics using tritiated thymidine and autoradiography was reported by Epstein and Maibach in 1965 (E5). A detailed discussion of this study is in order since many of the concepts involved in such studies are well illustrated. Reference to Fig. 3 will make the discussion easier to follow.

Epstein and Maibach carefully defined renewal time of the viable epidermis as "the average time needed for all basal cells to reach the horny layer." On the other hand, transit time is defined as "the time needed for a basal cell to reach the granular layer." Actually what was meant by transit time was the appearance of the *first* labeled cells at the granular layer, and this is the *minimum* transit time although this was not explicitly stated. As the authors pointed out "if all cells move at the same rate, transit time will equal renewal time." Otherwise, transit time will always be less than the renewal time. The results of their study were as follows.

Forty minutes after injection (before any mitotic divisions could have

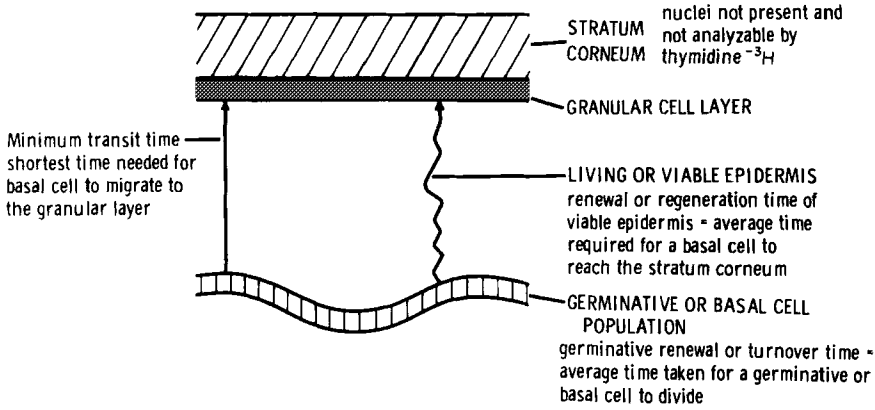


FIG. 3. Epidermal cell population kinetics. The germinative (DNA-synthesizing) cell layers reduplicate themselves in an average amount of time called the renewal or generation or turnover time. These cells make their way to the granular layer in a random fashion. The shortest time needed for the first cells to arrive at the granular layer is called the *minimal* transit time, and the average transit time is called the renewal, regeneration, or turnover time of the viable epidermis. From the granular layer to the surface, the cells are firmly attached one to the other and the cells all move in unison so that in transit through the stratum corneum there is only one transit time which is both minimal and average, and it is also the renewal, regeneration, or turnover time of the stratum corneum. Total epidermal regeneration or renewal, or turnover, includes both the renewal of the viable epidermis and the stratum corneum.

taken place), the basal cell layer and also a few cells just above the basal layer were labeled, thereby demonstrating that in normal human epidermis the germinative cell population includes the basal layer and a few cells above the basal layer. Actually more recent work (P4) has demonstrated that about 40% of the labeled cells (i.e., those synthesizing DNA) may be just above the basal layer.

The number of labeled basal cells was 5% of the total number of basal cells. Epstein and Maibach calculated that the time needed for renewal or turnover of the basal or germinative layer varied from 4.2 to 8.4 days with a mean of 6 days and for renewal of the entire viable epidermis from 12.4 to 25.6 days with a mean of 17.7 days. Unfortunately, inherent in these calculations was the value for the DNA synthesizing time of the germinative or basal cell layer. Epstein and Maibach assumed a value of 7 hours "following the almost universal finding that the T_s (DNA synthesis time) of normal mammalian cells is 6 to 8 hours." However, Sherman *et al.* (S10) had previously found a DNA synthesis time for mouse ear epidermis of 30 hours, and the DNA synthesis time

for normal human epidermal cells as subsequently determined by Weinstein and Frost (W11) is 16 hours. Correcting their average values for this difference would give an estimate of about 14 days for basal cell turnover and about 40 days for turnover or renewal of the entire viable epidermis.

Labeled nuclei first reached the granular layer and were lost to view by 10–14 days, and this represents the minimum transit time for a cell after production to reach the dead stratum corneum.

Epstein and Maibach also noted that the number of labeled cells increased steadily due to cell division for approximately 7 days and then declined rather steadily until none could be seen by about 42 days. The half-life of disappearance of labeled cells measured from the slope of the curve after 7 days was about 13 days, which fitted well with their previous calculations of 17 days for renewal of the viable epidermis. The slope of the disappearance curve, however, has two components: one is the rate of removal of the cells at the surface (which is the desired figure), and the other is the disappearance of labeled nuclei because of repeated cell division which makes the grain count so low that the cells can no longer be observed. This slope then cannot be used for the purpose of determining generation time and is also grossly in error for this purpose. It does, however, show that the cells do not stay static. Cells which have incorporated thymidine either make their way up to the surface and are sloughed off or they continue to divide and lose their labeled DNA by repeated dilution through several cell divisions. They do not divide once, for example, and then just stay in the basal layer without undergoing further divisions.

Two other pieces of evidence also indicate that Epstein and Maibach's calculations of 6 days for basal cell regeneration and 17 days for viable epidermal cell regeneration were incorrect. First, if the basal cell layer regenerates in 6 days, only three cell layers would be formed by the time the total viable epidermis was supposed to be regenerated (17 days), and histology shows clearly that the viable epidermis is about 6 cell layers thick. Second, they noted a marked discrepancy in the time it took for a cell to reach the granular layer, some cells still not having started 7 days after the injection. In view of this finding the epidermal renewal time should be much longer than the transit time (minimum) of 14 days and could not be almost the same. In summary, on the basis of the above study: (1) Epidermal minimum transit time is 10–14 days from the basal to the granular layers. (2) Basal or germinative cell renewal time is approximately 14 days. (3) Viable epidermal cell renewal time is approximately 40 days. (4) Total epidermal regeneration is approximately 54 days (adding the time needed for a cell to traverse the stratum corneum and the viable epidermal cell renewal time).

In 1965 Weinstein and Van Scott (W12) studied normal epidermis and psoriasis by the same methods. They noted in normal epidermis that labeled mitoses were not seen until 12 hours after injection, thereby implying a G_2 period (period between DNA synthesis and the onset of mitosis) of about 12 hours. Transit time (minimum) was 12–14 days in agreement with the previous study. In psoriatic epidermis, the lowest three cell layers were labeled; this implies DNA synthesis in all these layers. Labeled mitoses were seen at 3 hours, an observation implying that the G_2 period was much shorter in psoriatic germinative cells than in normal germinative cells (12 hours). Transit time (minimal) was 2 days; in addition, unlike the case in normal stratum corneum—where no nuclei and, therefore, no label can be seen—the psoriatic cells could be followed through the stratum corneum, and the first ones reached the outermost layers in 4 days. Weinstein and Van Scott also noted random movement of cells toward the surface, an indication that the generation time would be quite a bit longer than the transit time (minimal) of 2 days. In this study the minimal transit time, which is what the authors measured, was treated as though it was the average transit time and the authors spoke of "the turnover time of the normal stratum malpighii determined in this study (13–14 days)." As pointed out above, the turnover time or renewal time of both the psoriatic and normal epidermis, had they been calculated, would have been much longer than the minimal transit times actually measured. This criticism aside, this excellent study clearly showed an increased number of proliferating germinative cells in psoriasis and showed that the production of new cells was rapid enough to reduce the minimal transit time through the viable epidermis from the normal of 12–14 days to the psoriatic 2 days, or about 7 times faster. Figure 4 illustrates these findings for psoriasis and for some other scaling diseases.

In addition to the decreased transit time, there was evidence that the germinative cell in psoriasis might have a reproductive cycle quite different from that of the normal germinative cell; in subsequent studies, Weinstein and Frost (W10, W11) showed that there was indeed a great difference, the psoriatic germinative cell turning over in 37.5 hours as compared with 457 hours for the normal germinative cell. This situation is illustrated in Fig. 5, in which the cell cycle is generalized and represented as a circle with sectors representing the various periods. These periods are: (1) mitosis which can be recognized morphologically; (2) G_1 or the postmitotic period; (3) S in which DNA synthesis occurs; and (4) G_2, or the premitotic period. The duration of each of these periods in individual germinative cells can be obtained by preparing autoradiographs at short intervals after exposure to tritiated thymidine, and determining the fraction of cells in mitosis, the fraction of cells labeled,

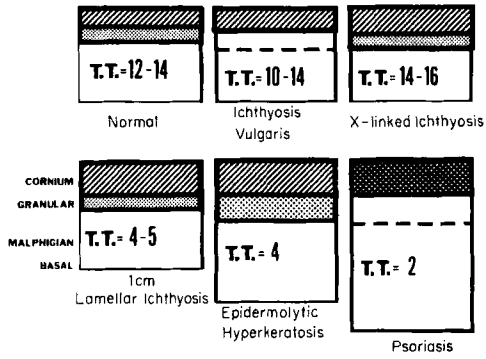


FIG. 4. Transit times of hyperproliferative skin diseases. These are minimal times for a cell to go from the basal or germinative cell layers to the stratum granulosum. Also shown is the relative thickness of the entire epidermis in these conditions and the presence or absence of a "granular layer." The presence of a "granular layer" in lamellar ichthyosis with a rapid transit time and its absence in ichthyosis vulgaris with a normal transit time should be noted. T.T. = transit time (days). (We are indebted to Dr. P. Frost and Dr. G. Weinstein for preparing this slide and allowing its use.)

and the fraction of cells that were labeled and undergoing mitosis. Those cells in mitosis which were not labeled represent cells in G_2, whereas, those mitosis which were labeled represent cells that were undergoing DNA synthesis at the time of exposure. By plotting the fraction of labeled mitoses as a function of time after exposure to tritiated thymidine, the duration of the S period and the G_2 period can be determined directly. Since the number of cells in any period of the cell cycle is proportional to the amount of time those cells spend in that period, the duration of the total cell cycle can be calculated from the relationship between the number of cells in DNA synthesis to the total number of germinative cells and the time spent in DNA synthesis to the total cell cycle. G_1 is calculated by subtraction of G_2, S, and M from the total cycle time.

These results indicate that the total germinative cell cycle time in psoriasis is decreased by a factor of about 12. This is accomplished by the psoriatic cell spending half as much time in S, G_2, and M and only one-fifteenth as much time in G_1. Psoriasis, therefore, differs from normal skin in having a greater number of cells in the proliferative cell cycle, a greater number of cells undergoing proliferation at any one time, and a shorter period of time required for individual dividing cells to replicate.

Some of the disadvantages in the use of tritiated thymidine for such studies are: (1) the injection of microcurie amounts of radioactive materials; (2) the necessity for carrying out numerous biopsies; (3) the

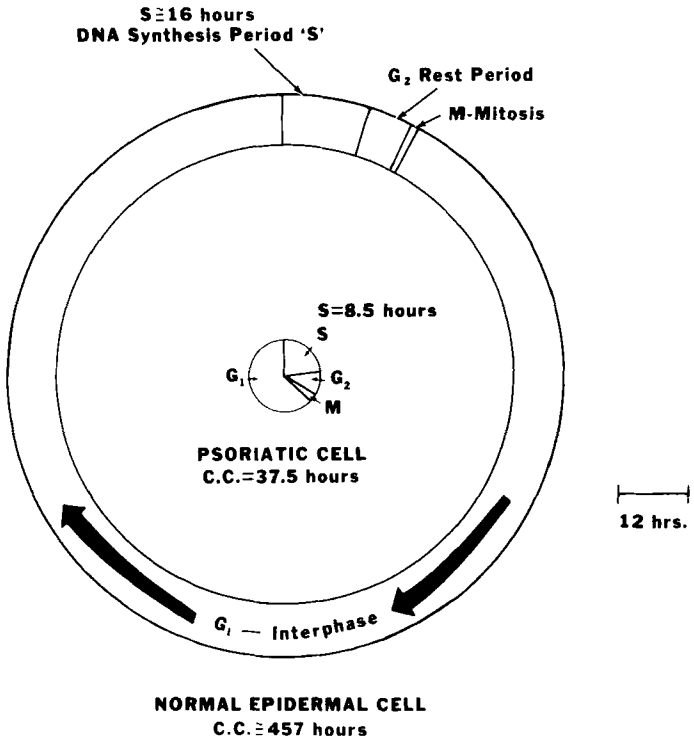


FIG. 5. Cell cycle kinetics in normal and psoriatic germinative cells. This schematic diagram of the cell cycle is made to illustrate the relative times taken for a psoriatic and normal germinative cell to divide, and the proportions in the 2 cycles are accurate with respect to a time scale of 12 hours for the distance shown on the scale at the right. C.C. = total cell cycle time. The other periods of the cycle are labeled in the figure. The great difference between psoriatic and normal cells, especially in the time spent in G_1 (interphase, or post mitotic period), can be appreciated. (We are indebted to Dr. G. Weinstein and Dr. P. Frost for preparing and allowing the use of this illustration.)

delay of several weeks before the autoradiographs can be developed and analyzed; (4) the need for a fully equipped histopathology and autoradiography setup; (5) the inability to study the stratum corneum in the normal or in diseased states without parakeratosis.

The only other method of studying the cell cycle is with the use of colchicine and its congener Colcemid. This technique is based on the observation that colchicine in sufficient concentration stops all the dividing cells in metaphase. By exposing a cell population for a given known period of time and determining the percentage of cells in metaphase one can determine the turnover time of the tissue. This technique requires

no special expensive equipment and can be done without a several-week waiting period; the results are easy to analyze. The main disadvantage of the method is that the concentration of colchicine is critical; if too high, cell death occurs, and if insufficient some dividing cells escape metaphase arrest. Although this technique is probably useful in *in vitro* studies where the time of exposure of the cells and the concentration of the drug can be precisely controlled, it probably should not be used for *in vivo* studies, although such studies have been reported (F7, F8, F9). An excellent study comparing the use of colchicine with the use of tritiated thymidine for studying cell kinetics was published by Bertalanffy (B19).

Another approach to epidermal cell population kinetics (but not the cell cycle) has been reported by Porter and Shuster (P9). Scotch tape strippings of the surface stratum corneum were obtained at various intervals after the intradermal injection of ^{14}C-labeled glycine and methionine and counted in a liquid scintillation spectrometer. This technique is similar to the original one of Rothberg *et al.* (R18) without the purification and quantitation of the specific activity of the injected amino acid. Obviously the results obtained depend not only on how fast cells are moving to the surface, but also on the level within the epidermis at which the amino acid is incorporated. In the psoriatic lesion activity reached the surface after only 1 day (free AA?) and reached a sharp peak at day 3 for glycine and day 7 for methionine. This would be consistent with a preferential incorporation of glycine into proteins at the level of the granular layer and an average transit time through the stratum corneum of 3 days. Similarly preferential incorporation of methionine at the basal layer an average transit time of 7 days would lead to the observed findings and would be consistent with the *minimal* transit times of 4 days from basal layer to surface reported by Weinstein.

The data on the arrival of radioactivity at the surface in normal epidermis and in the uninvolved epidermis of patients with psoriasis, based on this technique, are much harder to interpret, especially since symmetrical "best fit" curves have been drawn through points which obviously represent not one process with a normal distribution, but several different processes (proteins) into which these amino acids have been incorporated and which arrive on the surface at different times. In general, however, the *average* transit time as derived from this study for normal epidermis, would seem to be too long for passage through the stratum corneum (19–24 days) and too short for passage through the entire epidermis (23–35 days). It is more likely that the minimal time for glycine arrival at the surface (19 days) represents the transit time through the stratum corneum and this transit time would be both mini-

mal and average since unlike in the viable epidermis where random migration occurs once the cells reach the stratum corneum they presumably all travel in unison. The time for renewal of the entire epidermis as calculated from the methionine data is probably too short because methionine is incorporated not only at the basal layer, but also higher up in the epidermis, and it is exceedingly difficult to determine which part of the surface activity curve is actually due to methionine incorporated into proteins in the basal layer.

Finally several additional methods for studying selected aspects of epidermal cell kinetics have been described. Baker and Kligman (B5) described a technique for estimating the turnover time of the human stratum corneum. They applied a fluorescent chemical, tetrachlorosalicylanilide, topically to normal skin and determined the period of time required for the fluorescence to disappear when examined under ultraviolet light. The dye penetrated only to the base of the stratum corneum and did not leach out. In normal epidermis of the back, the fluorescence disappeared in 10–19 days. Simplicity in this study is its great advantage. However, exposure to this chemical resulted in allergic sensitization of 15% of the experimental subjects and many other tests were invalidated because of irritation from the vehicle. This precludes its use as a general method. Reizenstein *et al.* (R8) studied epidermal turnover time in psoriasis by following the loss of intravenously injected radioiron in the shed scales. Peak levels were reached in 2–4 days and activity continued to be found for over 15 days. It is difficult to interpret these data until we know how epidermis incorporates the iron and whether or not it is reutilized, as has been suggested by Cavill and Jacobs (C2) and Cavill *et al.* (C3).

3.4. The Biochemistry of Acanthosis (Thickening)

The most striking aspect of psoriatic skin under the microscope is the tremendous epidermal hypertrophy accompanied by its equally well developed papillary blood supply. As discussed previously, in psoriasis the lower 3 cell layers are the germinative or dividing cell population. Most likely the number of cell layers that can divide is determined by their distance from a blood vessel, since in elegant studies of the hair follicle (whose papilla resembles in many ways those of the psoriatic lesion) Van Scott *et al.* (V5) have shown that this critical distance is approximately 180 μ. Does this increase in germinative population necessarily imply a thickened epidermis?

In interesting studies on the guinea pig, Christophers and Petzoldt (C6) have shown that although ear skin is thicker than back skin and although the dorsal surface of the ear is thicker than the ventral surface,

the mitotic rate is the same in all areas and the difference in thickness is due solely to differences in the amount of time the cells need to get to the horny layer (8 days for the back and 14 days for the ear). Kurban and Azar (K8) have reported on a most unusual case, where the skin continually peels from the entire body surface. Incubation of skin from this patient with tritiated thymidine followed by autoradiography indicated a greatly increased rate of cell proliferation, yet the epidermis was not histologically thickened. These studies show that acanthosis is not necessarily an indication of increased cell proliferation, and some factor having to do with maturation may also be involved in determining thickness of the epidermis.

Is the increased epidermal thickness accompanied by an increase in size of the epidermal cell (hypertrophy)? Or are there just more cells (hyperplasia)? Despite histologic (H2) and electron microscopic (B36) impressions to the contrary, biochemical evidence indicates that the cell size in the psoriatic lesion is no different than normal. This conclusion is based on measurements indicating that both normal and psoriatic epidermis contain approximately two-thirds water by weight (H2, M15, S17) and, therefore, have the same wet:dry weight ratio in conjunction with DNA measurements which have been made in normal and psoriatic skin.

Reinberg *et al.* (R5) and Mier and McCabe (M12) found no difference in DNA between normal and psoriatic epidermis on a weight basis using phosphorus assays and differential extractions for DNA measurements. Using sensitive micromethods on human epidermis, Santoianni and Ayala (S2) found 300 ng of DNA-P per milligram wet weight in normal human epidermis, or about 1% of the dry weight. Dermis contained about one-fifth as much DNA. De Bersaques (B14) using similar analytical techniques made measurements on separated epidermal living layers and the horny layer; his figures are shown in Table 5. They indicate that the amount of DNA is the same on a dry weight basis in normal and psoriatic living epidermis; however, in normal epidermis there is practically no DNA still remaining in the horny layers whereas in psoriasis lesions (scales) approximately one-third of the cellular DNA is still present within the parakeratotic nucleus. Basal cell carcinoma offers an interesting contrast since the average cell would seem to be only about one-third the size of the average normal epidermal cell (or the cells have markedly hyperdiploid nuclei).

De Bersaques also noted that upper spinous layers have less DNA than the basal layer + lower spinous layers; this means either that the cells are larger as they ascend and contain more protein or that DNA is being removed from the cells during their ascension, and not just at the granular

TABLE 5
DNA CONTENT OF HUMAN EPIDERMAL TISSUE[a]

Tissue	DNA (μg/mg dry weight)	
	Basal and spinous cells	Horny layers
Normal epidermis	13	0.7
Normal plantar epidermis	15	1.5
Psoriasis	14.5	5.5
Basal cell carcinoma	39.8	—

[a] From de Bersaques (B14).

layer. Of importance in this context is the study of Kint (K4). Using Feulgen staining and histophotometry, he found that malpighian cells in normal epidermis have less DNA than do the basal cells. Since these measurements do not rely on dry weight as do those of de Bersaques, they mean either that the basal cell nuclei are hyperdiploid and the malpighian cells diploid or that the basal cells are diploid and the malpighian cells lose some of their DNA. Since by comparison with neighboring lymphocytes the basal cells appeared to be diploid, the latter is the preferred interpretation; and we must conclude from both of the above studies that although it looks as though the dissolution of the nuclei occurs quite suddenly in the "granular" or intermediate layer, this is untrue and actually the normal loss of DNA from the epidermal cell starts some distance below the "granular" layer while the cells are in the malpighian layer and while they still appear to possess a normal nucleus.

3.5. THE BIOCHEMISTRY OF PARAKERATOSIS (PERSISTENT NUCLEI IN THE STRATUM CORNEUM)

3.5.1. *General Remarks*

It has already been observed in the discussion on light and electron microscopy that normally nuclear and cytoplasmic organelles are removed from the cell prior to its becoming a "horn" cell. This can also be verified by autoradiography since tritiated thymidine incorporated into epidermal DNA cannot be followed into the normal stratum corneum. It disappears at about the level of the "granular layer," presumably because the DNA has been broken down to molecules which are sufficiently small to be washed out during preparation of the autoradiographs (F26). As noted above, however, this process probably starts some distance below the "granular" layer.

Parakeratosis is not unique to psoriasis. It is found in many situations

where increased epidermal proliferation is occurring. Recently Christophers and Braun-Falco (C4) have shown quite clearly in guinea pig epidermis that although parakeratosis is always a sign of increased epidermal proliferation, the reverse is not true; i.e., increased cell production even to the level of that seen in psoriasis can be present without the presence of parakeratosis. These experiments were done utilizing three different procedures which stimulate epidermal proliferation (stripping of the horny layer with Scotch tape, topical application of vitamin A acid, and topical treatment with hexadecane). All these treatments resulted in increased epidermal thickness (3–5 times normal) and increased mitotic activity with decreased transit time of the cells to the surface. Only with complete stripping off of the stratum corneum did parakeratosis result.

These findings imply that there must be another explanation for the parakeratosis found in psoriasis aside from the increased cell division, decreased transit time, and lack of time necessary for dissolution of the nucleus to take place. Comparable findings, i.e., increased cell proliferation without parakeratosis, have been reported by Frost *et al.* in congenital lamellar ichthyosis (F20) and by Kurban and Azar in familial continual skin peeling (K8). One explanation which has been advanced is that the DNA is chemically altered or more firmly bound to protein since, according to Jarrett and Spearman (J3) and Steigleder *et al.* (S21), it is not as easily digested by DNase as is normal DNA.

3.5.2. *Removal of Nucleic Acids from Epidermal Cells during Maturation*

The process of nucleic acid breakdown presumably starts a little before entry of the cell into the "granular layer." Histochemically, however, most lysosomal enzymes are strongest at the level of the "granular layer" (B28). The amount of DNA in psoriatic epidermis is equivalent per unit weight to normal epidermis, but about one-third of the nuclear DNA is still present in the parakeratotic horny layer of psoriasis while practically none is found in normal stratum corneum. RNA is much increased in psoriatic epidermis (M12, R5), and it also can be found in increased amounts in the psoriatic scale (Table 6).

Presumably in psoriatic scale both nucleic acids have been only partially removed either because of inadequate enzymes to break them down, a decreased amount of time for the enzymes to act, an abnormally strong attachment of the nucleoprotein which makes them resistant to enzymatic attack, or to a combination of the above.

Histochemical studies of RNase and DNase activities in normal and

TABLE 6
COMPONENTS OF HORNY LAYERS[a]

Component	Normal[b]	Callus[b]		Psoriasis scale[b]	
	(1)	(1)	(2)	(1)	(2)
DNA	10	5	—	138	400
RNA	70	22	—	336	250
Pentose (orcinol)	65	45	32	458	260
Deoxyribose	7	5	5	24	32
Xanthine	34	41	53	93	127
Hypoxanthine	19	26	33	68	90
Uracil and uridine	0	0	—	109	140
Ribose	—	—	0	—	0
Free reducing substances	—	—	348	—	391
Uric acid	—	—	9	—	19

[a] Values are expressed in milligrams per 100 g of dry tissue.
[b] Data from (1) Hodgson (H22); (2) Wheatley and Farber (W14, W15).

psoriatic skin have not been too satisfactory. In 1962 Steigleder and Raab (S22) reported finding increased RNase activity in the psoriatic horny layer. Using a different technique, Jarrett (J2) found both RNase and DNase which were active at an acid pH and located around epidermal nuclei. Most recently, Winter and Freund (W18) made antibodies to DNase and RNase obtained from a commercial chemical company and then stained cryostat sections using the antibody and indirect immunofluorescence. They found intense RNase activity both perinuclear and in nucleoli especially in the granular layer of normal epidermis. The reaction was greater in psoriatic epidermis and also was found in the cell cytoplasm and in the horny layer. DNase was perinuclear and especially strong in the basal layer of normal epidermis as well as in the granular layer. DNase in psoriatic skin was similar to the nonpsoriatic except for more activity in the stratum corneum. This latter study is an ingenious way to approach histochemistry. It is unfortunate, however, that better antigens were not used, i.e., purified epidermal RNase and DNase, since Liss and Lever (L7) found that epidermal RNase did not cross-react with bovine pancreatic RNase.

Tabachnik (T1) in studying guinea pig skin found an RNA and DNA content which was about equal to human epidermis, i.e., a DNA content of 1.24% of the dry weight of the epidermis and an RNA:DNA ratio of 1.16. Thereafter, he and Freed (T2) reported finding RNase and DNase in guinea pig skin and that 50% of the DNase and 75% of the RNase could be removed by washing the skin surface. Rat and albino

rabbit *but not man* also had DNase on the skin surface. The characteristics of the DNase from guinea pig skin surface were like pancreatic DNase I with an optimum at pH 7.0. Guinea pig epidermis contained both DNase I and DNase II (optimum pH 5.0), but only DNase I could be washed from the surface. In contrast rat epidermis contained only DNase II (T3). He also reported increased activities after β-irradiation which destroyed sebaceous glands and after various sorts of epidermal injury which resulted in increased epidermal proliferation (T4, T5, T6), thereby, implying that the enzymatic activity was truly epidermal. Most of the DNase II activity was particle bound in homogenates. Despite the increased nuclease activities after injury he was unable to find evidence of the pyrimidine breakdown products, thymidine or uridine, or of ribose or deoxyribose release.

Santoianni and Rothman found DNase II (active at pH 5), but no DNase I in rat and human epidermis (S3). It was not present in the dermis. Magnesium ions at concentrations above 1 m*M* and polyvalent anions above 5 m*M* inhibited the activity. The activity found in human epidermis was 7.6 μg of deoxypentose-P liberated per hour per milligram of supernatant protein (or 0.4 μg of deoxypentose-P per hour per milligram of dry tissue or 0.5 μg of deoxypentose-P per hour per microgram of DNA-P). This is equivalent to approximately 70 ng of DNA split per minute per milligram of dry tissue, or 8 ng of DNA split per minute per microgram of DNA in the tissue. Rat epidermis and rat spleen contained approximately 8 times as much DNase II as human epidermis.

RNase activity was also found in the above study. The pH optimum was 7.5, and activity was inhibited by zinc ions, cupric ions, and heparin. The activity found in human epidermis was 7.0 units per milligram of protein or 0.46 units per microgram of DNA-P where a unit is an increase in absorbancy at 260 nm of 1.0 in a 0.5-hour incubation. According to Tabachnik (T3) this increase represents the release of 2 μg of RNA-P or 21 μg of RNA; the activity would, therefore, be 28 μg of RNA-P released per hour per milligram of protein, or 294 μg of RNA released per hour per milligram of protein, or about 5 μg of RNA released per minute per milligram of protein, or 50 ng of RNA released per minute per milligram of dry tissue, or 5.5 ng of RNA released per minute per microgram of DNA. Rat epidermis had 1.5 times as much activity as human.

Liss and Lever (L7) purified RNase from psoriatic scale. They found an activity in the crude extract of about 7 units per 15 minutes per milligram of protein (10 μg RNA hydrolyzed per minute per milligram of protein, recalculated); this is twice that found by Santoianni for normal epidermis. The purified RNase had a molecular weight of about

14,000 and a pH optimum of 6.6. Starting with an RNA substrate having a purine:pyrimidine base ratio of 1:1 the RNase preferentially split out pyrimidines so that the core RNA left after digestion had a purine: pyrimidine ratio of 2.8:1. Immunologically it was not related to bovine pancreatic RNase.

Figure 6 is a metabolic map of nucleoprotein catabolism, and Table 6 shows some of the components that can be recovered from scrapings of normal skin, from callus, and from psoriatic scales. In conjunction with the preceding it shows that about 5% of the RNA and less than 1% of the DNA is left in the normal horny layer while up to one-third of the RNA and DNA is still present in the cells of the parakeratotic horny layer of psoriasis. Xanthine and hypoxanthine can be found in psoriatic scales and presumably result from catabolism of nucleic acid purines. Uric acid, although present in the scales, probably comes from the blood, since xanthine oxidase has not been found in human epidermis (B15, B17). Pyrimidine breakdown products have not been found. This might

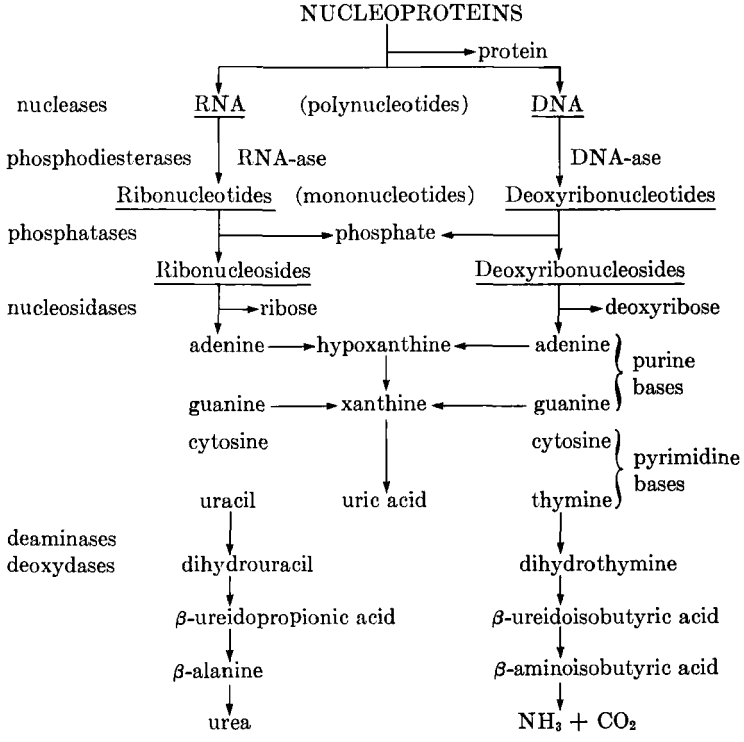


FIG. 6. Catabolism of nucleoproteins.

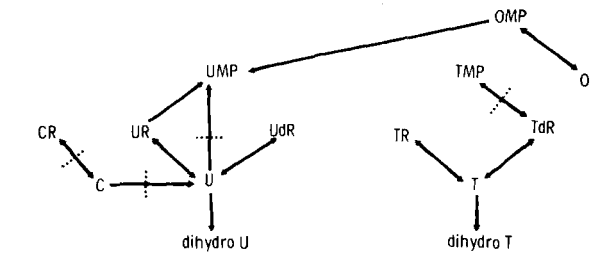
be due to the fact that they are preferentially removed by RNase and that RNase is very active in psoriatic scale or to the active formation and breakdown of dihydrothymine and dihydrouracil.

The remarkably increased amount of "pentose" which has been found using the orcinol reaction is misleading since actually no ribose was detected. The most recent study on the identity of this material indicated that it is probably uridine diphosphoglucose (UDPG) rather than a "pentose" (B12). These authors found 140 μg per 100 mg of scales in psoriatic lesions versus 40 μg per 100 mg of normal horny layer, which confirms earlier findings shown in the table. This finding may also explain the "uracil" found, since Hodgson (H22) showed that much of this was in fact uridine and might also come from some compound such as UDPG.

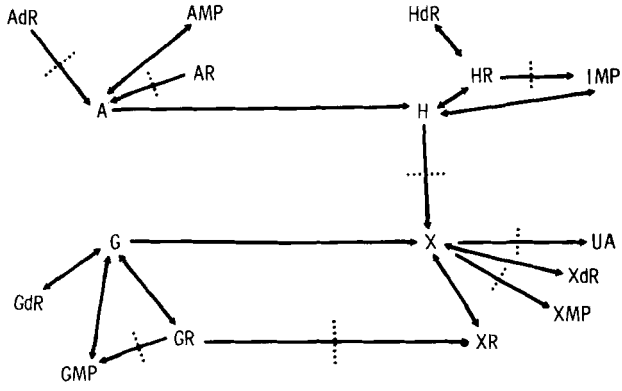
On the catabolic side of RNA and DNA metabolism aside from the nucleases which have already been discussed, Zaruba *et al.* (Z2) have reported on the presence of phosphodiesterases specific for 3′ and 5′ linkages in skin of newborn mouse. Phosphodiesterases of at least three types present in epidermis have also been mentioned by de Bersaques (B17). Zaruba *et al.* (Z3) reported that phosphodiesterase activity was higher in psoriasis lesions than in normal epidermis or in adjacent uninvolved skin of psoriasis.

De Bersaques (B13) reported on the presence in human epidermis of a purine nucleosidase and did an extensive investigation in 1967 (B15) of purine and pyrimidine catabolism using labeled substrates and autoradiography on thin-layer plates after incubation of the substrates with epidermal preparations. Some of his results are shown in Fig. 7. Of note are the absence of xanthine oxidase and the lack of thymidine kinase. The latter enzyme must surely be present in intact tissue, however, since thymidine is a standard label for epidermal DNA synthesis. The lack of xanthine oxidase probably explains why xanthine and hypoxanthine can be found in psoriatic scale extracts, and the presence of active catabolism of the pyrimidines might help explain why they are not found in scales. Pyrimidine metabolism in guinea pig and mouse epidermis has been examined also by Baden (B2). Radioactive thymidine and uridine was injected into the animals; after enough time for them to be incorporated into epidermal nucleic acids and for the process of nuclear dissolution to start, evidence was sought of reutilization or further metabolism of these compounds. Neither reutilization nor further metabolism was found when the nucleic acid was hydrolyzed during formation of the stratum corneum, and Baden concluded that the compounds must be washed away in the systemic circulation.

Studies by Pillarisetty and Karasek (P6) on the synthesis of pyrimi-



(A)



(B)

FIG. 7. (A) Pyrimidine metabolism *in vitro* in human epidermis. C, cytidine; U, uracil; T, thymine; O, orotic acid; R, ribose; dR, deoxyribose; MP, monophosphate. (B) Purine metabolism *in vitro* in human epidermis. A, adenine; G, guanine; H, hypoxanthine; X, xanthine; UA, uric acid; I, inosine; R, ribose; dR, deoxyribose; MP, monophosphate. In (A) and (B), dotted line indicates reaction not found. Adapted from de Bersaques (B15).

dines de novo in newborn mouse skin indicated an ability to form orotic acid and pyrimidines which was comparable to that of other tissues.

Reutilization of Nuclear Components. The question whether the constituents of nucleic acids may be "salvaged" and used again is not a simple one, and some lines of evidence do point to this possibility. Direct evidence in oral mucosa and epidermis of the rat for DNA reutilization has been presented by Cutright and Bauer (C18). This is in direct contrast to the study of Baden quoted above. In addition, studies utilizing radioactive iron injected intradermally in humans indicated reutilization of iron by epidermal cells since the half-life of the injected iron was found to be 67 days (C2, C3). This is far greater than the usually

accepted 28-day turnover time of the epidermal cells. However, as pointed out in the section on cell kinetics, this figure of 28 days may be grossly too low.

3.6. Epidermal Lysosomes

The fact that in normal epidermis various hydrolytic enzymes (e.g., nonspecific esterase, acid phosphatase, β-glucuronidase) can be found localized to the stratum granulosum or intermediate layer of the skin has been recognized for some time. Also known and thoroughly reviewed by Braun-Falco (B28) were the histochemical findings of increased activities of these enzymes in psoriatic epidermis and their distribution throughout the whole of the horny layer as well as the intermediate (but nongranular) layer. He stated that there were no intrinsic clues to psoriasis to be found in these observations since similar although less marked changes were found in other diseases which were also characterized by "hastened Keratinization." Ellis (E4) in reviewing the enzymes of the epidermis noted that although the majority of these activities were just below the horny layer in normal epidermis, there also was activity seen as small cytoplasmic granules in the basal and malpighian cells. Winter's studies on DNase and RNase (W18) also localized these activities within epidermal cells beneath the stratum granulosum, and especially in basal cells for DNase.

Brody's electron microscopic studies (B35, B36, B37, B38, B39, B40, B41) disclosed small granules within the epidermal basal cells as well as many higher up; especially in psoriasis there were in the intermediate layer and in the parakeratotic stratum corneum larger vesicular bodies which could have been lysosomes. Using acid phosphatase staining as a lysosomal marker with electron microscopy, Olson and Nordquist (O4) definitely established membrane-bound acid phosphatase activity to be present in human epidermis even in basal cells. A comparison of normal versus psoriatic epidermis using these techniques (B31) revealed both intra- and extracellular acid phosphatase activity. Intracellular activity was found free in the cytoplasm bound to keratohyalin granules and also inside membrane-bound granules. In psoriasis these activities were much increased and were found throughout the parakeratotic horn instead of only at the stratum granulosum and lowermost horny layers as in normals. Tabachnik in 1959 (T1) had found in guinea pig skin that proteolytic activity, i.e., autolysis of the tissue occurred only at acid pH. DNase activity as previously noted is only of the acid pH type in human epidermis. Naphthylamidases and peptidases were recently studied in detail both biochemically and histochemically by de Bersaques (B16). He found that psoriatic epidermis contained more than the normal

amounts and that several different activities might be measured depending on the substrate used. He could not confirm the earlier observation of Paschould *et al.* (P1) of a dipeptidase inhibitor present in psoriasis. Hopsu-Havu and Jansen (H27, J1) have recently reviewed and studied protease activity in normal epidermis, and Ockerman (O1) has reported finding several hexosidases which are active at acid pH in normal human epidermis.

Some of these enzymes were found in the blister fluid produced artifically with cantharidin and the activities were most likely released from the lysosomes of epidermal cells (S14). There are, however, considerable differences between the reactions of liver lysosomes and epidermal lysosomes. Dicken and Decker (D1) found that while Triton X-100 caused release of latent acid phosphatase activity from epidermal lysosomes; acid pH, hypotonic solutions, and freezing and thawing which are effective disrupters of liver lysosomes did not disrupt epidermal lysosomes. Smith *et al.* (S16) found that capsaicin and cantharidin also would not lyse epidermal lysosomes and they substantiated Dicken's finding that Triton X-100 did cause lysis.

The evidence seems to indicate that epidermal lysosomes are more stable than liver lysosomes when isolated. Also in Dicken's study only 3.6% of the total acid phosphatase activity of mouse epidermis and 8.3% of the total acid phosphatase activity of human epidermis was found in the lysosome fraction, the great majority of the activity being free in the supernatant. It is of course possible that all of the free activity was due to disruption of the lysosomes during homogenization, but perhaps this free activity actually corresponds to the diffuse staining seen histochemically just below the stratum corneum. This may indicate that normally lysosomal activity is present in several different forms. Some may be packaged in lysosomes at all layers of the epidermis and only becomes active when released or needed while some may be produced only at the intermediate layer where cell contents are undergoing dissolution and this activity may be active, free enzyme when originally made. This latter type may correspond to the acid phosphatase activity measurable with *p*-nitrophenol phosphate as substrate while the lysosomal may be that measurable with α-naphthyl phosphate as substrate. Gerson (G3) has shown in the oral mucosa of the rabbit that as the area of keratinization is approached the lysosomal (α-naphthyl phosphate) activity increases while the nonlysosomal (*p*-nitrophenol phosphate) activity is unchanged. The above studies of acid phosphatase activity where over 90% of the activity was found in the nonlysosomal fractions may have indeed been measuring mainly nonlysosomal acid phosphatases, and this problem needs further study.

The place and importance of these enzymes in the process of normal keratinization and in psoriasis is still obscure; however, the fact that vitamin A-acid has been used to treat psoriasis successfully and is a lysosomal labilizer (F21) may indicate that they are of great importance.

3.7. Keratohyalin Granules: The Story of the "Histidine-Rich Protein"

The presence of keratohyalin granules is correlated with the presence of a normal stratum corneum while the absence of keratohyalin granules is correlated with abnormal formation of the horny layer as in psoriasis. Even in the very early lesion of psoriasis (2 days old) there is parakeratosis and loss of the granular layer (keratohyalin granules) (H15). It is also well known that in a single lesion there will be areas of abnormal horn formation with parakeratosis and no underlying granular layer lying right next to an area of normal horn formation with an underlying granular layer (G9). Because of these histological considerations a connection between the keratohyalin granule and the normal formation of horn has been assumed to exist. In addition, in observing the response of lesions to treatment Gordon *et al.* (G10) found a very early return of the granular layer, and Fry *et al.* (F22, F23) also have noted a return of the granular layer prior to any significant drop in the mitotic counts.

Keratohyalin granules have been studied histochemically for many years with the result as stated by Steiner in 1958 (S25) that "keratohyalin does not contain SH, SS, glycogen, mucoids, or nucleic acids" and, therefore, "its role in keratinization remains obscure."

Electron microscopy has been singularly unhelpful in this problem showing only a dense "glob" of irregular outline with fibrils seemingly going into it and possibly with ribonucleoprotein particles around it (B34).

Some recent advances in understanding this structure have taken place. Fukuyama *et al.* (F27, F29) showed that some radioactive amino acids, namely histidine, arginine, serine, glycine, and cystine are preferentially incorporated into the epidermis at the level of the granular layer. Also Fukuyama and Epstein (F28) showed that histidine was actually incorporated into the granules using autoradiography and the electron microscope. Using histochemical means, Reaven and Cox (R2) found a positive reaction for histidine in the keratohyalin granules, and further studies (C11) showed histidine incorporation into keratohyalin with autoradiography.

Bernstein *et al.* (B11) in whose laboratory the original observations on the localization of the amino acids were made have recently reviewed

their biochemical work on the formation of the "histidine-rich protein." It shows quite clearly that a specific protein having a molecular weight of about 30,000 is specifically synthesized at the granular layer and is presumably part of the keratohyalin granule. This protein is rich in threonine (10.7%), aspartic acid (15.4%), glutamic acid (12.0%), alanine (10.1%), glycine (16.8%), arginine (10.0%), histidine (8.3%), and serine (6.0%), and contains no sulfur. It "appears to exist in its native state as a part of a molecule with a molecular weight in excess of 200,000 containing bound copper and a urocanic acid-like moiety possibly involved in a peptide linkage" (B11).

Of great interest is the fact that although the synthesis of the 30,000 molecular weight precursor is puromycin sensitive, polymerization to form the large aggregate is not and, therefore, probably involves a mechanism other than the usual ribosomal one. As Bernstein *et al.* (B11) point out, the relationship of this protein formed in the granular layer, and presumably in part of the keratohyalin granule, to the process of formation of the keratin in the fully cornified cell is still not clear. The reasons for this are (1) the lack of sulfur in the "histidine-rich protein"; (2) the failure of the "histidine-rich protein to become insoluble in urea at a time when tritiated histidine is demonstrable in the cornified layer (6 hours after injection in newborn rats); and (3) the fact that in histidinemia, where urocanic acid cannot be formed and where it is absent from the epidermis, keratohyalin appears morphologically normal. This work on the formation of a particular functional protein at a high level in the epidermis has been most exciting and has provided a starting point for further work directed at the formation of "keratin." Recent studies by Ugel (U1) show that this protein can be preferentially extracted from bovine hoof, and this finding may speed up work in this area.

Voorhees *et al.* (V6) have recently reviewed the situation in psoriasis with regard to the formation of the "histidine-rich protein." As has been stated previously, keratohyalin granules are absent from the intermediary layer in psoriasis, and these authors have demonstrated a lack of synthesis of the "histidine-rich protein" and a return toward normal with treatment and with the return of the keratohyalin granule. How the defect in formation of this protein is related to the increased epidermal proliferation is not known, but presumably on grounds of economy they should be related to one underlying abnormality.

3.8. Arginase: A Mystery

In 1948 Van Scott reported finding arginase activity in human skin, especially epidermis (V2), and in 1951 (V3) he reported finding activi-

ties of 20 nanomoles per minute of urea formed from arginine per milligram of skin (reviewer's calculations) with an epidermal to dermal ratio of approximately 20–25:1. Arginase was not present in sweat despite the fact that sweat contained 85 mg of urea per 100 ml with a skin urea content of 9–18 mg/100 ml. Psoriatic lesions, warts, and arsenical keratoses had very high activities and the greatest concentration was in the granular layer. Most of the activity was "potential," i.e., the skin preparation had to be preincubated at 50°C with manganese to activate the enzyme. In further studies Rothberg and Van Scott (R17) indicated that scales from both psoriasis and exfoliative erythroderma (both of which have parakeratotic scales and fast cell turnover) had extremely high enzyme activities. Of further interest was the fact that mucous membrane, a tissue with a presumably rapid cell turnover, had a low level of arginase while plantar callus with its thick stratum corneum had higher activities than normal epidermis.

These observations plus the report by Rothberg (R15) that hair roots which are mitotically very active, had activities which were *low* strengthened the possibility of a relationship between arginase activity and keratinization rather than cell proliferation. Ornithine, the other product of the arginase reaction was not found to be incorporated into any of the epidermal proteins of psoriatic lesions, and so the function of the arginase was unknown. This problem was reinvestigated in 1961 by Crounse and Rothberg (C14), and despite extensive studies they could find no evidence that the rest of the urea cycle existed in human epidermis except possibly for the formation of argininosuccinate from citrulline and aspartic acid. Whatever the function of arginase was, it did not appear to be connected with the formation of urea.

On the other hand, evidence from the work of Rogers and Moore (R11) and Satoh *et al.* (S4) indicates an increased arginase activity in Shope papilloma virus-induced tumors of rabbit skin, which are hyperplastic lesions. Also recent work with acanthosis producing applications of hexadecane to guinea pig skin demonstrated a direct relationship between the degree of cellular proliferation or thickening of the epidermis and the amount of arginase in the tissue (B43, R14). Arginase, therefore, seems to be related both to the formation of a horny layer and to cell proliferation. Its role in normal epidermis and the reasons for its elevation in psoriasis remain completely unknown.

3.9. Chalone: The Search for Regulators of Epidermal Mitotic Activity

Psoriatic epidermis with its great mitotic activity and increased thickness bears several resemblances to epidermis at the edges of a wound.

In addition it shares with the wound situation the property of being able to be turned off or of returning to a "normal" state; i.e., it is transitory and, therefore, must be controlled by a "regulator" which turns the mitotic process on or off.

Figure 8 is based on a study reported by Bullough and Laurence (B48). They had earlier shown (B46, B47) that in order to get a burst of epidermal mitotic repair activity in mouse ears it was necessary to wound the epidermis itself. The hypodermis and hair follicles had their own regulators and did not influence epidermal mitotic activity. In the study illustrated, the authors studied the epidermis of the mouse ear on the side away from the area of injury (about 1 mm distance). They hypothesized that if during this repair process the injured but viable epidermal cells at the edges of the wound produced a mitotic stimulator or "wound hormone," this substance would diffuse across the ear and stimulate the epidermis of the opposite side in two definite areas lying closest to the wound edges. On the other hand, if there were normally an inhibitor constantly being produced by uninjured epidermis which is removed when the wound is made, then the lack of inhibitor should be felt most strongly directly across from the middle of the wound, and this lack of

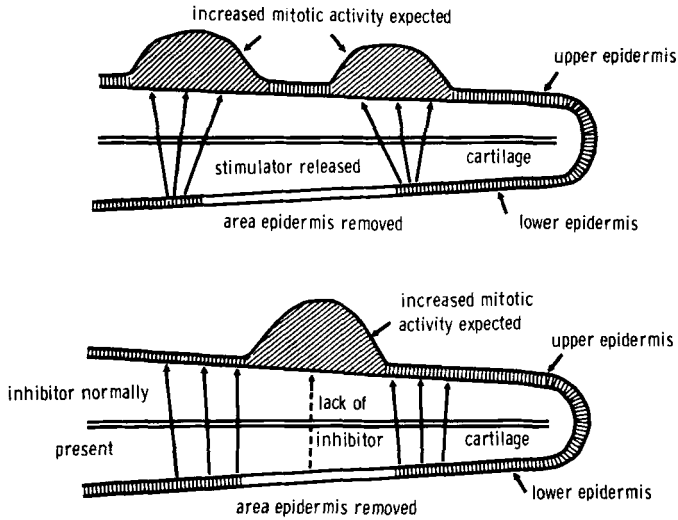


FIG. 8. Mouse ear epidermis response to injury. Diagrammatic illustration of the two possible responses of the epidermis of the mouse ear on the side away from the area of epidermal injury. As explained in the text, the lower representation (i.e., that which would be expected if an inhibition of epidermal proliferation was normally present and was removed by the wound) was the situation actually found. Redrawn from Bullough and Laurence (B48).

inhibitor would manifest itself as a mitotic burst. This is what they found, and they labeled this inhibitor "chalone," i.e., an internal secretion produced by a tissue to control, by inhibition, the rate of cell production in that tissue (B44).

Using the mouse ear epidermis *in vitro* as an assay system and counting mitoses arrested by Colcemid in the medium over a 4-hour period, the same workers showed that the epidermal chalone is not species specific, but is tissue specific (B49, B52); that it requires epinephrine for effect (B48), and perhaps glucocorticoids (B50); and that it is most likely a glycoprotein with a molecular weight of about 30,000 (B25). This work has been recently reviewed and the additional comment made that epidermal chalone is present in active form in human urine (B45).

Other work indicating the existence of such a chalone mechanism includes that of Simnett *et al.* (S13) on mouse lung; Bullough and Laurence in the VX2 epidermal tumor (B51) and melanomas of mice and hamsters (B49); Mohr *et al.* on mouse melanoma (M14); and that on rat chloroleukemia by granulocytic chalone (R22). This last "chalone" is said to have a molecular weight of 4000, and inhibition of DNA synthesis was shown by tritiated thymidine uptake rather than by counting Colcemid-induced mitotic arrest. If this is so, chalone might act on more than one process to produce mitotic inhibition.

In an attempt to define whether "chalone" acts at the G_2 phase (Bullough's antephase) or earlier in the cell cycle, Baden and Sviokla (B3) did experiments using both the mitotic arrest technique with Colcemid and scintillation counting of tritiated thymidine uptake into DNA isolated from epidermis exposed to chalone. The number of experiments was small (3) but they found no difference in thymidine incorporation while confirming the decreases in mitotic figures arrested by colchicine. This supported the concept of a G_2 effect. Epinephrine in their experiments had no effect.

More recently Marrs and Voorhees (M9) have repeated Bullough's work and confirmed the presence of an epidermal inhibitor which is heat labile, trypsin labile, nondialyzable, and dependent on epinephrine. In addition the most purified fraction from alcohol precipitation of the water extracts gave 8 bands on polyacrylamide gel.

Our own very preliminary work on this factor indicates the presence in human epidermis of a tissue specific, species nonspecific, partially heat labile, nondialyzable, factor which inhibits thymidine incorporation into DNA and does not depend on epinephrine. Epidermal extracts from psoriatic lesions did contain the factor in amounts equal to or greater than normal (M8).

If it is true that such a substance plays a role in regulating epidermal

mitotic activity and if it is present in higher than normal amounts in psoriatic epidermis, then it must either diffuse away faster from the psoriatic lesion or the psoriatic cells must be relatively insensitive to it. Some presumptive evidence that the carbohydrate-containing cell coats of the epidermal cells in psoriasis may be deficient and, therefore, "leakier" or less capable of providing adequate cell-to-cell contact has been presented by Mercer and Maibach (M11) using electron microscopy with special stains for extracellular glycoproteins.

3.10. Cell Metabolism

3.10.1. *General Metabolic Activity*

Frienkel in 1960 (F19) studied normal human skin biopsy specimens *in vitro* using radioactive glucose as a precursor. Her main findings were that (1) most of the glucose was converted to lactate (at least 75%); (2) about 5% was used along the pentose shunt indicating an active formation of NADPH and pentose for lipid and nucleic acid synthesis; (3) about 2% went to complete oxidation by means of the Krebs cycle; (4) about 5% was converted to glycogen.

A more complete study of normal epidermis, noninvolved epidermis of patients with psoriasis, and psoriatic lesions was done by Herdenstam (H16). All samples were taken from the gluteal region with a keratome. Using incubation techniques with radioactive glucose, chromatographic separation of the products, and radioautography and liquid scintillation counting of the separated products, he found the following: (1) The oxygen uptake was significantly greater for the uninvolved skin of patients with psoriasis than for normal and the psoriatic plaque was even greater (2.2 times the normal). His figures for oxygen uptake of normal skin are comparable to what others have found (Table 7). (2) CO_2 production by normal-appearing skin of psoriatics was approximately twice as great as normal skin, and the psoriasis lesion produced 4 times as much CO_2 as normal skin. The observation that CO_2 production increased by a factor of 4 in the psoriasis lesion while O_2 uptake was only twice as great implies that the hexose monophosphate (pentose) shunt is increased out of proportion to the Krebs cycle in the psoriatic lesion. (3) Incorporation of labeled glucose into protein was 6 times as great in the psoriatic lesion (mainly aspartic, glutamic, alanine, and leucine-isoleucine) as in normal skin. (4) The free amino acid pool of psoriatic lesions was smaller than in normal skin and much of it was found extracellularly. (5) The incorporation of labeled glucose into lipid was 6–8 times as high as in normal skin, and this was true for both the sterol and fatty acid fractions. (6) Lactate formation accounted for

TABLE 7
OXYGEN CONSUMPTION BY EPIDERMIS

Preparation	Reference	Q_{O_2} (μl O_2 used per minute per mg dry weight)
Human—Normal	(H16)	1.5 (1)[a]
Human—Psoriasis, uninvolved	(H16)	1.75 (1)
Human—Psoriasis, involved	(H16)	3.30 (1)
Human—Psoriasis, uninvolved	(H8)	1.15 (2)
Human—Psoriasis, involved	(H8)	1.45 (2)
Human—Normal	(A3)	1.48 (3)
Human—Normal	(G5)	1.8 (4)
Guinea pig ear	(C16)	4.0 (5)
Guinea pig ear	(C15)	5.25
Rat ear	(C15)	3.69
Rat back	(B7)	0.88 (6)
Mouse ear	(C15)	2.95
Mouse ear	(F10)	4.98
Mouse back	(F10)	2.13

[a] Key to numbers in parentheses: (1) recalculated assuming 1/3 of the fresh weight equals the dry weight; (2) using Cartesian diver; (3) punch biopsy; (4) keratome slices from surgical amputations; (5) recalculated assuming 75% of the fresh weight is water; (6) full thickness adult skin.

86.7% of the glucose utilized by normal skin, 90.7% of the glucose utilized by uninvolved skin of psoriasis, and only 73.8% of the glucose utilized by the psoriatic plaque. In each case about 90% of the lactate was found extracellularly.

Using Herdenstam's data, I have recalculated the figures in terms of the percentages of the glucose utilized which go in each metabolic direction; these figures are shown in Table 8. The total utilization of glucose in the normal and psoriatic skins was similar (600 μg of glucose utilized per 100 mg of skin in 2 hours), but the distributions were quite different in the two cases.

Herdenstam's experiments are quite useful in pointing out several

TABLE 8
UTILIZATION OF GLUCOSE BY NORMAL AND PSORIATIC EPIDERMIS[a]

Epidermis	Percent of glucose going in each metabolic direction				
	Lactate	CO_2	Amino acid pool	Protein	Lipid
Normal	86.7	4	8.3	0.83	0.1
Psoriasis	73.8	15	5.3	5.0	0.7

[a] Based on Herdenstam (H16).

characteristics of epidermal metabolism which were first described by Barron *et al.* over 20 years ago (B7), and have been confirmed since then by others (A2, H16, Y1). Psoriatic lesions not only share these characteristics, but accentuate them. (1) Epidermal cells maintain an active pentose shunt. (2) Epidermal glucose metabolism leads overwhelmingly to lactate, i.e., predominantly an anerobic type of metabolism. (3) The oxygen uptake of the tissue cannot be explained by the glucose utilized.

For example, in the material presented by Herdenstam in normal epidermis, 4% of the glucose ends up as CO_2. Some of this, however, comes from the pentose shunt, not from oxidation in the Krebs cycle. If we disregard recycling and accept the $C_1:C_6$ (CO_2 from carbon 1 of glucose *vs.* CO_2 from carbon 6) ratio of 2 which Herdenstam found for both normal and psoriatic skin, approximately half of the glucose or 2% was going through the pentose shunt and half through the Krebs cycle (2% agrees very well with Frienkel). Calculations indicate that 600 μg of glucose or 3.3 μmoles was utilized during the 2-hour incubation procedure. If 2% of this (0.066 μmole) went through the Krebs cycle it would account for an oxygen uptake of 0.96 μmole. However, the figure for O_2 uptake is 4.5 μmoles, or approximately 5 times that needed to oxidize the glucose. Similarly, if one tries to calculate the amount of O_2 needed to burn the glucose that went through the Krebs cycle in psoriatic skin, it is probably not much more than in the normal. This is due to the fact that far more of the increased CO_2 production comes from increased pentose shunt activity. Since oxygen utilized by the psoriatic lesion was 9.9 μmoles there is probably more than 5 times as much oxygen being utilized than can be accounted for by glucose oxidation.

Cruickshank *et al.* (C16) found that guinea pig ear skin was able to maintain an oxygen uptake in the absence of glucose which was only 20% lower than the rate in the presence of glucose. This indicated that the epidermis uses endogenous sources (presumably lipid or phospholipid) to feed into the Krebs cycle and is able to curtail this suicidal activity when outside sources of energy (glucose) are presented. However, the work discussed previously would indicate that a good deal of endogenous supply to the Krebs cycle was occurring even in the presence of added glucose (H16). Further work on this problem by Cruickshank *et al.* (C17) disclosed that endogenous glycogen, glucose, and protein could account for less than half of the endogenous respiration rate of their guinea pig ear slices.

Incubation of guinea pig ear or human epidermis with radioactive phosphate and isolation of the resulting phosphate-containing compounds by chromatography and radioautography disclosed about 20 labeled

compounds in the presence of glucose. These included most of the glycolytic compounds plus AMP, ADP, ATP, GDP, UDPG, and UTP (B42). However, when such skin was not provided with glucose, Yardley and Godfrey (Y2) found an almost total lack of the phosphorylated carbohydrate intermediates and instead an increase (30-fold) in the formation of phosphorylcholine. This indicated that endogenous respiration does not come from carbohydrates, but rather from phospholipids. Since total ^{32}P incorporation into phospholipids was decreased by the lack of glucose, presumably there was a block in the formation of phospholipids at the stage where phosphorylcholine is incorporated into cytidine diphosphate choline. There must also be phospholipid breakdown to supply fatty acids for respiration. Recently this has been elegantly shown in cow snout epidermis by Long (L11). This epidermis is thick enough to allow several slices of viable epidermis to be analyzed. The results shown in Fig. 9 show clearly that, as glucose disappears and the tissue energy needs are not met, phospholipid is broken down with the release of fatty acids and cholesterol.

In general then, normal human epidermis is metabolically quite active and maintains an anerobic type of metabolism with approximately 90% of administered glucose ending up as lactate, about 5% being converted

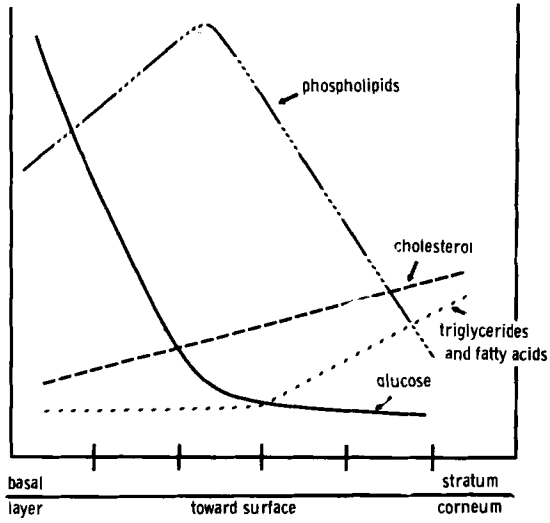


FIG. 9. Changes, with depth, of lipid and glucose in cow snout epidermis. Six horizontal slices of living epidermis were individually analyzed for the above constituents. Glucose is used up by the time the still living cell is only halfway to the surface, and it then must break down its own phospholipid to survive. Redrawn from Long (L11).

to glycogen, 2% to the Krebs cycle, and 5% along the pentose shunt. These figures are also accurate for monkey epidermis and hair follicles (A2). Oxygen uptake is due not only to the small amount of glucose reaching the Krebs cycle, but also to fatty acids deriving probably from phospholipids. In psoriasis, oxygen uptake is increased 2-fold, CO_2 production is increased 4-fold, and the incorporation of glucose carbons into protein and lipid rises 6- and 7-fold, respectively.

3.10.2. *Lipids and Phospholipids*

The total amout of lipid extractable from psoriatic scale is high (Table 9). Also the proportion of this lipid which is phospholipid is high. This could mean that the decreased transit time for the epidermal cell has not left enough time for the usual amount of lipid and phospholipid breakdown to occur. A study of the proportion of the various phospholipid classes by Gerstein (G4) disclosed that, although the amount of phospholipids was raised, the proportion of the various classes present was identical to normal; he, therefore, did not believe the elevation to be of great significance.

TABLE 9
LIPIDS IN PSORIATIC SCALE

Lipid	Callus[a]	Psoriatic[a]	Scale[a]
	(1)	(1)	(2)
Total lipid (% of dry wt)	2.4	8.1	3.75—12.2
Phospholipids (% of total)	0.41	1.24	0.7—5.0
Fatty acids, triglycerides (% of total)	43	38	>25
Nonsaponifiable (% of total)	57	62	
Sterols (% of total)	3.75	3.07	5—15
Free (%)	38	53	
Ester (%)	56	25	7—43
Free fast acting	15	22	

[a] Data from (1) Wheatley and Farber (W14); (2) Cornish *et al.* (C10).

Another abnormality noted in Table 9 is the decreased amount of esterified cholesterol present. This probably represents a greater contribution of epidermal lipid to the total in relation to lipid coming from the sebaceous gland since Nicolaides and Rothman (N1) showed that the epidermis is primarily responsible for the formation of free sterols.

3.10.3. *Soluble Proteins and Keratin*

In 1958 Roe (R10) presented a summary of her studies showing that psoriatic scales contained three soluble proteins: One protein contained no sulfur and formed 60 Å fibers when precipitated; it seemed to be

identical with tonofilaments present in normal epidermis. A second stained metachromatically and was presumed to contain chondroitin SO_4; it was, therefore, glycoprotein. The third was a nucleoprotein. Normal callus was found to contain two unrelated soluble proteins whereas normal epidermis contained all three, but with only small amounts of the glycoprotein. Other scaling dermatoses (2 cases) contained large amounts of the glycoprotein and of the major band contained in the callus, but not the other two proteins found in the psoriasis scale extract.

Baden and Freedberg (B1) found four soluble proteins in epidermis which did not come from serum. One of them was resistant to trypsin proteolysis. Matoltsy and Matoltsy (M10) in a study of soluble proteins from hair, nails, callus, normal epidermis stratum corneum, and scale from psoriasis and exfoliative dermatitis found that all tissues contained two fast-moving, sharply defined bands on polyacrylamide gel. In addition, callus contained two slower moving bands whereas normal stratum corneum contained three slower moving ill-defined bands. Psoriasis scale contained these five normal bands plus three extra slower bands. Marghescu (M6) carried out polyacrylamide gel electrophoresis on proteins extracted from psoriatic epidermis, psoriatic scale, normal epidermis, normal stratum corneum, serum, and leukocyte homogenates. He did find two bands in the psoriasis material which could not be found in the others but also noted that the major glycoprotein band (PAS positive) was probably coming from the leukocytes. Also Marghescu and Braun-Falco (M7) had previously shown that some esterase isozymes which travel as α_2-globulins on agar electrophoresis can be found in parakeratotic scale, but not in normal stratum corneum. They normally are found histochemically below the stratum corneum and do not reach the surface. In general, amid the plethora of proteins which can be extracted (K7), the search for a specific soluble precursor of keratin has not been rewarding nor has the attempt to reason backward from the insoluble final products. For reviews on this subject, see Rothberg (R16), Crounse (C13), and Bernstein *et al.* (B11). This last review also discusses attempts at *in vitro* protein synthesis using epidermally derived cell free preparations.

Soluble protein is increased in psoriatic lesions as compared to normal and also in the psoriatic scale as compared to callus (see Table 10) (F12, F13, F15, W14). Since the DNA content of the epidermis of normal and abnormal epidermis is the same (see above), this implies a greatly increased soluble protein pool per cell which persists right into the scale, but whether this represents keratin, enzymes, or other proteins is unknown. In addition Herdenstam (H16) found an increase in protein

TABLE 10
SOLUBLE PROTEINS AND NITROGEN-CONTAINING COMPOUNDS IN PSORIATIC SCALES[a]

Component	Normal[b]	Callus[b]		Psoriasis[b]	Scales[b]
	(2)	(1)	(2)	(1)	(2)
Total N (water soluble)		2480		1990	
Protein N		330		1069	
SH Protein		—	28[c]	—	125
Non protein N		2090		884	
Amino acid N	378	200		100	
Urea N		183		35	
NH_3 N		155		148	

[a] Values given as milligrams for 100 g day tissue unless marked otherwise.
[b] Data from (1) Wheatley and Farber (W14); (2) Flesch and Esoda (F12).
[c] Millimoles $\times 10^{-2}$.

formation from glucose in the psoriatic lesion together with a decrease in the amino acid pool, and this is consistent with the data in Table 10. Although the amount of SH-reacting soluble protein is also increased in the soluble proteins from psoriasis scales, Magnus (M2) has pointed out that this may be an artifact due to incomplete masking of the SH groups in the psoriasis protein. When the extracts were boiled in 10% lauryl sulfate, psoriasis scales, callus, and normal forearm stratum corneum all gave about the same amount of SH. Amino acid analysis of scales actually have shown low sulfur values (L8, L12).

3.10.4. *Enzymes of Carbohydrate Metabolism*

Hershey *et al.*, since 1954 (H17, H18, H19, H20) have reported on several glycolytic, pentose shunt, and Krebs cycle enzymes in human epidermis measured by Lowry's microtechniques. They have also compared enzyme activities in various structures of the skin (e.g., hair follicle, sweat gland, sebaceous gland, dermis, and epidermis), and have reported on their susceptibility to heat inactivation.

In regard to any abnormalities that might exist in psoriasis, Weber and Korting found increased activities of the pentose shunt enzymes glucose-6-phosphate dehydrogenase (G-6-PDH) and 6-phosphogluconate dehydrogenase (6-PGDH) in psoriatic scales (W6). Prior to this Weber had reported extensively on the relative activities of several enzymes in epidermis, in blisters, and in serum to show that the observed activities in the blisters did indeed come from the epidermis (reviewed in W3). Comaish (C8) reported increased levels of aldolase (3$\times$ normal) in the psoriatic lesion as compared with either normal skin or the uninvolved skin of patients with psoriasis.

Braun-Falco and Petzoldt (B30), Rassner (R1), and Halprin and Ohkawara (H2) presented extensive surveys of the glycolytic, pentose shunt, and citric acid cycle enzymes in normal skin, unaffected skin of psoriatic patients, and in the lesions themselves. Figure 10 is reprinted from this work (H2) and shows the reorganization of the cellular metabolic activity made necessary by the high synthetic and mitotic rate of the tissue.

In the figure, the width of the arrows is proportional to the activity of the enzyme determined in homogenates of psoriatic lesions as compared with homogenates of normal epidermis. A single line (e.g., phosphorylase breaking glycogen down to glucose 1-phosphate) represents the normal activity, and each additional line is a 25% increase over the normal. The

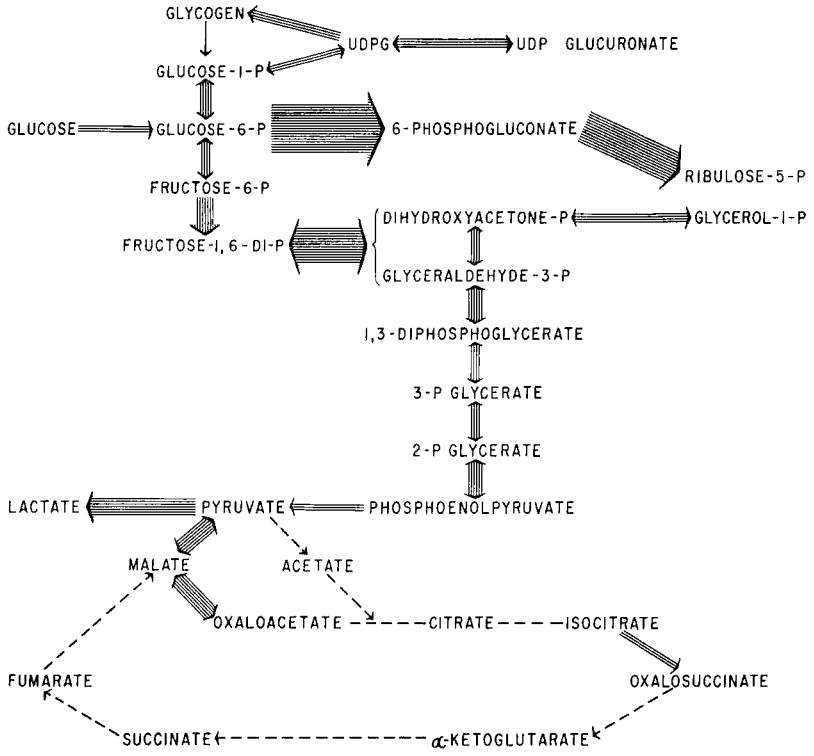


FIG. 10. Enzyme activities in psoriasis lesions as compared to normal epidermis. A single line in the arrow indicating a particular enzymatic reaction is equivalent to the normal activity of that enzyme (e.g., phosphorylase breaking glycogen down to glucose 1-phosphate). Each additional line in the arrow indicates a 25% increase in the enzyme activity. Reprinted from Halprin and Ohkawara (H2) by kind permission of the Williams & Wilkins Co.

very high increase in pentose shunt activity can be appreciated. The only enzyme not increased in this study was phosphorylase and since the enzymes leading to glycogen formation were increased, the cells become full of glycogen (3× normal).

Rassner's assays and our own were done on a weight of tissue basis. However, Rippa and Vignali (R9) in measuring 6-P-G-DH, G-6-PDH, aldolase, and phosphohexoisomerase in normal and psoriatic skin measured their activities on the basis of protein in the supernatant solution from the centrifuged epidermal homogenates. They found increases in only the pentose shunt enzymes based on specific activity (units per milligram of protein). The explanation is that in the psoriatic homogenate and supernatant there is twice as much soluble protein for the same fresh weight of tissue, and corrected on this basis many of the elevations in enzyme activity would not be apparent.

Since the DNA concentration is about the same in psoriatic and normal epidermis (B14, M12), each cell must have a correspondingly decreased amount of structural or nonsoluble protein within it to counterbalance the increased amount of soluble protein. Within the psoriatic lesion there is more soluble protein per cell, with the ratio of carbohydrate metabolizing enzymes to the rest of these soluble proteins unaltered except for the increase in the pentose shunt activity which rises preferentially even in relation to the increased soluble protein.

Hammar, using microdissection techniques, confirmed the increased activities of G-6-PDH, glyceraldehyde-3-phosphate dehydrogenase, enolase, pyruvic kinase, and phosphoglycerate kinase, but he could not find an increase in glycerolphosphate dehydrogenase (H10). In further studies of the border of psoriatic lesions, he showed an increase in glucose-6-phosphate dehydrogenase in areas that were histologically not yet involved; this evidence of biochemical change preceding histological is most interesting (H7) as are data on respiratory stimulation (H9).

Isoenzymes. Weber (W3), Grosfield *et al.* (G15, G16), and Marghescu (M5) have all examined the LDH isoenzyme patterns in normal and psoriatic skin. The pattern in both is that of an anaerobic tissue, i.e., a predominance of the cathodal bands 4 and 5. Psoriatic lesions tend to have increased activity and a slight shift to a more aerobic pattern.

NADPH-specific isocitric dehydrogenase shows at least 3, and probably 4, bands separable by starch gel electrophoresis in normal epidermis (F25, O3) whereas in psoriatic skin the most anodal bands disappear (F25) (Fig. 11). They reappear with treatment of the lesion. Basal cell carcinoma also shows no activity of this anodal band. We think the most anodal band is somehow linked to normal keratinization since the basal cell carcinoma has a rate of proliferation which is not much faster

FIG. 11. NADP-specific isocitric dehydrogenase isozymes in normal human epidermis and a psoriasis lesion. Epidermis is homogenized, and a small amount is placed in a well at the origin. The proteins in the homogenate are allowed to migrate when an electric current (anode at right) is passed through the agar plate for 2 hours. The enzymes are visualized by applying a solution of NADP, isocitrate, phenazine methosulfate, and nitroblue tetrazolium to the surface. The tetrazolium salt is reduced and deposits as the blue formazan on the gel wherever the enzyme is located. The most anodally migrating isozyme is not present in the psoriasis lesion.

than normal (W11) while both lesions share a lack of normal formation of keratin.

3.10.5. *Glycogen and Mucopolysaccharides*

Glycogen is formed by the epidermis from glucose through glucose 6-phosphate, glucose 1-phosphate, and uridine diphosphoglucose. Glucose enters the epidermal cell by a process of passive diffusion which seems not to be affected by insulin (H3, H4, K1), and normally about 5% is transformed into glycogen (F19). The usual enzymes involved in glycogen metabolism (glycogen synthetase, branching enzyme, debranching enzyme, and phosphorylase) are presumably present in epidermis, and recently Leathwood and Ryman (L4) have used this tissue for the diagnosis of the glycogen storage diseases.

Glycogen histochemically by PAS staining is present in human fetal epidermis even before it is present in the liver, but by the sixth fetal month, it disappears from the epidermis and is usually not seen in the normal adult epidermis (M16). Actually the glycogen concentration in normal adult epidermis is about 0.046% (H1), and it is just below the level which is picked up by the histochemical procedure. Even a slight rise will result in a positive PAS stain (O2), and the markedly increased staining found in the psoriasis lesion in fact represents about a 3-fold rise in glycogen content (H2). In the psoriatic lesion we found (H2) increased levels of glycogen formation and unchanged breakdown which presumably could account for the increase.

Other situations of skin injury both experimental and natural are also accompanied by glycogen accumulation, and it is difficult to understand why. Glycogen accumulation in basal cells does not accompany or precede mitotic activity (L9, P2), and in the malphighian cells it seems to

increase as in psoriasis when the metabolic activities of the cells are highest. If glycogen were functioning as an energy source, one would expect to find a depletion of glycogen in these situations rather than an accumulation. We have proposed (H2) that the glycogen might be a sign of increased mucopolysaccharide formation since increased uridine diphosphoglucose (UDPG) needed for increased mucopolysaccharide formation would also provide a substrate for increased glycogen formation. This would also fit with the finding of increased UDPG in psoriatic scales (B12). The biochemical evidence for mucopolysaccharide formation in epidermis is very limited.

Fetal pig epidermis contains hyaluronic acid and chondroitin 4-sulfate containing mucopolysaccharide (S15) as does human epidermis (M13), and some of the enzymes of uronic acid metabolism are present in human epidermis (F24). Also Barker *et al.* (B6) have shown uptake of radioactive sulfate into epidermis, and Braun-Falco *et al.* (B33) have shown that psoriatic epidermis is more active than normal epidermis in accumulating radioactive sulfate *in vitro*. There is also a suggestion that methionine sulfur may be preferentially accumulated in psoriatic lesions (B26, L6). There seems to be little doubt, therefore, that mucopolysaccharides are formed, and there is some suggestion that they may be formed in greater amounts in psoriatic skin lesions. The next section will consider the possibility that they are of importance in the cell membrane and intercellular contact.

3.11. Intercellular Cement

Histochemically, mucopolysaccharides outlining the epidermal cells can easily be seen (B28, G9, S23, S25, W19). In addition, Flesch and Esoda (F14) found evidence of a "glycoproteolipid" in normal stratum corneum and increased amounts in psoriatic scale. They could find no sialic acid in the material, it was resistant to hyaluronidase, and they proposed that a lack of proper breakdown of this material was important in the pathophysiology of the disease.

Bonneville *et al.* recently reviewed this subject (B27) and added electron micrographic evidence of their own indicating: (1) the existence of dense amorphous masses (of mucopolysaccharide nature) between stratum corneum cells of the psoriasis lesion which they did not see in normal skin and which were continuous with the middle plate of the desmosome; and (2) an increased number of keratinosomes in psoriasis (keratinosomes are the same as membrane coating granules, which are also the same as cytoplasmic lamellated structures that empty into the intercellular space at about the level of the granular layer).

The substance which is deposited might be mucopolysaccharide (H12)

or it might consist of lysosomal enzymes (W22). Bonneville *et al.* believe that these keratinosomes are structurally normal whereas Van de Staak *et al.* (V1) and Hashimoto and Lever (H12) believe that they are altered. Meanwhile Mercer and Maibach (M11) have found that there is a lack of the surface coat mucopolysaccharides around the psoriatic cells rather than too much. Perhaps some of these problems will be settled when the lysosomal enzymes responsible for breaking down these substances are examined in more detail in normal skin and in psoriatic lesions.

3.12. The Dermis

Recently an increase in the amount of hyaluronic acid in the psoriatic lesion was reported with no change in the amount of dermatan sulfate (F11). These studies were done using both cetylpyridinium chloride-salt fractionation and electrophoresis and were referred to dry weight of skin. Total glycosaminoglycans found equalled 792 μg per gram of psoriatic skin versus 532 for normal skin. The effect of this increase in hyaluronic acid, which is presumably dermal on the overlying epidermis, is not known although Hambrick *et al.* (H5, H6) have shown marked effects of heparin and glucosamine upon skin in tissue culture.

3.13. Zinc and Psoriasis

Zinc-deficient animals get a scaling dermatosis which has similarities to psoriasis (A4). In 1958 Braun-Falco (B28) noted that he and Rathjens had found less zinc by histochemical methods in psoriatic lesions than in normal skin, but he also noted that zinc therapy failed to cure psoriasis. Greaves and Boyde (G12) reported a low serum zinc level in patients with psoriasis, and this was confirmed by Voorhees *et al.* (V8) but refuted by Molokhia and Portnoy (M15), who used neutron activation analysis in contrast to the previous studies which relied on atomic absorption. Voorhees *et al.* (V8) analyzing whole skin found an increase in zinc in the lesion compared to noninvolved skin of the psoriatic patient, but Molokhia and Portnoy (M15), who separated epidermis from dermis, point out that this is probably attributable to the epidermis, which contains four to five times as much zinc as the dermis, making up a larger proportion of the biopsy in the involved skin. By contrast, their figures do show increased zinc in the epidermis of both the involved and noninvolved skin as compared with normal, and this would agree with Voorhees *et al.* finding of increased zinc in psoriatic scales.

In any case, treatment with zinc sulfate orally did not help despite a rise in serum and scale zinc concentrations (V8).

4. The Uninvolved Skin

There has been a continuing debate over whether or not the clinically uninvolved skin of the psoriatic is actually "normal." Is there a way of bringing out "latent" psoriasis? Since the genetic influence in this disease is fairly strong, one would expect all skin cells to have the same abnormality present within them; therefore, the uninvolved skin should have an observable abnormality inherent within it.

Histological differences between normal skin and the uninvolved skin of patients with psoriasis could not be found by Helwig (H15), although the measurements made by Gordon and Johnson (G9) do indicate a thickening of the uninvolved skin (Table 11). Capillary changes diagnostic of psoriasis were suggested (H24, T7), but these also were not confirmed (I1).

Biochemically a decrease in skin surface cholesterol-esterifying ability was mentioned (G2), but this also could not be confirmed (W16). Enzyme assays have shown no differences between normals and the normal-appearing skin of psoriasis (H2, R1) even when histochemistry suggested that there was a difference (W21). Wohlrab (W20) has reported an increased number of nuclei which are hyperdiploid in uninvolved epidermis.

More promising have been recent attempts to show a difference by the response of the skin to injury. Reid (R3) and Reid and Jarrett (R4) have shown an increased erythema response of normal-appearing skin of psoriasis patients to applications of vitamin A with increased release of acid phosphatase in the uninvolved but psoriatic epidermis. Braun-Falco *et al.* (B32) found an increased number of cells incorporating tritiated thymidine after Scotch tape stripping. While Hell and Hodgson (H13) noted increased uptake of thymidine *in vitro* by uninvolved skin of psoriatic subjects. There may in the future be a way of reliably

TABLE 11
MEASUREMENTS OF EPIDERMAL THICKNESS IN PSORIASIS AND NORMAL SKIN[a]

Sample	Height (μ) from basal layer to undersurface of stratum corneum		Height of dermal papillae	
	Range	Average	Range	Average
Normal skin	73–105	92	36–55	46
Psoriasis, uninvolved	105–167	127		
Psoriasis, involved	242–448	303	151–408	250

[a] From Gordon and Johnson (G9).

picking out psoriasis "carriers" if these studies can be reliably repeated by others.

Stankler (S17) has reported decreased levels of B_{12} in serum and skin including the apparently normal skin (see Section 5.7).

5. Blood Chemistry in Psoriasis

5.1. Blood Groups and Serum Factors (Table 12)

Wendt (W13) reviewed the literature up to 1968 and added his own observations on 423 patients with psoriasis as contrasted with 907 controls from the same area. He found no difference in the distribution of ABO (A1, A2), D, Ss, and Kk blood groups or in the distribution of Hp, Gc, Gm (1), and Gm (12) serum factors. In prior studies from England (H11) and Scotland (M1), no significant difference had been found in ABO groups. In Germany a prior report had indicated a predominance of O (P8), but a more recent report found no difference (D2). This second report from Germany, therefore, confirms a lack of linkage of psoriasis to any of the ABO blood groups. Similarly negative findings were previously reported for haptoglobin (S20) and Gm (1) (B10).

Positive correlations were found by Wendt for blood group M and serum factor Gm (2). If confirmed by further studies this association would lead to another area of investigation of the hereditary factors responsible for psoriasis.

TABLE 12
Blood Groups and Serum Factors in Psoriasis[a]

	Poehlmann (P8)		Dorn (D2)		Hargreaves and Hellier (H11)		Wendt (W13)	
Blood group	Ps	C[b]	Ps	C	Ps	C	Ps	C
O	57.5	41.5	40.0	40.0	49	47.6	40.9	37.2
A	28.0	42.3	42.0	40.0	40.5	41.5	42.8	46.4
B	11.6	10.2	12.5	15	9	7.8	10.1	11.8
AB	3.0	6.1	5.5	5.0	1.5	3	6.2	4.6
M							36.9	28.8 ($p = 0.02$)
MN							45.9	52.1
N							17.2	19.1
Gm (2+)							30.5	19.9 ($p = 0.0005$)
Gm (2−)							69.5	80.1

[a] All figures are % and have been rounded off to the first decimal place from the original tables.

[b] Ps = psoriatics; C = controls.

5.2. Serum Proteins

Tickner and Mier (T8) using paper electrophoresis found a significant fall in total protein and albumin with significant rises in the mean values of α_2- and β-globulin in 86 patients. The β-globulin tended to drop as the disease became more widespread and in individual patients the changes were slight. On the other hand, Muller-Sutter *et al.* (M17) found a tendency for α_2-globulins to be decreased in psoriasis and to return to normal with treatment. Laugier and Zimmer (L2) used gel electrophoresis and found that in the majority of cases globulins were normal. They also studied 27 subjects in 10 families and found no familial correlation between the presence or absence of psoriasis and the presence or absence of hyper or hypoproteinemia. Seven members of one family studied by Schuster *et al.* (S6) showed no correlation between the presence or absence of psoriasis and the serum proteins. In addition no difference was found in one patient studied both during an active state and restudied during remission. The same group reported no significant abnormalities in serum proteins in a group of 18 patients (L3).

Lipoproteins studied by the same group were said to be normal as all patients with abnormal findings had evidence of atherosclerosis. Shapiro *et al.* (S8) found slight decreases in low density and very low density lipoproteins by ultracentrifugation. They did not consider these changes as significant and in a further report on 20 patients treated with a variety of agents which changed the serum lipoprotein pattern (low fat diet, entozyme, lipan, trypsin, hesperidin, triamcinolone, and heparin) there was no correlation between the clinical course of the disease and the effects on serum lipoprotein (S7).

In accordance with the findings on serum lipoproteins are those on serum cholesterol. As would be expected in Tickner and Mier's (T8) study on 86 psoriatics, the serum cholesterol tended to follow changes in the serum β-lipoproteins and was elevated in only 20% of the subjects with psoriasis. In the North Carolina study by Abele *et al.* (A1) of a large kindred with psoriasis there was no significant elevation of cholesterol or any correlation with the presence or absence of disease in the 258 members examined for cholesterol and who were over 21 years of age.

Block *et al.* (B22, B23, B24) in a series of publications since 1958 has indicated that there is no significant difference in the way patients with psoriasis handle proteins and amino acids as compared with normals. These studies were done on patients under rigorous hospital conditions on controlled diets. Nitrogen uptake in two patients with psoriasis was exactly balanced by nitrogen loss in urine, feces, and scale. Sulfate ex-

cretion in the psoriatics was the same as in the control patient (B22). Also amino acid analyses of blood and urine have shown no differences in the content or handling of amino acid by psoriasis patients (B23, B24, H28).

In summary, none of the above investigations have given any evidence of a primary abnormality in the production or utilization of serum proteins, lipoproteins, or glycoproteins. Also the feeding of amino acids (R19) or alterations in the protein level of the diet (Z1) do not change the disease process.

5.3. Serum Uric Acid

In most studies no significant increase in serum uric acid values have been found (A1, T8, W1), but in some an increase has been reported (B8, S24). The work of Eisen and Seegmiller (E3) is the only report concerned with the metabolic formation of uric acid using radioactive glycine. They did show an increase in the formation of uric acid in extensive psoriasis and a reduction to normal levels with treatment. In addition, the excretion of pseudouridine and uracil was increased in extensive psoriasis (E2) (Table 13). There was a direct correlation in the above studies between the serum uric acid level versus the extent of skin involvement, the excretion of pseudouridine versus the extent of skin involvement, and also the excretion of pseudouridine versus the uric acid excretion ($R = 0.81$). These findings imply increased nucleic acid synthesis and increased nucleic acid breakdown in the skin, access of the purine breakdown products to the blood stream and from there to the liver(?) for transformation into uric acid and finally to the kidney for excretion.

TABLE 13
Uric Acid, Uracil, and Pseudouridine Excretion in Psoriasis[a]

	Normal (mg/day)	Psoriatic (mg/day)
Uracil excretion	4–6	10–13.5
Pseudouridine (5-ribosyluracil)	45 (median)	64 (median for patients with over 20% of the body involved)
Uric acid	357 and 459	312–773 (average 570)
% of administered glycine-^{14}C excreted in 7 days in urinary uric acid	0.34 and 0.37	0.52, 0.61, 0.84, 1.09

[a] Data from Eisen *et al.* (E2, E3).

5.4. Serum Glucose Levels and Diabetes

This subject has been well reviewed by Sidi *et al.* (S12). There is extensive evidence against there being any abnormality in blood glucose in psoriasis or any connection between diabetes and psoriasis. Studies attesting to the lack of clinical effect on the disease process of agents designed to lower blood sugar were also reviewed in the above publication.

5.5. Serum Levels of Inorganic Ions

Serum sodium, potassium, chloride, and bicarbonate values were all within the normal range in a series of 44 patients studied by Reinberg *et al.* (R6). Also, according to Sidi *et al.* (S12), serum phosphorus, calcium, and magnesium are normal. Zinc has already been discussed, and Molokhia and Portnoy (M15) will undoubtedly soon have comparison values of copper and manganese in the serum of psoriatics versus normals.

5.6. Enzyme Changes in Sera and Erythrocytes

Weber (W4) reported finding elevated activities of glucose-6-phosphate dehydrogenase (G6PDH) in the sera of patients with psoriasis. Also he found that the elevation correlated in quantity with the severity of the disease process (Table 14). This finding was confirmed by Holzmann (H25) and Salfield and Vogelsberg (S1), but not by Ruzicka *et al.* (R20). The two levels shown for each category of patient in Ruzicka's study is due to the fact that he used two sets of reagents from different sources and reported them separately. The reason for the discrepancy between Ruzicka's results and the other three is not known. In addition to the above enzymes, Holzmann (H25) also measured aldolase levels, and found a slight increase in psoriasis. Salfield and Vogelsberg (S1) compared levels of aldolase, glyceraldehyde-3-phosphate dehydrogenase, enolase, pyruvic kinase, malic dehydrogenase, lactic dehydrogenase, glutamic–pyruvic transaminase, and glutamic–oxaloacetic transaminase in normals, psoriatics, and patients with eczema. Although he found elevations in the psoriatic sera (especially with aldolase and enolase over 50% above normal), he found similar elevations in the sera from patients with eczema.

The most plausible explanation is that the serum enzymes are a reflection of leakage of the enzymes into the serum from the damaged epidermal cells since the above enzymes are precisely those that are most elevated within the psoriatic epidermis. Another source for these enzymes might be the erythrocyte, but all authors agree (H25, M3, R20, W5)

TABLE 14
SERUM GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE IN PSORIASIS

Author	Material	Enzyme activity (mμ/ml) G6PDH	6PGDH
Weber (W4)	Psoriasis—mild	0.92	—
	—moderate	3.8	—
	—severe	7.4	—
	Nonpsoriatic dermatological disease	1.0	—
Weber and Ganzer (W5)	Psoriasis	—	0.98
	Normal	—	0.50
Holzmann (H25)	Psoriasis	2.9	2.3
	Normal	1.1	1.1
Salfield and Vogelsberg (S1)	Psoriasis	1.11	—
	Normal	0.60	—
Ruzicka *et al.* (R20)	Psoriasis, male	3.41	—
		2.36	—
	Normal, male	1.77	—
		2.97	—
	Psoriasis, female	2.17	—
		2.67	—
	Normal, female	3.06	—
		4.07	—

that G6PDH and 6PGDH are not elevated in the psoriatic RBC. A comprehensive study by Holzmann (H25) (Table 15) finds no increase in erythrocyte enzymes; in fact the only significant difference from normal was a decrease in phosphohexoisomerase activity of the psoriatic erythrocyte. It is difficult to assess this finding since this enzyme is usually present in large amounts and is not thought to play a regulatory role in glycolysis. In other work on this enzyme, Holzmann and Morsches (H26) have found that phosphohexoisomerase of RBC, WBC, platelets,

TABLE 15
ERYTHROCYTE ENZYMES IN PSORIASIS[a,b]

Subject	HK	PHI	PFK	ALD	PGM	PK	G-6-PDH	6-P-6-DH
Normal	20	608	165	50	892	388	131	67
Psoriatic	22	209	146	57	877	404	132	71

[a] Values are expressed as milliunits per 10^9 cells.
[b] From Holzmann and Morsches (H26).

and epidermis of both normals and psoriatics all give the same one band on agar gel electrophoresis. Along with the decreased activity of phosphohexoisomerase, Holzmann (H25) also found an increase in G-6-P, ADP, and ATP in the psoriatic red cell and an abnormal increase in red cell G-6-P when the cells were incubated in the presence of glucose and chloroquine. These findings need confirmation.

Bielicky *et al.* have reported increased utilization of glucose and production of ribose by incubated psoriatic erythrocytes using the orcinol reaction (B21). The figures are not very convincing (i.e., 6–10% pentose shunt in normal erythrocytes versus $13 \pm 6\%$ in psoriatics). In addition because of the "known" tendency of antimalarials to make psoriasis worse, they gave 750 mg of chloroquine a day to psoriatics and measured the change in pentose shunt activity of their erythrocytes. There was no significant change. Also only 22% of the patients got worse whereas 39% got better during chloroquine administration for 6 weeks. Other evidence against there being anything similar to G-6-PDH deficiency disease in psoriasis is the report by Karasek and Farber (K3) that glutathione levels in RBC's of psoriatic patients are the same as levels in normals and there is no difference in their response to antimalarials or phenylhydrazine.

Malina *et al.* (M4) have reported an investigation of lactic dehydrogenase isozymes in psoriatic erythrocytes; although the authors state that the differences in LD_1, LD_2, and LD_4 are statistically significant, they are not impressive (Table 16).

Another enzyme found to be present in increased amounts in psoriatic serum as compared to normal is a phosphodiesterase studied by Zaruba *et al.* (Z3). Again this enzyme is found in elevated amounts in psoriatic epidermis and presumably the increase of enzyme in the serum is the result of leakage from the epidermis. With this enzyme, which is presumably bound to microsomes and mitochondria, it is a little more difficult to understand exactly how it leaks out of the cell.

In summary, there are elevations of several cellular enzymes in the sera of patients with psoriasis and presumably the psoriatic epidermal

TABLE 16

SERUM LACTIC DEHYDROGENASE ISOZYMES IN PSORIASIS: PERCENT OF TOTAL LDH ACTIVITY IN EACH ISOZYME[a]

Subject	LD_2	LD_1	LD_3	LD_4
Psoriatic	39.1	37.1	19.5	4.1
Normal	35.4	31.5	22.5	9.8

[a] From Malina *et al.* (M4).

cell is somewhat "leakier" than normal epidermal cells. Also there may be a biochemical difference in the initial phosphorylation and utilization of glucose within the psoriatic red cell but this has not been clearly established as yet.

5.7. Vitamins

Vitamin B_{12} levels were measured in serum and skin by Stankler (S17). These results are shown in Table 17 and indicate a decreased amount of B_{12} in the lesions and even in clinically uninvolved skin. Presumably the B_{12} is more rapidly utilized in the metabolically active lesion. This skin utilization is not reflected in a significant decrease in serum B_{12}.

Shuster *et al.* (S11) measured B_{12} levels in the sera of 20 patients with psoriasis and found normal levels in all but one. Folic acid levels were decreased below normal in 21 out of 28 patients, urinary FIGLU excretion was elevated in 19 of 30, and in 5 of 9 patients sternal marrow puncture revealed megaloblastic erythropoiesis, thereby implying a true folate deficiency. Neither serum iron deficiency nor lack of absorption of folate could be implicated as a cause of the folate deficiency, and presumably it is related to the skin lesions.

Grignani *et al.* (G14) studied several of the enzymes of folate metabolism in human epidermis—both normal and psoriatic. Increased levels of folate reductase were found in the psoriatic lesion, and further enzyme could be induced by treatment of the patients with amethopterin. By contrast, formate-activating enzyme, 5,10-methylenetetrahydrofolate dehydrogenase, serine hydroxylase, and cyclohydrolase were normal in the psoriatic lesion. Formiminotetrahydrofolate transferase could not be measured either in normal or psoriatic skin. The activities of the above enzymes as well as the absence of the transferase are similar to the findings for small bowel but not to other tissues studied. How these findings

TABLE 17
Serum and Skin Vitamin B_{12} Levels in Psoriasis[a]

	Serum[b]	Skin[b]	Number of patients
Nonpsoriatics	384	8597	16
Psoriatics			
Uninvolved skin before Rx	408	6176	10
Uninvolved skin after Rx	425	8056	10
Involved skin before Rx	408	4187	10
Involved skin after Rx	425	6581	10

[a] From Stankler (S17).
[b] Serum in pg/ml; skin in pg/gm.

relate to the folate deficiency found in the work of Shuster *et al.* (S11) remains to be clarified.

6. Reflections and Speculations

Psoriasis has been with us for many hundreds of years. Recognition of the fact that in some families the incidence is very high while in others it is nonexistent, coupled with the fact that the disease can range in severity from a minor nuisance to a life-threatening involvement of the entire integument, makes one think that probably there are several different diseases masquerading under one red scale. Most likely all have to do with an abnormality in the same underlying mechanism, but to different degrees and perhaps at different points along the pathway from gene to product.

What might the underlying mechanism be? At least two things are definitely wrong in the psoriatic lesion—one is the control of the rate of proliferation of seemingly normal epidermal cells and the other is in the formation of properly packaged keratin fibers, a process which seems to be dependent on the keratohyalin granule and the histidine–arginine-rich protein. Enough evidence indicating that the two defects are dissociable has now accumulated as to make it unlikely that the one defect is due to the other.

There must, therefore, be another more basic defect which causes both of the above, and in speculating about what this might reasonably be, two possibilities come to mind: one is that both defects may be the result of chalone deficiency—either because it is not made in adequate amounts or because it is ineffective even though made;* the second is that both defects might be due to decreased levels of arginine or histidine. These two possibilities result from taking the most likely candidates at the present time for each of the defects and assuming that it is responsible also for the second defect.

How might chalone interfere with proper keratinization as well as with proliferation? There are many conceivable ways in which this glycoprotein could shut off the synthesis of those enzymes required for DNA synthesis and cell division and at the same time (perhaps in a reciprocal manner) unblock the synthesis of those enzymes needed for synthesis of the keratohyalin protein. This would be the normal state. A lack of chalone would then lead to unblocking of DNA synthesis and blocking of formation of keratohyalin, i.e., psoriasis.

Similarly, arginine and histidine are presumably needed for formation of the keratohyalin protein, and perhaps they are needed also for the protein which normally blocks the DNA coding for formation of DNA

* See Note Added in Proof, page 388.

synthesis and cell division enzymes. A lack of arginine or histidine due to elevated arginase or histidase would have the effect of unleashing cell division while preventing keratohyalin formation.

All of the above is of course quite speculative, but it should be noted that it is only the advances of the past few years which have allowed even speculation as to the mechanisms behind this disease.

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Note Added in Proof

A theory proposed by O. H. Iverson (Chalones of the skin. *In* "Homeostatic Regulators," pp. 29–52. Ciba Found., Churchill, London, 1969) implicating the adenyl cyclase system within the cell wall as the receptor site for chalone has received support from the finding that adenyl cyclase activators and/or dibutyryl cyclic-AMP inhibit epidermal mitoses [Voorhees, J., Duell, E., Kelsey, W. H., Englehard, K., and Sibrack, L., The epinephrine-chalone, adenyl cyclase, phosphodiesterase cascade in the *in vitro* control of epidermal growth. *Clin. Res.* **19**, 365 (1971)]. In addition, a low level of adenyl cyclase in psoriatic lesions was reported by Reba Wright on June 16 in San Francisco at a meeting of the American Society of Biological Chemists.

MULTIPLE ANALYSES AND THEIR USE IN THE INVESTIGATION OF PATIENTS

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1. Introduction

Until a few years ago the vast majority of tests performed by clinical chemistry laboratories were performed at the discretion of the clinicians responsible for the patient concerned. The conventional techniques of history taking and clinical examination were followed by a choice of investigations by the clinician. Some simple tests would be performed whatever the patient's signs or symptoms, and these could be described as screening tests; that is, they were tests performed irrespective of the patient's clinical condition. The routine screening of urine by tests for sugar and protein has been a feature of clinical examination for several decades. In many hospitals, the determination of blood hemoglobin level is now in the category of a screening test. Performing tests for which there is no obvious clinical reason is not solely concerned with tests of urine or blood. The taking of blood pressure and body temperature, listening to the heart sounds, and routine chest radiography must also be placed in this category of screening tests. However, added to these tests are often one or more tests chosen at the discretion of the clinician and based upon his desire to check a tentative diagnosis.

It is customary and good clinical practice to ask for certain tests in groups. The requesting of serum calcium, phosphorus, and alkaline phosphatase in suspected bone disease is a common trio of tests. Interpre-

tation is helped by knowing all three at the time of interpreting any one. Groups of tests described as "liver function" tests are a further example. Some tests are inevitably reserved for use if certain commoner tests are abnormal. Creatinine clearance may be performed as a result of a urea which is regarded as high enough to suspect renal disease. Protein-bound iodine determination may follow the finding of a high serum cholesterol. Electrophoresis of the serum to ascertain the pattern of globulin concentrations commonly follows the finding of a high total globulin concentration. Thus, some of the first discretionary tests asked for by the clinician are commonly described as screening tests. In this case, the use of the word screening is in respect of tests which, if positive, are not necessarily diagnostic, but give an indication that further more complicated tests are worthwhile. To prevent confusion in the dual use of the word screening, it is suggested that its use be confined to the first example, tests performed irrespective of the patient's clinical condition. The use of the term "preliminary discretionary test" is advocated for the other type of test.

Grouping of tests has become an important part of laboratory investigation. In addition, screening tests and preliminary discretionary tests have also become a convenient and integral part of patient investigation.

The introduction of automatic methods of analysis in clinical laboratories has led to increased possibilities in all these aspects of laboratory investigation. In the early days of the use of the AutoAnalyser, it could be shown that the purchase of a two-channel machine for the simultaneous analysis of blood urea and blood glucose cost much less than the purchase of two separate machines, one for each analysis. In many laboratories this heralded an addition to patient investigations based upon analytical convenience. When requesting either test the result of the other was also provided. Later, analytical expediency as well as conventional clinical grouping resulted in the Technicon development of a five-channel AutoAnalyser producing results on urea, sodium, chloride, potassium, and carbon dioxide-combining power. It became very clear to laboratory workers when using such equipment that if a discretionary request for any one of these determinations was made, then it was most convenient to perform all five. "Exception" analysis or reporting caused more work than performing the analyses. The ease of performing such analyses also, in many hospitals and clinics, stimulated a screening approach. For example, blood serum of all patients entering surgical wards is analyzed on this five-channel apparatus.

The combination of test groups, screening tests, and preliminary discretionary tests received further stimulus with the introduction of the simultaneous multichannel (SMA) machine by the Technicon Co. The first (SMA 8/30) was analytically unsatisfactory, the second, the

SMA 12/30, and later the SMA 12/60 were considerably improved in design and analytical performance. The introduction of the SMA was heralded by the company with three reasons why it should be used. The first was analytical convenience, "exception" analysis was inconvenient and uneconomic. The second was that the unsolicited information produced by the equipment led to new or additional diagnoses unsuspected by the clinician. The third was that the preliminary discretionary tests could be completed within hours of the patient's admission to hospital rather than the days required by the usual process of discretionary analysis. The combined results were called a biochemical profile. In biochemical profile it was postulated that we had a combination of discretionary analyses, grouping of tests, and screening, as well as analytical convenience.

It was obvious that this was only the beginning of such developments. The development of the Autochemist (Aga Co.) by Gunner and Ingmar Junger in Stockholm indicated that they could perform 25 analyses on each specimen of serum, automatically and at a rate of 100 specimens or more each hour.

2. Biochemical Profile Analysis in Hospital Patients

Biochemical profile investigations at the Queen Elizabeth Hospital, Birmingham, began in 1967. We were stimulated by a desire to know the answer to the following questions:

1. What is the actual cost of performing biochemical profiles?
2. Do such techniques increase or decrease patient bed stay?
3. What is the effect of the profile analysis on the routine laboratory workload and other clinical services?
4. What are the problems of staffing a laboratory solely devoted to profile analysis?
5. Does this technique of investigation lead to unsuspected new or additional diagnoses?
6. How many unexplained abnormal results are produced?

The literature contained two studies concerned with biochemical profile techniques—those of Byran *et al.* (B1) and Young and Drake (Y1). These studies, although giving pointers to the answers to these questions, did not, for a variety of reasons, fully answer the questions we had posed. In addition, they were carried out in a medical setting different from that experienced in this country.

2.1. Analytical Techniques

With the active support of the Department of Health and Social Security, an investigation of biochemical profile techniques was started

in this laboratory in February, 1967. The project was housed in a specially built laboratory with a total floor area of 548 sq. ft. The laboratory equipment consisted primarily of single-channel AutoAnalysers. In the beginning we confined profile analysis to patients newly admitted to seven of the wards in the hospital. The wards included patients with medical and surgical conditions. Sixteen biochemical determinations were initially chosen: glucose, creatinine, urea, sodium, potassium, total CO_2 content, albumin, globulin, alkaline phosphatase, bilirubin, zinc sulfate turbidity, calcium, serum glutamic-oxaloacetic transaminase (SGOT), iron, uric acid, cholesterol.

Because of technical difficulties, the total carbon dioxide content was removed from the group of tests (W1). The zinc sulfate turbidity test produced a low yield of information, and the test had to be performed manually; its performance was discontinued early in the survey.

On the patient's admission to hospital, a set of punched cards was produced in the registration department, containing his identification information. One of these cards was sent to the biochemistry department at 17:00 hours on the day of admission. Between 8 and 10 (i.e., 1 or 2 hours after a hospital breakfast) each morning except Sundays, laboratory staff collected 20 ml of venous blood from each patient admitted to the seven wards during the preceding day or overnight.

Prior to this investigation, virtually all patients entering these wards had some biochemical or hematological tests. This investigation required extra blood but not additional venipunctures, and the reason was explained to the patients. Initially, an attempt was made to take the blood under fasting conditions, but this proved to be impractical.

The serum constituents were determined, using either AutoAnalyser or work-simplified manual methods. While blood specimens were being prepared for analysis, three punched cards were automatically reproduced for each patient from the punched card delivered from the registration department the previous evening. Each of two cards had space for the entry of eight biochemical results and the third card had space for the coded answers to questions which the clinicians were asked to answer on receipt of a duplicate report form.

These cards were used to produce, automatically, a laboratory worksheet and the reports of all patients from whom blood had been collected. Figure 1 shows the report form, of which only the top part was returned to the ward; results considered to be abnormal were indicated with a red marker by a senior member of the laboratory staff. The normal limits used for this purpose were mainly based on the work of Roberts (R1) in Birmingham, using AutoAnalyser methods and specimens from blood donors selected in a random fashion (Table 1).

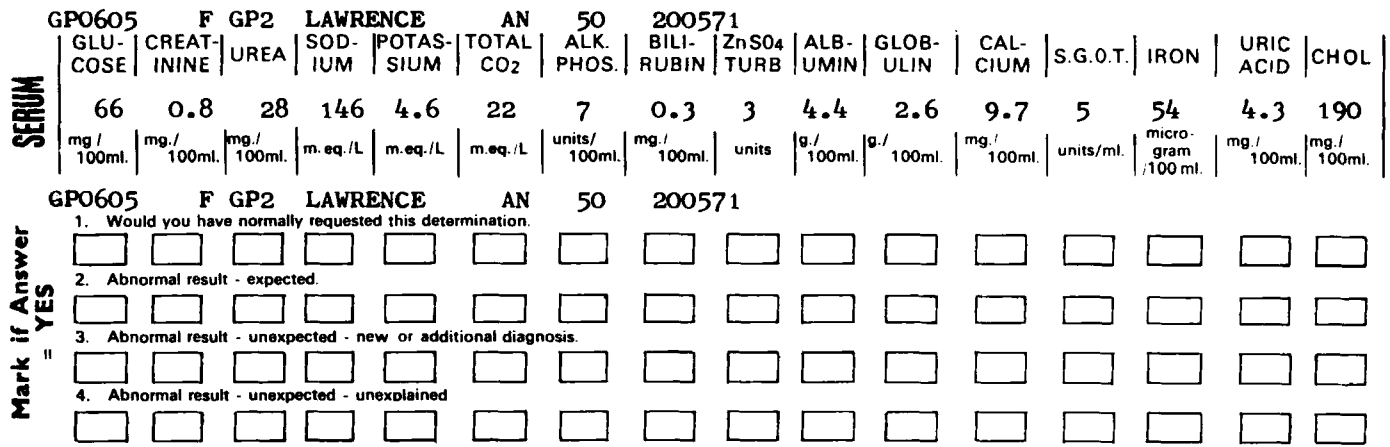

FIG. 1. Laboratory worksheet.

TABLE 1
CRITERIA FOR MARKING ABNORMAL RESULTS

Test	Criteria for abnormal result
Glucose	150 mg/100 ml
Creatinine	1.3 mg/100 ml
Urea	45 mg/100 ml
Sodium	134–147 mEq/l
Potassium	3.6–5.0 mEq/l
Alkaline phosphatase	14 King-Armstrong units/100 ml
Bilirubin	1.0 mg/100 ml
Albumin	3.3 g/100 ml
Globulin	2.0–3.5 g/100 ml
Calcium	9.0–10.5 mg/100 ml
SGOT[a]	35 units/ml
Iron	60–200 μg/100 ml
Uric acid	Females: 7.0 mg/100 ml
	Males: 7.5 mg/100 ml
Cholesterol	100–300 mg/100 ml
Hemoglobin	12.0 g/100 ml
ESR[a]	15 mm/hour

[a] SGOT, serum glutamic-oxaloacetic transaminase; ESR, erythrocyte sedimentation rate.

The wards received the report forms at 17:00 hours on the day the specimens were collected (except in the case of specimens taken on Saturday, which were separated and refrigerated; they were analyzed the following Monday).

2.2. EVALUATION OF INVESTIGATION

Initial evaluation of the scheme was based on a questionnaire completed on every patient by the clinician, who was either the consultant or registrar associated with him. The questionnaires were sent to the clinicians at weekly intervals over a period of 8 months, attached immediately beneath a duplicate of the profile results.

Question 1. "Would you normally have requested this determination on this patient?" had to be answered on all the tests. In the second question the clinician was asked, if the result was abnormal (that is, outside the limits listed in Table 1), to place it in one of three categories: (1) an expected abnormal result; (2) an unexpected abnormal result leading to a new or additional diagnosis; (3) an abnormal result, unexpected and unexplained at the time of completing the questionnaire.

The answers were coded and punched into the third punch card. At intervals the results were analyzed by computer.

2.2.1. *Analysis of Replies*

Of the first 2166 questionnaires, 95.6% (2071) were analyzed. The results are shown in Table 2, which lists the total number of analyses for each determination on these 2071 patients, and shows how many of the tests would normally have been requested and were normal. Also shown are the tests which would not normally have been requested, and these are divided into four categories: (1) normal; (2) expected abnormal; (3) abnormal, leading to a new or additional diagnosis; (4) abnormal, unexpected, and unexplained at the time of completing the questionnaire.

Table 2 shows that on the 2071 patients, 31,439 tests were performed. Of these 77% (24,203) would not normally have been requested. For individual determinations there was a wide variation in the percentage of patients that would not normally have had a particular determination. Thus 96% of patients would not normally have been tested for serum uric acid, while 35% would not normally have had urea and electrolyte determinations.

Of the 7236 tests that would normally have been requested, 81.9% (5926) were normal; while of the 24,203 tests that would not normally be requested, 92.2% (22,307) were normal, 2.4% (576) were expected abnormalities, 0.9% (225) were unexpectedly abnormal but led to a new or additional diagnosis, and 4.5% (1095) were unexpectedly abnormal and could not be explained at the time of completing the questionnaire.

Examination of the individual determinations shows that of the 225 results that would not normally have been requested but led to a diagnosis, nearly half (105) were either glucose or iron, while of the 1095 results that would not have been requested but were abnormal, unexpected and unexplained, half (542) were in one of four determinations: glucose, globulin, calcium, or iron.

An unexpected abnormal result that would not have been requested normally occurred in 42.9% (888) of the patients, in 8.3% (172) of patients there was a result leading to a new diagnosis that would not have been requested, but in 36.1% (748) of patients, there was an unexpected abnormal result not explained at the time of completing the questionnaire.

2.2.2. *General Aspects*

Answers to the questions raised at the beginning of the project have been obtained.

A costing of the analysis showed that all the tests can be performed at

TABLE 2

RESULTS FROM ANALYSIS OF QUESTIONNAIRES

Test	Grand total	Normally requested		Not normally requested				
						Abnormal		
		Total	Total normal	Total	Total normal	Total expected	Total diagnostic	Total unexplained
Glucose	2069	258	200	1811	1630	25	33	123
Creatinine	2071	307	223	1764	1620	73	12	59
Urea	2068	1342	1139	726	702	5	4	15
Sodium	2070	1336	1253	734	703	3	0	28
Potassium	2067	1332	1181	735	701	3	3	28
Total CO_2	1054	119	89	935	869	16	4	46
Alkaline phosphatase	2063	306	199	1757	1612	37	13	95
Bilirubin	2068	237	177	1831	1722	18	9	82
$ZmSO_4$ turbidity	1452	86	75	1366	1338	9	3	16
Albumin	2064	535	409	1529	1485	17	7	20
Globulin	2070	539	409	1531	1363	44	10	114
Calcium	2069	242	170	1827	1660	30	15	122
SGOT	2064	161	109	1903	1869	16	4	14
Iron	2065	136	57	1929	1482	192	72	183
Uric acid	2066	71	54	1995	1846	59	23	67
Cholesterol	2059	229	182	1830	1705	29	13	83
Total	31439	7236	5926	24203	22307	576	225	1095
Total as percent of grand total	—	23.0	18.8	77.0	71.0	1.8	0.7	3.5
Test normally requested as percentage of total					81.9			
Tests not normally requested as percent of total					92.2	2.4	0.9	4.5

a cost of 73 np (14/6d). If a larger unit having a higher output had been used, this cost could have been lowered even further.

In the wards studied there has not been any significant increase or decrease in patient bed-stay.

The introduction of profile analysis has reduced the routine laboratory workload, and the laboratory was not overloaded with additional secondary biochemical determinations such as glucose tolerance curves.

In respect to personnel, the project has been most successful, and the staff have enjoyed working in a laboratory that is almost completely automated.

Shortcomings in the design of the experiment have come to light, particularly in regard to the completion of the questionnaires. Investigation of patients is a dynamic, not a static process, and one that may result in diagnostic decisions at any time during, or even after, the patient's stay in hospital.

3. Biochemical Profile Analysis in a General Practice

Patients from one general practice were studied (C1). This was an urban group practice of about 10,000 patients cared for by four doctors, two of whom took part in the study.

Except in emergency, each practitioner follows up his own patients. The practice has open access to hospital laboratory and radiological services. The study was confined to adult patients attending the surgery, at which an appointment system was already in use. Before each surgery started the doctors knew the number of patients due to attend, normally from 15 to 30. By numbering these patients the doctors randomly selected four to be studied at each surgery. Two of the patients were allocated to a control group, while the other pair had 20 ml of blood taken by venipuncture after the doctor had described the purpose of the scheme. The blood serum was separated at the surgery by a part-time clerical helper using a centrifuge. The serum and 2 ml of blood in a sequestrene tube was placed in a special container and sent by first-class mail to the laboratory for multiple analysis. Specimens were normally analyzed the morning after venipuncture, except those collected on Friday; these were stored in the refrigerator for analysis the following Monday.

The general practitioners determined the Westergren sedimentation rates in their surgery. The remaining analyses were performed on single-channel AutoAnalyzers at the laboratory. The serum was analyzed for glucose, creatinine, urea, sodium, potassium, alkaline phosphatase, bilirubin, albumin, globulin, calcium, aspartate aminotransferase (SGOT), iron, uric acid, and cholesterol. Hemoglobin was measured on the sequestrene blood.

At the time of consultation the following details were recorded of all

patients in the test and control groups: (1) name, address, year of birth, and sex; (2) reason for consultation; (3) presumptive diagnosis, including any other conditions present but not responsible for the consultation; (4) presumptive course of action: (a) discharged, (b) follow-up by the general practitioner, or (c) referral to hospital; (5) discretionary investigations normally asked for; and (6) present drug therapy.

On return of the multiple blood test results, the test group was reviewed in order to see how many patients required further follow-up, blood tests, or referral to hospital. In addition, for every patient in the test group a questionnaire was completed by the general practitioners 3–6 months after the blood test. On this questionnaire any results marked by the laboratory staff as abnormal were placed in one of three categories: (1) an expected abnormal result, (2) an unexpected abnormal result leading to a new or additional diagnosis, or (3) an unexpected abnormal result, unexplained at the time of completing the questionnaire.

Nearly 600 patients have been studied, 296 in the test group and 293 in the control group. The action of the general practitioners at the time of consultation is shown in Table 3. In 35.1% of the control group and

TABLE 3
PROPOSED ACTION AT TIME OF CONSULTATION

	Control group	Profile group
Patients discharged	103 (35.1%)	59 (19.9%)
Patients followed up by practitioner	175 (59.8%)	229 (77.3%)
Patients referred to hospital	15 (5.1%)	8 (2.7%)
	293	296

19.9% of the test group the patients were discharged—that is, not asked to reattend; while 59.8% of the control group and 77.3% of the test group were asked to reattend the general practitioners. There was therefore a higher rate of reattendance in the test group.

Fifteen patients were referred to hospital from the control group and eight from the test group. The general practitioners' diagnoses on patients referred to hospital are shown in Table 4. Examination of the diagnoses of patients in the control group referred to hospital gives no indication that performing a blood profile could have made such a consultation unnecessary.

Of the 293 patients in the control group 33 (11.3%) had blood taken for biochemical or hematological tests within the profile group; these tests requested would represent 1.2% of the total tests if all the control group had full profiles performed. In the patients profiled the practitioners had

TABLE 4
DIAGNOSES OF PATIENTS REFERRED TO HOSPITAL

Control group	Profile group
Foreign body in finger	Before return of profile
Otitis media	Rheumatoid arthritis
For insertion of intrauterine coil	Brachial neuritis
Asthma	Chronic bronchitis
Chronic bronchitis	Varicose veins
Multiple sclerosis	Osteoarthritis
Cataracts	Inguinal hernia
Mitral stenosis	Polyneuritis
Lump in breast	? Acoustic neuroma
Carpal-tunnel syndrome	After return of profile
Lump in breast	Uremia (urea 309 mg/ml)
Fractured finger	Known diabetes mellitus (glucose 410 mg/100 ml
Carcinoma of lung	Known pyelonephritis (urea 175 mg/100 ml)
Sciatica	
Gastric ulcer	

noted that 3.9% of the total tests performed would normally have been requested.

On return of the profile results, 23 (39%) of the 59 patients originally discharged had to be recalled, 17 requiring further investigation.

Of the 229 patients asked to return to the surgery before the profile results were known, 64 required further investigation and 3 were referred to hospital (see Table 5).

Of the 4645 tests performed in the profile group, 4464 would not have been requested; 74 of these unrequested tests led to a new diagnosis in 50 patients—that is, in 16.9% of patients in the profile group. The diagnoses resulting from the unrequested tests and the range of hemoglobin in those found to be anemic are shown in Table 6. In addition to the data given in this table one patient, a man born in 1901, was

TABLE 5
ACTION ON RETURN OF PROFILE RESULTS

	Patients originally discharged	Patients already being followed up
Discharged	36	—
Follow up by general practitioner		
no tests	6	162
further tests	17	64
Referred to hospital	0	3
Total	59	229

TABLE 6
DIAGNOSES RESULTING FROM PROFILE TESTS

Iron-deficiency anemia (40 patients)						
Hemoglobin[a] (g/100 ml)	6–	7–	8–	9–	10–	11–
No. of patients	1	1	10	7	14	7
Diabetes mellitus (6 patients)						
Glucose level (mg/100 ml)	155	155	157	273	310	345
Year of birth of patient	1922	1926	1888	1886	1903	1891[b]

Renal Disease (4 patients)		
Creatinine (mg/100 ml)	Urea (mg/100 ml)	Year of birth of patient
1.0	51	1909
1.4	51	1898
1.9	72	1888
14.4	309	1891

[a] In three patients the hemoglobin was requested but the result was unexpected and diagnostic, and the serum iron was diagnostic and not requested. One woman with a hemoglobin of 12.5 g/100 ml and serum iron 50 μg/100 ml was diagnosed as a case of iron deficiency.

[b] This patient had unsuspected uremia.

found to have myxedema (cholesterol 358 mg/100 ml). In his case the follow-up protein-bound iodine was low. The general practitioner recorded a new diagnosis when the result was significant in management of the patient. A further 64 patients (21.6%) had an unexplained abnormal result which would not have been requested.

Unsuspected biochemical abnormalities such as hypercholesterolemia in 17 patients and hyperuricacidemia in four patients were recorded as unexplained abnormal results, not as diagnostic results, since treatment was not considered necessary for such patients. In 57 (19.3%) of the patients in the profile group the serum potassium levels were over 5 mEq/l; most of these were thought to result from hemolysis and were therefore marked as expected abnormalities.

With regard to the effect on treatment of the patients, the profile results led to 27 patients (9.1% of those profiled) having iron therapy. In some patients, drugs likely to cause intestinal bleeding were withdrawn or replaced with other drugs; one patient was found to have bleeding hemorrhoids, which were removed surgically. In the 14 patients in whom the hemoglobin has so far been repeated after therapy, the initial

hemoglobin results varied from 8.7 to 11.7, mean 10.4 g/100 ml, with rises after treatment of 1.0 to 6.7, mean 3.5 g/100 ml.

One patient with myxedema started thyroxine therapy, and five patients with diabetes mellitus were given diet with or without hypoglycemic agents.

After the profile results, three patients were referred to hospital; two were known to have diabetes or renal disease, but the biochemical results disclosed an unsuspected deterioration in their condition, and the third patient required inpatient treatment for renal failure. As a result of tests performed subsequent to the profile, a further two patients were referred to hospital, one, already mentioned, for hemorrhoidectomy and the other, a subject with back pain and a previous colectomy for carcinoma, for reassessment when found to have a persistently raised serum calcium.

The whole scheme worked well, including the bleeding of patients by the general practitioners, centrifuging and dispatch of specimens by the part-time clerical help, and return of reports by post. This confirms the possibility of performing such procedures on a larger scale. The main problem with the blood specimens was hemolysis, which resulted in nearly one-fifth of the patients in the profile group having serum potassium results over 5 mEq/l.

Unrequested results led to a new diagnosis in 16.9% of the patients in the profile group, many of whom required an alteration in treatment. Iron deficiency anemia was the major finding and, while the indication for screening for anemia in the general population remains uncertain (C2), all the patients found to be anemic in this series were attending the general practitioner with a variety of symptoms, and when reassessed after treatment all had a rise in hemoglobin. They should therefore be treated for anemia until treatment is shown to be of no benefit. Diabetes, renal disease, and one patient with myxedema accounted for the remaining diagnoses considered to be clinically significant; other unsuspected biochemical abnormalities such as raised serum cholesterol or uric acid were found, but these were not classified as a new diagnosis because treatment was not considered necessary.

There are many advantages in performing such tests in general practice rather than hospital. It is our experience that in hospital practice the finding of an abnormality like diabetes mellitus in a patient admitted for routine surgery can lead to delay in treatment of the surgical condition, not only inconveniencing the patient but also increasing the cost of hospital treatment. If such abnormalities were detected previous to hospital care, therapy might be given and so prevent any delay after ad-

mission to hospital. Further, results of certain tests may be considered irrelevant to the present diagnosis if such tests are performed in hospital, but subsequent results may prove the information valuable—for example, raised uric acid in a patient subsequently developing arthritis. Under present conditions results of such tests performed in hospital are not adequately conveyed to the general practitioner. If tests altered by hospital investigation, like protein-bound iodine, are added to the profile, then the earlier such a test is performed in the course of the investigation the less likely is it that a false result will be produced. Thus in a random survey of protein-bound iodine in 424 patients on admission to the Queen Elizabeth Hospital, 23 (5.4%) had high abnormal results due to recent radiological investigations.

In hospital or health screening clinics the problem arises of managing patients with abnormalities of doubtful significance detected on unsolicited tests. If such tests are performed under the auspices of the general practitioner, however, since he and his partners assume continuous care for such patients, a conservative policy can be adopted without the need for unnecessarily alarming the patient. The general practitioner should also know of any drug therapy the patient is receiving which may be important in certain tests.

Performing many tests on a specimen of blood taken at one point in time has the advantage that if an abnormality is detected other results may help to exclude or confirm the presence of certain diseases and therefore save the need for follow-up investigations. Thus, if a raised cholesterol is found, the profile can be examined for the presence of diabetes mellitus, the nephrotic syndrome, or liver disease.

Normal results are of considerable value; they may reassure the doctor or patient that certain diseases are not present. Thus in an anemic patient a normal urea should exclude uremia as the cause of anemia. The normal results may also be used to assess the suitability of patients for various treatments such as hepatotoxic drugs and certain forms of surgery. In later years previous results may be useful as a baseline if disease subsequently develops.

4. Unexplained Abnormal Results

It is evident from the foregoing description of profile investigation of patients at various stages in medical care that this type of investigation combines screening with grouped analysis and preliminary discretionary tests in such a way that they can be performed at relatively low cost. The tests we now use are only the beginning of such methods of investigation of patients. The introduction of enzyme, protein, and polypeptide profiles within the next few years will considerably improve the con-

tribution of clinical chemistry laboratories to unsuspected diagnoses. However, the present challenge is not that of unsuspected diagnoses or presymptomatic diagnoses, but the area of unsuspected, unexplained abnormal results. If further developments in profile analysis is advocated with the area of ignorance that surrounds our current approaches, then clinical chemistry will be performing a disservice to the patients.

The problem of the unsuspected, unexplained abnormal result must be solved. It is postulated that there are four reasons for the large number of unexplained abnormal results found in our original surveys.

4.1. Lack of Understanding of What Are Normal Values

If a normal biological population is surveyed for sixteen independent factors and each factor is a normally distributed (Gaussian) factor, then, describing any result outside ± 2 SD of the mean as abnormal will result in 40% of the population having at least one abnormal result.

This was not the major reason for our high finding of unexplained abnormal results. Many of the determinations were not normally distributed, and in general a much greater than ± 2 SD range was used. In addition, there was a high number of abnormalities in the older age groups, sometimes in one of the sexes. It is true that many of these abnormalities were just outside our declared normal range, as in Table 1. Detailed examination of the results, when divided according to age and sex, indicated how little we understood changes in the so-called normal values with these two factors. This could be illustrated with urea, glucose, albumin, calcium, uric acid, creatinine, alkaline phosphatase, bilirubin, cholesterol, potassium. All results from patients varied with age, some with age and sex. The vast majority of the abnormal results were only just outside the so-called normal range. This point will be illustrated with the determination of serum alkaline phosphatase.

A result of less than 15 units/100 ml was regarded at the start of this survey as being a normal result. If we accept, for the purposes of argument, that this is so and in addition accept any result greater than 20 units/100 ml as indisputably abnormal, then we are left with a "borderline" group between 15 and 19 units/100 ml inclusive. Table 7 shows how the number of such results increases with age, particularly in the female. Table 8 lists the age of patients by decade in our survey. It will be seen that we dealt with patients the majority of whom were over 50 years of age. It is a disturbing fact that few normal surveys in clinical chemistry contain results on subjects above 50 years of age, and many survey only males. In fact, the basis of many quoted normal values is a survey of male medical students usually at the beginning of their third decade of life. To interpret changes from such values in both sexes and

TABLE 7
ALKALINE PHOSPHATASE RESULTS ACCORDING TO AGE GROUP AND SEX OF THE PATIENT

Age group (years inclusive)	Female[a]			Male[a]			Combined[a]		
	A	B	C	A	B	C	A	B	C
20–29	95	3	2	95	4	1	95	3	2
30–39	90	6	4	86	13	1	89	9	2
40–49	91	6	3	90	7	3	87	7	6
50–59	83	11	6	89	7	4	87	8	5
60–69	75	16	9	85	10	4	82	12	6
Over 70	74	14	12	80	13	7	77	14	9

[a] A, Percentage of patients in the age group with alkaline phosphatase lower than 15 units; B, percentage of patients with values 15–20 units inclusive; C, percentage of patients with results greater than 20 units.

at all ages of patients as abnormal changes due to a disease state is akin to assuming that gray hair is indicative of disease, because it rarely occurs in male medical students. Alternatively how can we be convinced that the rise in the number of patients in the borderline group is not due to disease? There are two relevent comments on such a possibility. First, if this is so, and we are really detecting true abnormality or disease in our population, then it occurs in approximately 1 in 3 of our older age groups, particularly in females, and thus the alkaline phosphatase is a very nonspecific test in such groups. Second, and probably the most important comment, if such a situation exists then surely some patients would remain in the nondiseased group. This does not happen; after the menopause the whole distribution is shifted to a higher mean with a very

TABLE 8
AGE DISTRIBUTION OF PATIENTS IN THE PROFILE SURVEY

Age group (years inclusive)	Females		Males		Combined	
	No.	%	No.	%	No.	%
10–19	61	2.0	82	2.6	143	4.6
20–29	146	4.9	132	4.4	278	9.3
30–39	163	5.4	146	4.9	309	10.3
40–49	226	7.6	273	9.1	499	16.7
50–59	262	8.8	458	15.3	720	24.1
60–69	228	7.6	459	15.3	687	22.8
Over 70	138	4.6	215	7.2	353	11.8

similar standard deviation. We postulate that this is a physiological change with age, not a general disease state in the population.

More detailed information on such changes will be published elsewhere; suffice it to say at this time that our knowledge of changes in "normal values" with age and sex is meager, and the usual approach is naive.

4.2. Laboratory Error

The second reason for unexpected, unexplained abnormal results is laboratory error. There are two types: the random error and the systematic error.

The random error may be caused by the incorrect labeling of a specimen with reference to the patient's identity; the accidental switching of specimens within the laboratory; clerical error at any stage of the process of reception, analysis, or reporting. It is difficult to quantitate such errors, but all reasonable attempts at assessment indicate that such errors occur much more frequently than laboratory heads admit. Figures of 5% have been quoted and are too high. Figures of 1% are much more realistic but may be too low.

Systematic error can be more easily assessed and is certainly responsible for the movement of large numbers of results from the normal to abnormal range.

Much has been written about the quality of laboratory results and their control. Even in the most efficient and well run laboratories, systematic errors do occur, and these may carry whole groups of patients from the so-called normal to the abnormal range. Two examples will be quoted from our own laboratory.

Example 1. The daily mean value of all results for a particular determination often contains useful information on drifts in precision. Such techniques of quality control have been discussed in detail elsewhere (W3). Some years ago such statistical analyses of daily means of all potassium results on patients with a urea of less than 80 mg/100 ml indicated that on Saturday mornings the mean was frequently below 4.3 mEq/l, whereas during the week it rarely fell below 4.4 mEq/l. It required considerable investigation to explain this phenomenon. The results on Saturday morning were correct, it was during the weekdays that the results were too high. A centrifuge was overheating and blood being separated using the centrifuge was heated thus driving the potassium out of the cells into the serum. On Saturday morning, because technicians wished to have a half day's break, the blood was centrifuged for shorter periods, thus reducing the effect.

Example 2. Sera left overnight in plastic AutoAnalyzer sample cups

may give low calcium values. This is due to absorption of calcium onto the walls of the plastic cup (W2). The absorption is brought about by a rise in the pH of the serum. The phenomenon is of practical importance because as much as 10% of the calcium in the serum may be absorbed.

The poor precision of some hospital departments of clinical chemistry has been described in many national and international surveys. Lack of precision may lead to characterization of a normal result as abnormal. Such lack of precision may be due to lack of scientific ability on the part of those obtaining the result, but it must be emphasized that even in the best of laboratories such "drifts" in precision do occur. A clear and true indication of laboratory precision is important in all patient investigations; it is even more important when unsolicited information is being provided.

4.3. Lack of Understanding of Biochemical Changes in Disease

We have found that the use of profile analysis has brought to the investigation of patients a maturity that could not be accomplished by the simple discretionary use of investigations. The discretionary use of serum uric acid determination following a tentative diagnosis of gout has, in our experience, led to a poor understanding of the changes of serum uric acid in disease. In the past we have restricted serum uric acid investigations to those patients with suspected gout, providing clinicians with "normal values" determined by another laboratory by an unspecified method. Only the determination of uric acid on all patients entering the hospital led to a clear understanding of what was "normal" and of the uric acid changes in many diseases other than gout.

By analogy the discretionary situation was akin to restricting clinicians to abdominal examination of patients only when abdominal abnormalities were suspected and depending upon others to describe the "feel" of the normal abdomen. Only when a clinician has felt the abdomens of patients with and without abdominal abnormalities does his maturity as a clinician develop. Similarly the provision of profile analysis has been of considerable benefit to a much wiser understanding of biochemical changes in disease.

Our work has indicated a number of areas of biochemical investigation where profile analysis has been responsible for increased understanding. This will be the subject of a separate communication.

4.4. Detection of Presymptomatic Disease

Inevitably, in a small number of patients, we detected biochemical abnormalities that could not immediately be associated with clinical

symptoms, but were later associated with a clinical diagnosis. Examples are the discovery of high alkaline phosphatase levels in a patient who later presented with carcinoma of the liver, high serum transferase values in patients later presenting with hepatitis, patients with abnormal liver functions who later showed reactions to drugs such as chlorpromazine, high urea values in a patient taking phenacetin but with no renal symptoms. Such results were frequently characterized by our clinical colleagues as "unexpected, unexplained, abnormal result."

5. Conclusion

Profile analysis is now an accepted part of clinical chemical investigation. The performance of tests on patients without clinical indication is a form of screening which is acceptable to the patient, who almost inevitably has to have a blood test. Such investigations lead to a number of patients having new or additional diagnoses, many of which lead to an alteration of treatment.

The performance of such investigations as early in patient care as possible is strongly advocated.

This method of investigation has been started at a time when there is considerable ignorance regarding normal ranges, precision of analysis, biochemical changes in a wide spectrum of disease, and biochemical changes indicating presymptomatic disease. A plea is made for increased understanding in these areas before profile analysis is extended further.

By the performance of profile analysis on patients we can add to our knowledge of chemical changes in disease. Profile analysis is a challenge to clinical chemistry, not a soporific to further thought.

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BIOCHEMICAL ASPECTS OF MUSCLE DISEASE

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1. Diseases Affecting Muscles

Disease of the voluntary muscles can result from a very large variety of different causes. A comprehensive classification of the neuromuscular disorders was drawn up by the Research Group on Neuromuscular Diseases of the World Federation of Neurology a few years ago, and an internationally agreed upon version was published in 1968 (W3). In this classification muscle diseases are divided and subdivided according to their etiology. Many are very rare and have not yet been the subject of biochemical investigations. The major biochemical efforts have been directed toward the genetically determined myopathies, especially the muscular dystrophies and, to a lesser degree, the myotonic disorders and some of the more common muscle disorders of established neurogenic origin.

1.1. The Muscular Dystrophies

Case reports of conditions, which today would be denoted as muscular dystrophies, have appeared for over a hundred years, but only comparatively recently has a satisfactory classification of the different varieties been achieved, based on both clinical and genetic criteria. The basic classification of Walton and Nattrass (W6) with some subsequent modifications is widely accepted. However, occasional cases occur that do not fit exactly into the existing scheme, therefore minor modifications may still be required [see Walton and Gardner-Medwin (W5)]. It hardly requires emphasizing that in biochemical investigations on muscular dystrophy the clinical type of the case under study should be known and specified in the reported findings, since there may be important biochemical differences between the varieties. Unfortunately, this has not been the case in a few reports in the literature.

The most commonly investigated type of muscular dystrophy is the Duchenne type, a severe X-linked form, which appears in boys during the first few years of life, progresses relentlessly, and leads to death in the second or third decade. There are two other main varieties which have been often studied; both are generally less severe than the Duchenne type, afflict both sexes, and usually commence at a later age. These are limb-girdle muscular dystrophy, transmitted as an autosomal recessive character, and the fascioscapulohumeral type, which has usually an autosomal dominant inheritance. These and the other forms are discussed fully by Walton and Gardner-Medwin (W5). Special mention, however, should be made of myotonic dystrophy, the disorder involving muscular atrophy accompanied by myotonia; the latter term denotes the continued active contraction of a muscle persisting after the cessation of voluntary effort or stimulation. In myotonic dystrophy the muscle involvement is

accompanied by many other bodily changes, and the biochemical disturbances are probably widespread. In addition, the myotonic phenomenon itself may have a predominantly biochemical basis.

1.2. Inherited Animal Myopathies

Although numerous changes in the chemical constituents of blood and urine have been observed in patients with muscle diseases (see below), the biochemical abnormalities can be adequately studied only by direct investigation of the diseased muscle. Inevitably, the procuring of a sufficient number of samples of biopsied muscle from patients and suitable controls is often difficult and is a serious impediment to research along these lines; many workers, indeed, find it virtually impossible to obtain such material. Moreover, the tissue donated is often from relatively advanced cases, where the degree of muscular degeneration is so extreme as to mask the earlier and more meaningful changes. The recognition of Duchenne type muscular dystrophy in the very early stages by serum enzyme changes is of some help in this respect, but the provision of adequate quantities of suitable muscle is still a difficulty. Moreover, the ability to detect many female carriers of Duchenne dystrophy, which is a valuable aid to genetic counseling, must eventually influence this situation. The application of ultramicro methods of analysis to muscle samples (M10) would assist in obtaining the most information from available material, but these have not been widely used.

There exist, however, a number of genetic myopathies in animal species [for a full account, see Bajusz (B2)] which can be much more easily used as subjects for investigation of muscular disease, and the majority of workers have turned their attention to these. It cannot be assumed that the fundamental genetic effect in any of these is identical with that of any of the human muscular dystrophies; often, in fact, the mode of inheritance and many features of the disease suggest otherwise. Nevertheless, it is now obvious that muscle degeneration resulting from various causes may show many similar features, and the study of animal myopathies provides information on such common features of muscle pathology. Such information may ultimately be useful in helping to control the process of muscle degeneration. It is also possible, of course, that the animal studies may provide valuable clues as to the nature of the underlying defect in the human muscular dystrophies.

The best known of these animal diseases is the hereditary myopathy of the mouse, which appeared in a strain 129/Re at the Jackson Laboratory, Bar Harbor, Maine, and is described by Michelson *et al.* (M15). It is characterized by progressive weakness (most evident in the hind legs), atrophy, and a reduced life-span; the affected animals have not only a reduced muscle mass, but also a smaller skeletal size than normal. In-

heritance is by an autosomal recessive mechanism, and the affliction can usually be recognized at 2–3 weeks of age. A practical disadvantage in the use of this strain is that the dystrophic animals are unable to breed normally. Colonies may be maintained by mating of heterozygotes but, since these are not distinguishable from normal homozygotes except by the production of dystrophic offspring, this is a wasteful procedure unless the surplus of phenotypically normal mice can be utilized. The difficulty may be overcome by transplantation of ovaries from dystrophic into normal females (J4). Recently, however, a report (M14) has appeared of inherited muscular dystrophy in another strain of mice (WK/ReJ), which has a later onset and slower progression and in which affected males and females can breed.

An inherited muscular dystrophy in the chicken, discovered by Asmundson and Julian in 1956 (A4), is also transmitted as an autosomal recessive characteristic. Affected birds are unable to raise their wings or to rise when placed on their backs on a flat surface. Egg production is, however, normal, making it a relatively easy matter to breed a dystrophic line. The chicken, therefore, has been particularly useful in studying the development of myopathy and the relationships of pathological and developmental changes in muscle. It is of interest that, in contrast with the mouse disease, a number of morphological and biochemical abnormalities can be seen in the heterozygotes in this species.

A hereditary myopathy, accompanied by cardiomyopathy, in an inbred line of Syrian hamsters, also autosomal recessive, was first described in 1962 (H7). Although the hamster has not been used as widely as the mouse and chicken, some workers have preferred the dystrophic hamster to the mouse on account of its larger size, which is particularly useful where relatively large amounts of muscle are required for biochemical studies.

The White Pekin duck may carry an inherited myopathy (R2), but this appears to be less well characterized and not as commonly used in the study of muscle disease. Further instances have been reported in domestic turkeys and in lambs (B8).

The phenomenon of myotonia can be studied in a hereditary disease in goats, known for several decades in the United States. This condition is most comparable to congenital myotonia or Thomsen's disease in man, although they differ in some respects.

1.3. Experimentally Induced Muscle Atrophy

Among the many possible consequences of a dietary deficiency of vitamin E, there is observed in many species a profound atrophy of the skeletal muscles with corresponding weakness. This nutritional muscular

dystrophy, which was first observed in the rat, has been frequently studied also in the rabbit, guinea pig, chicken, and many other species, including farm animals; the precise nature of the pathological changes may vary with the species. In the more susceptible species, such as rabbits, muscular dystrophy can be induced within about a month by a vitamin E-deficient diet. Nutritional muscular dystrophy has been studied widely as a model of muscle pathology, particularly before the introduction of the hereditary animal myopathies. The exact causal relationship between the lack of vitamin E and the muscular atrophy is still not clear. There are also complex dietary relationships between vitamin E and other nutrients, such as selenium and the sulfur-containing amino acids. Under certain conditions, some of the consequences of the deficiency of vitamin E can be reversed by other antioxidants, but it is not generally accepted that the biological activity of vitamin E is solely a consequence of its antioxidant properties. The similarities between nutritional muscular dystrophy and human muscular dystrophies has frequently led to the speculation that there may be a disturbance in metabolic pathways involving vitamin E in the latter, but there is no good evidence for such a defect.

It has been known for a hundred years or more that a muscle will atrophy if its motor nerve is damaged. This procedure has provided a relatively easy means of inducing muscle wasting, and countless morphological and chemical studies have been carried out on muscles after section or crushing of the corresponding nerve. A valuable review of this subject was provided by Gutmann (G17). There is normally a rapid loss in muscle weight after the nerve is cut, and a progressive decrease in the diameter of the individual fibers, and more-widespread changes may be seen after a longer period of denervation (D7). An initial transitory hypertrophy occurs in the denervated diaphragm muscle. The precise mechanism by which the nerve normally maintains the muscle fibers in a healthy state is still not fully understood. In particular, it is a matter of controversy whether this is mediated by the nerve impulse and acetylcholine release or whether additional, so-called "trophic," factors (K12) are involved. Experimentally denervated muscle can be expected to be related most closely to the various neurogenic muscle diseases among the human conditions, although the sudden and complete severance of the link between nerve and muscle is not strictly comparable with the relatively slow denervation process normally found in the human diseases. Denervation is less suitable as a model for the so-called primary myopathies, although, as discussed below, the involvement of nerve lesions in these conditions cannot be excluded and, indeed, is strongly postulated by some workers.

One difficulty encountered in the study of chemical changes in denervated muscle is the choice of a suitable control muscle for comparative purposes, discussed, for instance, by Goldspink *et al.* (G8). The use of the unoperated contralateral limb muscle as the control, as is often the case, is open to certain objections, and sham-operated animals probably provide the most satisfactory solution of this problem.

It is well known that if a muscle is immobilized by one of the various means (limb splint, isolation of a spinal segment, or cutting of a tendon), atrophy will occur although the motor nerve is intact. Such disuse or underuse atrophy has been often studied; the identity or otherwise of the changes with those of denervation has been discussed [e.g., see Brooks (B12) and Klinkerfuss and Haugh (K8)].

2. Morphological Changes in Diseased Muscle

2.1. Other Cell Types

All quantitative chemical and metabolic studies on samples of diseased muscle must take into account that any piece of muscle tissue will contain not only muscle fibers, but also connective and adipose tissue, nerve and blood vessels. Changes in the relative amounts of these components in pathological specimens may be important in two respects. Gross increases in the amounts of fat or of connective tissue are frequently seen in samples of diseased muscle, leading to a marked reduction in the proportion of true muscle tissue in the samples. It would, therefore, be misleading to express the quantities of the muscle fiber constituents as a proportion of the total wet or dry weight of the muscle, and a reference base must be used which reflects, at least approximately, the amount of true muscle tissue. A technically simple and reasonably satisfactory solution to this problem is to use as reference base the nitrogen of the muscle which will dissolve in dilute alkali at room temperature (L2). Such "noncollagen nitrogen" includes virtually all the nitrogen of the muscle fibers but excludes the alkali-insoluble collagen and elastin of connective tissue. In normal muscles "noncollagen nitrogen" is usually well over 90% of the total nitrogen, but may fall to less than one-half in muscles in an advanced state of degeneration. This reference base has been used by many workers in this field, but others have used less satisfactory criteria.

Second, it must be borne in mind that any observed increase in an enzyme activity or other feature in diseased muscle could result from a proliferation of nonmuscle tissue associated with the muscle. In particular, one must consider the possible contribution of metabolically active fat and connective tissue cells and the presence of invading macrophages. These are a characteristic feature of many kinds of diseased muscle and are rich in many enzymes. Examples are mentioned below

where the contribution of these various tissue elements may be particularly suspected. The complications due to these factors may generally be minimized by confining the studies to muscle taken in an early stage of disease, when such secondary morphological changes are relatively smaller.

2.2. Fiber Regeneration

There is well established evidence from both light and electron microscopy that damage or disease of muscle may often be accompanied by attempts on the part of the tissue to form new muscle fibers. Such regeneration can be demonstrated, for example, by crush injury of a muscle, and Walton and Adams (W4) showed that human dystrophic muscle had not lost the ability to regenerate under these circumstances. It is commonly accepted that signs of regeneration occur during the progress of human muscular dystrophy, although it appears that its occurrence and efficiency become less common as the disease develops (M5). Regeneration is a predominant feature in polymyositis (M6) and may proceed to completion. It has been described in mice with hereditary dystrophy (R8), but is not, however, normally seen in denervated muscle.

The various steps involved in regeneration are probably similar to the stages of myogenesis observed during the growth of muscle in tissue culture. In the latter process (R8) single-celled myoblasts multiply by mitosis and fuse to form myotubes, which eventually give rise to the mature fibers. There is some doubt concerning the origin of the myoblasts which appear in degenerating muscle. The weight of evidence suggests that they originate from the degenerating muscle, and it was widely believed that they were formed by the accumulation of cytoplasm around the nuclei from the degenerating fibers. It now seems a likely possibility, however, that they originate from the so-called satellite cells; these are individual mononucleate cells which lie within the basement membrane of the muscle fibers.

There is much evidence that the metabolic patterns of such immature muscle cells differ from those of mature fibers. The presence of substantial numbers of regenerating fibers in diseased muscle could therefore be responsible for biochemical changes in such muscle. This again emphasizes the importance of considering all biochemical changes within the framework of the morphological alterations.

3. Muscle Fiber Types

Any assessment of metabolic changes in diseased muscle must also take into consideration the fact that all the fibers, even within a single muscle, may not have the same metabolic pattern. Muscles have long been

classified as "red" or "white" on the basis of their color, and these were recognized to contain different types of fiber. It is now clear that most muscles contain a mixture of fiber types, the color of the muscle being determined by the predominating fiber type. Histochemical studies have revealed numerous differences between the different types of fiber. Mostly, two types have been distinguished, so-called red (type I) and white (type II) fibers, in a variety of muscles and species. The type I fibers are generally smaller and tend to be rich in oxidative enzymes such as succinate dehydrogenase and cytochrome oxidase and in myoglobin; mitochondria and fat droplets are more numerous, and the fibers are evidently geared to a highly oxidative pattern of energy production. Enzymes of the glycolytic pathway and certain associated enzymes, on the other hand, have relatively low activity in type I fibers. Type II fibers are generally larger in diameter and relatively poor in oxidative enzymes, but have a high level of enzymes concerned in glycolysis. Detailed quantitative studies on enzymes in typical red muscles, such as the soleus, and white muscles, such as the extensor digitorum longus, have recently been published (B6, G7). Myosin from white muscle was shown to have a higher adenosine triphosphatase activity than that from red muscle (S13), and other differences between the two myosins have since been reported (K15). The higher adenosine triphosphatase activity of white muscle myosin is the possible reason why white muscles are, in general, "fast" muscles, whereas red muscles are "slow." The calcium-binding activity of the sarcoplasmic reticulum, which is responsible for the relaxation of contracted muscle, is also higher in white muscle (H2).

The existence of different fiber types in human muscle was clearly demonstrated by Dubowitz and Pearse (D16).

It is now generally accepted, however, that a classification into two fiber types is an oversimplification. Stein and Padykula (S21) distinguished a third, intermediate fiber type in rat muscles, and the existence of such a type is now recognized by many workers. Other classifications have been proposed; thus Romanul (R4), on the basis of phosphorylase activity, divided fibers into eight types which were grouped into three main classes.

The developmental aspects of the differentiation of fiber types has been studied by a number of workers. It has become clear that at an early age all or most muscles tend to resemble more closely red muscles, both in their speed of contraction and in their metabolic pattern. Bass and co-workers (B5) have recently detailed the developmental changes in glycolytic and oxidative enzymes in red and white muscle in the chicken; their results illustrate the much greater changes which occur in white muscle.

It has become evident from much recent work that differences between these two general groups of muscle are very dependent upon the nature of the nerves which supply them. A valuable review of the influence of nerve upon the properties of muscle is provided by Guth (G15). Clear evidence for the neuronal regulation of specific muscle characteristics was reported by Buller and co-workers (B16), who showed that after cross union of nerves to slow and fast mammalian muscles, the fast muscles have a slower contraction and the contraction time of the slow muscles is shortened. Later, Drahota and Gutmann (D8) showed that biochemical properties of red and white muscle could be influenced in a similar manner by cross-innervation. They found that the glycogen and potassium content of fast muscles is higher than that of slow muscles, and that after reinnervation of a slow muscle (soleus) with nerve normally supplying a fast muscle there was an increase in these constituents in the soleus muscle. Marked reversal of enzyme activities in the fibers of soleus (slow) and flexor hallucis longus (fast) muscles following cross-innervation was demonstrated histochemically by Romanul and Van der Meulen (R6) and by Dubowitz and Newman (D15). Quantitative enzyme studies on the cross-innervated soleus (G9) have given similar results. Comparable changes in other biochemical characteristics of muscle after cross-innervation have recently been described by a number of authors (G16, M17, P23). Of particular interest are the qualitative changes in myosin observed by Samaha *et al.* (S2). It is thus clear that the nature of the nerve supply exerts a profound and complex regulatory effect upon the protein synthetic machinery of the muscle fibers, although the mechanism of this influence is not yet understood.

The existence of multiple fiber types in skeletal muscle raises the possibility of their differential involvement in muscle disease; in particular, it is of interest to know whether either of the two main types is preferentially affected. This question, as it concerns human neuromuscular diseases, is discussed at length by Engel in a recent review (E7). In Duchenne dystrophy, atrophy appears to be relatively nonselective. In muscular dystrophy in the mouse, Brust (B15) observed that a slow muscle (soleus) was less affected than the fast gastrocnemius, although Susheela *et al.* (S26) noted greater atrophy in type I fibers. After denervation there is a more rapid atrophy of type II fibers (B1), but Romanul and Hogan (R5) found that each type of fiber showed a more rapid decrease in the activities of the enzymes which were normally higher in that particular fiber type; this was shown to be true of some but not all enzymes by Goldspink *et al.* (G8). In disuse atrophy of muscle, Klinkerfuss and Haugh (K8) found a decrease in size of fibers rich in oxidative enzymes, but no significant change in fiber diameter of

phosphorylase-rich fibers over the period of observation. Atrophy of skeletal muscle in patients with Cushing's syndrome is more marked in type II fibers (P20).

4. Contractile Proteins in Diseased Muscle

The proteins which constitute the contractile machinery of the muscle fibers account for 50–60% of the total muscle protein (H4). It is now known, particularly from the work of H. E. Huxley and colleagues, that the contractile units, the myofibrils, are made up of two sets of filaments: thick filaments consisting of myosin and thin filaments of actin. Contraction results from the sliding of thin filaments between thick ones. Tropomyosin and troponin appear to be associated with the actin filaments and are concerned in the regulation of contraction (E1). A number of minor myofibrillar proteins have also been described. Apart from myosin, however, little attention has been given to possible changes in myofibrillar proteins in muscle diseases. Vignos and Lefkowitz (V3) demonstrated that there was a decrease, relative to noncollagen nitrogen, of the amount of myosin in human dystrophic muscle; the change was more pronounced in the adult than in the childhood form of muscular dystrophy. Studies on possible changes in the nature of myosin in diseased muscle have not produced a consistent picture. Thus Smoller and Fineberg (S11) reported differences between the proportions of many amino acids in myosin isolated from normal and dystrophic mouse muscle. In addition, fewer sulfhydryl groups in the abnormal myosin were capable of reacting with *p*-chloromercuribenzoate and *N*-ethylmaleimide, and the protein showed a greater tendency to aggregate. Oppenheimer *et al.* (O3), also working with mice, found that myosin from normal and dystrophic mouse had a similar ultracentrifuge pattern, but light-scattering seemed to indicate a greater degree of polydispersity in the myosin from the dystrophic mice; however, myosin ATPase and the ability to combine with actin were normal. Other workers (M19) have been unable to detect any difference between myosins from normal and dystrophic chick muscle.

Two rare diseases, "central core disease" (S6) and "nemaline myopathy" (S7) are said, from microscopic evidence, to affect the myofibrils, but little work has been done on the biochemistry of these conditions.

5. Energy Metabolism

Muscle tissue can readily metabolize both carbohydrates and fat, the breakdown of the latter being particularly important in the resting state.

The enzymes involved in energy release and transfer are generally among the most abundant and most easily measured [see Czok and Bücher (C3)]. In view of this and of the importance of an adequate energy supply for contraction and many metabolic activities, it is not surprising that many workers have looked for possible changes in energy metabolism in diseased muscle.

5.1. Glycolysis

The first study of the glycolytic pathway in dystrophic muscle was carried out by Dreyfus *et al.* (D10); specimens of abdominal muscle from patients were compared with the corresponding normal muscle, obtained during appendectomies. The overall rate of glycolysis (referred to noncollagen protein) was low, being as little as 20% of normal in advanced cases. Assays of individual enzymes, notably α-glucan phosphorylase, phosphoglucomutase, and aldolase, revealed low activities. Later investigators have demonstrated decreases in other glycolytic enzymes (H6, K6); these changes were summarized by Pennington (P8). Vignos and Lefkowitz (V3) reported, however, that the rate of glycolysis is essentially normal in the adult forms of muscular dystrophy. The above-mentioned alterations in glycolytic enzymes, nevertheless, are not a specific characteristic of Duchenne muscular dystrophy, since comparable changes are observed in neurogenic muscle disease (D5) although the loss of phosphorylase occurred earlier and was more marked in muscular dystrophy. There is a marked decrease in aldolase activity in denervated rat muscle (G13). In the mouse dystrophy, glycolysis was reported to be normal (M8); aldolase is actually elevated in older dystrophic mice (S17).

Considerable interest was aroused by the finding of Wieme and Lauryssens (W16) in 1962 that there is a change in the electrophoretic isoenzyme pattern of lactate dehydrogenase in diseased human muscle. The major isoenzyme of lactate dehydrogenase in most normal muscles moves slowest on electrophoresis (LDH 5), but in myopathic muscle the proportion of LDH 5 may be considerably reduced. This finding has been confirmed and extended by numerous workers, utilizing various techniques for isoenzyme differentiation (e.g., B10, E5). The abnormal pattern is seen in most, but not all, cases of Duchenne dystrophy and in a variety of other muscular disorders. It may be evident in the very early stages of Duchenne dystrophy (P2) and is seen even in some female carriers of the disease (E3).

It is generally accepted (L1) that the slower moving isoenzymes of lactate dehydrogenase are more important in anaerobic glycolysis, and

white muscles tend to contain a higher proportion of these than do red muscles. Hence the changes in LDH isoenzyme pattern in diseased muscle may be considered together with the fall in other glycolytic enzymes.

As first pointed out by Dreyfus *et al.* (D11), the abnormal LDH isoenzyme pattern in diseased muscle resembles that of normal fetal muscle. In fact, in dystrophic chickens the normal adult isoenzyme pattern is never attained in the affected birds (D3), and possibly this is true of some human muscle diseases. An increase in the specific activity of many other glycolytic enzymes during development has been reported (B5, K3).

In seeking for an explanation of the decline in the amounts of glycolytic enzymes in diseased muscle, one possible causative factor is the diminished activity of the muscles. It has been shown by Dawson and Kaplan (D3) and by Kendrick-Jones and Perry (K3) that the rate of increase in certain muscle enzymes during development is influenced by the use of the muscles; the latter workers also showed (K2) that exercise or stimulation can increase the concentration of some muscle enzymes. However, it does not seem likely that muscular activity is the sole explanation for the enhancement in glycolytic enzymes during development or its lack for their decline in disease; thus Mann and Salafsky (M1) have recently reported that the characteristically high levels of glycolytic enzymes in white muscle will develop even when the muscle is totally immobilized. The possible regulatory role of (as yet hypothetical) "trophic" factors transmitted by the nerve has still to be investigated.

The isoenzyme composition of muscle aldolase also shows a change in the direction of the fetal pattern after denervation of the muscle (S4).

It is not intended to discuss in this review the conditions involving well-recognized defects in enzymes concerned in muscle glycogen metabolism, which have been dealt with adequately in a previous volume (S22). A recent addition to this category, however, is described by Hug *et al.* (H13), who were unable to find any detectable activity of the cyclic 3′,5′-AMP dependent phosphorylase kinase kinase in the muscle of a girl patient.

5.2. Respiration and Oxidative Phosphorylation

In contrast with the fall in the activity of muscle glycolytic enzymes in human muscular dystrophy, Dreyfus and his colleagues (D10) found little or no decrease in the concentrations of certain enzymes involved in oxidative breakdown of fuel, notably succinate dehydrogenase, cytochrome oxidase, fumarase, and aconitase. In the mouse myopathy, the concentration of cytochrome oxidase is increased (W12); elevated levels of respiratory enzymes have been reported also in myopathy resulting from vitamin E deficiency (D6) and in genetically dystrophic chickens

(M2). Pennington (P5) found a similar activity of succinate-tetrazolium reductase in mitochondria from normal and dystrophic mouse muscle. Succinate dehydrogenase activity has been recently reported to be normal in muscle from patients with dystrophia myotonia (B7). Muscle mitochondria from hamsters in the late stages of hereditary muscular dystrophy have a low rate of oxygen uptake with pyruvate and fumarate as substrates, but not with succinate (J2).

The relative stability of oxidative enzyme concentrations in myopathic muscles may be viewed in relation to the observations that these enzymes show comparatively small changes in development. As mentioned previously, mature slow muscle, in which these enzymes predominate, more closely resembles immature muscle than does fast muscle. In fast muscles, a decrease in the concentration of certain respiratory enzymes may occur during growth (B5).

Arising from earlier observations (V4) that the ATP:ADP ratio is lower in skeletal muscle from patients with Duchenne muscular dystrophy than in normal human skeletal muscle, Olson and co-workers (O1) explored the possibility that this inability to maintain high energy phosphate concentrations might be due to a defect in oxidative phosphorylation. However, mitochondria from Duchenne or limb-girdle patients, utilizing glutamate as substrate, showed normal P:O ratios. Recently, in more extended studies [Ionasescu *et al.* (I2)], the normal P:O ratio in Duchenne dystrophy was confirmed, but a significantly low respiratory control index was observed, signifying "loose-coupling" of oxidative phosphorylation. Similar findings were recorded in severe amyotrophic lateral sclerosis, but in other clinical forms of muscular dystrophy and in amyotrophic lateral sclerosis with mild muscle lesions there was evidence for decreased P:O ratio but normal respiratory control. The latter authors, however, used whole homogenates rather than isolated mitochondria in their studies: this may induce complications, as pointed out by Peter *et al.* (P17), who have discussed the difficulties inherent in these measurements.

Oxidative phosphorylation was found to be normal in muscle mitochondria from dystrophic mice (W18), but low P:O ratios have been reported in muscular dystrophy in the Syrian hamster (L6).

An interesting hypermetabolic myopathy was discovered and biochemically explored by Luft *et al.* (L7). There was no evidence of hyperthyroidism, and mitochondria from biopsied muscle had a high rate of respiration and a loosely coupled state of oxidative phosphorylation. A few other cases of unusual myopathies with loosely coupled mitochondria have since been described (e.g., S12), although it does not seem that this is a single disease entity.

5.3. Pentose Phosphate Pathway

Although it is doubtful whether the chief function of the pentose phosphate pathway of glucose oxidation is the provision of energy in the cell, it will be convenient to consider it at this point. McCaman (M10) observed that the activity of glucose-6-phosphate dehydrogenase, the first enzyme of this pathway, is enhanced in dystrophic mouse muscle; subsequently (M11), this was shown to be true also of experimentally denervated muscle. Heyck *et al.* (H6) demonstrated an increase in this enzyme and also in 6-phosphogluconate dehydrogenase, the second enzyme of the pathway, in human dystrophic muscle. The biological significance of this relatively nonspecific change is not clear. Garcia-Bunuel and Garcia-Bunuel (G1) observed a close parallel between the increase in glucose-6-phosphate dehydrogenase activity and collagen content of denervated muscle and postulated that the connective tissue was the source of this enzyme. They further suggested that the connective tissue cells may have an important function in providing the muscle fibers with pentoses, derived from this pathway, for nucleic acid synthesis. There is, however, histochemical indication for the presence of this enzyme in muscle fibers.

6. Lipid Metabolism

It is now accepted that fatty acids are the chief energy source during sustained activity of muscle. The fibers take up circulating free fatty acids and possibly also fatty acids liberated by the action of lipoprotein lipase, present on the capillary endothelium, upon circulating triglycerides. Fat droplets are commonly observed in normal muscle fibers; they are presumed to have an energy storage function and to be hydrolyzed intracellularly, although Wallach (W1) was unable to detect in rat skeletal muscle a lipase which hydrolyzed triglycerides containing long-chain fatty acids. Lipids other than triglycerides are present in muscle, as in other tissues, as constituents of membranes. Muscle itself is capable of synthesizing phospholipids de novo (P13).

A few studies have appeared on various aspects of the composition of muscle lipids in diseased muscle. It has long been known from histochemical studies that in many patients with muscular dystrophy there is a huge increase in adipose tissue between the fibers, but it seems doubtful whether there is an increase in true muscle fat. Pennington *et al.* (P14) could find no marked abnormality in the fatty acid composition of the infiltrated fat in a case of Duchenne dystrophy when compared with normal adipose tissue. Hughes (H15, H16) has carried out extensive studies on the concentration of various classes of lipids in the various

cell fractions from dystrophic mouse muscle, and also in muscle from Duchenne dystrophy. Duchenne muscle contained a normal amount of plasmalogen, but an increased quantity of sphingomyelin. Evidence was presented also for a decrease in normal muscle sphingomyelin concentration during development; thus a further instance is provided of the resemblance of dystrophic muscle to immature muscle. Kunze and Olthoff (K17) confirmed the increase in sphingomyelin but found a decrease in plasmalogens, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. Susheela (S25) could find no abnormality in the free fatty acid content of dystrophic human muscle or in the level of free fatty acids in the patients' blood. The same worker and colleagues, however, previously observed a high free fatty acid content of muscle from dystrophic mice (S26). This may find explanation in the *in vitro* studies of Lin and co-workers (L3), which showed a decrease in fatty acid breakdown and increase in synthesis in dystrophic mouse muscle. Tanaka *et al.* (T2), however, could not find any difference in the *in vivo* incorporation of labeled acetate into the fatty acids and cholesterol of tissues of dystrophic and normal mice.

The fatty acid composition of muscle lipids may show quantitative alterations in diseased muscle. Thus lecithin isolated from human dystrophic muscle had an increased amount of oleic but diminished linoleic acid (T1). Changes have been recorded also in the fatty acid composition of lecithin from denervated muscle (P1). Recently it has been reported (K16) that the fatty acid pattern of muscle phosphatides from patients with the autosomal dominant form of myotonia congenita differed markedly from that of the autosomal recessive form and from the normal. Tani and his co-workers (F7) have made a detailed study of the phospholipids of normal and dystrophic mouse tissues. In normal mice phosphatidylcholine and phosphatidylethanolamine from skeletal and heart muscles had a very high content of 20–22-carbon polyunsaturated acids, in comparison with those for other tissues; the most abundant was docosahexaenoic acid. In dystrophic mice there was a sharp decrease in the proportion of docosahexaenoic acid in the phosphoglycerides from skeletal and heart muscles, suggesting the likelihood of important alterations in muscle membranes. Somewhat similar studies have been reported by Owens (O5), who also observed a fall in the proportion of docosahexaenoic acid, mainly in the phosphatidylcholine + choline plasmalogen fraction.

Recent isotope incorporation studies by a number of workers have indicated increased turnover of muscle membrane lipids following denervation. These include increased incorporation of labeled phosphate and glycerol into glycerophosphatides (B17), increased turnover of

proteolipids (L8), and increased specific activity of a ganglioside after injection of labeled *N*-acetylmannosamine (M7). The possibility was suggested (L8) that the increased sensitivity to acetylcholine of the denervated muscle membrane may be related to the increased proteolipid turnover.

As mentioned above, Wallach (W1) could find no evidence for a lipase which hydrolyzed triglycerides containing long-chain fatty acids in skeletal muscle. In a further paper (W2), he showed that in dystrophic mouse muscle there was an enhancement of the activity of lipases hydrolyzing monopalmitin, monomyristin, monolaurin, and tricaproin. An interesting skeletal muscle disorder has been described recently by Engels' group (E9). The patients failed to show a ketonemia or ketonuria on prolonged fasting, which invoked episodes of muscle symptoms. Orally administered medium-chain triglycerides produced ketonemia and ketonuria. It was considered possible that the muscle symptoms resulted from a defect in the metabolism of long-chain fatty acids in the liver and perhaps also in the muscles. The defect in the liver would deprive the muscle of circulating ketone bodies, an important energy source in starvation.

It is convenient to mention here that Fitzpatrick and Pennington (F6) studied the metabolism of acetoacetate-^{14}C by dystrophic mouse muscle *in vitro*. They found no significant difference between normal and dystrophic muscle in the production of $^{14}CO_2$, but the incorporation of ^{14}C into nonvolatile compounds was much higher in the dystrophic muscle. In nutritional muscular dystrophy in the chicken, Jenkins (J3) found that acetoacetate oxidation by muscle homogenates was unaltered, but the utilization of β-hydroxybutyrate was impaired.

A deficiency of coenzyme Q in dystrophic mice has been recently reported by Folkers and his colleagues (L4), following an earlier finding by the same group (F1) that this disease is alleviated by treatment with hexahydrocoenzyme Q_4.

7. Protein and Amino Acid Metabolism

7.1. Protein Turnover

Although there may be some exceptions, it appears likely that the majority of the proteins in muscle fibers are synthesized *in situ*. Preparations of ribosomes can be made from muscle which are capable of synthesizing protein under conditions similar to those used for other tissues (Y1). Varying estimates have been made of the rate of turnover of muscle proteins (P10); older estimates of protein half-lives are probably too long owing to error resulting from the reutilization of the labeled

amino acid, and more recent work based on measuring the rate of synthesis after continuous infusion of a labeled amino acid (W8) has suggested half-lives of 5–6 days. Work by one group (D9) has led to the conclusion that myofibrils may have a fairly constant life-span of 30 days, although there was evidence that individual myofibril proteins could be replaced during the life-span of the myofibril. Protein turnover studies in muscular dystrophy of the mouse by Simon *et al.* (S9) and independently by other workers have demonstrated that there is a sharp increase in the rate of protein turnover in the muscle. Thus the muscle wasting which characterizes the disease is associated with an increased rate of protein breakdown rather than a general decrease in the rate of protein synthesis, although this is not necessarily true of all the individual proteins. It has not been practicable, for obvious reasons, to carry out this type of investigation in human muscular dystrophy. In contrast with the mouse dystrophy, it is agreed (G3, S10) that in denervated muscle there is an increased rate of protein degradation but also a decrease in the rate of synthesis. In the dystrophic chicken embryo amino acid incorporation into muscle protein was similar to normals but was higher 1 week after hatching owing to the more rapid decrease of incorporation in the normals during development (W14).

The cause of the elevated rate of protein turnover in dystrophic muscle is as yet unknown. It is not clear, for example, whether it can be accounted for by the presence of regenerating fibers or whether, on the other hand, an increased turnover precedes or accompanies degeneration of the mature fibers. The decreased rate of protein synthesis in denervated muscle, in which regeneration is not normally seen, would suggest that regeneration may be largely responsible for the accelerated synthesis of protein in dystrophic muscle.

7.2. Protein Synthesis *in Vitro*

In Duchenne muscular dystrophy, it has been reported (M18) that polyribosome preparations from muscle incorporate phenylalanine-^{14}C into polypeptides at an elevated rate either in the absence or the presence of polyuridylic acid as messenger. The magnitude of the increase did not appear to be related to the severity of the histological changes. These findings with human dystrophic muscle accord with the accelerated protein turnover in the dystrophic mouse, and suggest some difference in the translating activity of the polysomes. Studies on cell-free preparations from dystrophic mouse muscle by Srivastava and Berlinguet (S18) also indicated an enhanced protein synthetic activity. Srivastava reported, however (S16), that there was a decrease in polysome concentration in dystrophic muscle and that ribosomes from dystrophic muscle responded

more to the addition of poly U than the controls and suggested that there might be less messenger RNA in dystrophic muscle. The pH 5 enzyme fraction from the dystrophic muscle was, however, much more active for the synthesis of protein than that of the normal. On the other hand, the activity of the amino acid-activating enzymes has been found to be lower in dystrophic mouse muscle (P4).

7.3. Protein Catabolic Enzymes

Although the factors and mechanisms concerned in the intracellular catabolism of proteins are little understood, it seems reasonable to assume that the breakdown of muscle proteins in the living fibers involves the action of the proteolytic enzymes, or cathepsins, present in the muscle. Acid cathepsin activity has long been recognized in muscle homogenates, and Iodice *et al.* (I1) purified two cathepsins from muscle with maximum activity at an acid pH, a cathepsin A and a cathepsin D. There is evidence (S20) that acid cathepsin may occur in lysosomes in muscle, as is the case in liver and some other tissues. Muscle homogenates are capable also of breaking down protein at an alkaline pH (K13, P7), but the enzyme or enzymes involved have not been purified; in the author's experience, alkaline cathepsin activity associated with structural elements in muscle is difficult to obtain in soluble form. Alkaline cathepsin activity was found to be higher in a slow muscle than in a fast muscle (H1). Acid cathespin, however, is higher in extensor digitorum longus (white) than soleus (red) muscle in the rat (G7).

Increased concentrations of muscle cathepsins have been recorded in many types of muscle atrophy. Acid cathepsin activity increases in dystrophic mouse muscle (W12) and in muscle from Duchenne-type dystrophy (P6); in the latter investigation, the elevation tended to be greater in the more advanced cases. Elevated acid cathepsin has been recorded also in denervation atrophy and vitamin E deficiency. Alkaline cathepsin activity has been shown to be higher in the dystrophic mouse (P7) and in Duchenne dystrophy (P12).

One is led to consider both the reason for the increased activities of these protein-degrading enzymes in diseased muscle and also their possible importance in the process of muscle atrophy. Acid and alkaline cathepsins may require separate consideration in respect to both of these questions. A number of other acid hydrolases, like acid cathepsin, display elevated activity in diseased muscle; such enzymes are probably also lysosomal, and the increase in their activity may signify increased numbers of lysosomes in diseased muscle. The suggestion frequently has been made that the increase in acid cathepsin and other hydrolases is a result of the macrophage invasion of the muscle, but Weinstock and his col-

leagues have presented evidence of several kinds (e.g., W13) that there is an increase in the muscle's own acid cathepsin.

Another possibility is that the acid cathepsin changes are another instance of the above-mentioned dedifferentiation phenomenon which occurs in diseased muscle; Weinstock and Lukacs (W11) showed that acid cathepsin of normal chicken muscle decreases on maturation. However, alkaline cathepsin in mouse muscle tends to increase with age of the animal (P7).

It seems reasonable to suppose that the elevated cathepsin activity in dystrophic muscle, by enhancing muscle protein breakdown *in vivo*, is the cause of the increased rate of protein turnover; the increased synthesis could then be seen as an adaptive response to the accelerated breakdown. There is yet, however, no real evidence that this is so; the factors which control the breakdown of protein in muscle fibers are probably complex and little understood (P10). A further possibility, that the proteins of atrophying muscles are in some way more susceptible to breakdown by proteolytic enzymes, seems unlikely; Kohn (K9) reported that myosin from denervated rat muscle was digested normally by trypsin, and this was found to be true also of myosin from dystrophic mice and chickens (K1), whereas Pollack and Bird (P21) stated that the autolytic activity of denervated muscle was not increased relative to the breakdown of hemoglobin by the muscle.

7.4. Amino Acid Uptake by Muscle Fibers

The first step in protein metabolism in muscle is the uptake of amino acids from the blood by the fibers, via the extracellular space. Experiments with injected amino acids and with isolated muscle preparations by many workers have demonstrated that muscle fibers can accumulate amino acids from the medium. Experiments with α-aminoisobutyric acid, a nonmetabolizable amino acid, have been particularly useful in enabling accumulation to be studied independently of incorporation of the amino acid into protein. An increased accumulation of amino acids has been observed in the dystrophic mouse (B3), in vitamin E deficiency (D4), and in denervated muscle (D4). The authors of the last-mentioned observation concluded from their evidence that the increased accumulation was associated with increased active transport into the muscle cells, not with a change in passive permeability of the membranes. Nichoalds *et al.* (N1) found that puromycin, which abolished protein synthesis, had no effect upon the accumulation of glycine-^{14}C by control or vitamin E-deficient muscle. More recently, Goldberg and Goodman (G4) observed a decrease in the accumulation of α-aminoisobutyric acid by soleus and plantaris muscles within 3 hours of denervation; subsequently,

the accumulation increased. These authors discussed the possible effects of muscular activity upon the amino acid uptake by muscle.

An analysis of the free amino acids in the muscle of the dystrophic chicken showed increased amounts of all the amino acids except hydroxyproline (P19); the dipeptides carnosine and anserine were decreased.

7.5. Plasma Proteins

The most well-established change in the composition of the plasma proteins in human muscle diseases is an increase in the α_2-globulin fraction. Oppenheimer and Milhorat (O2) observed this in all types of human myopathy which they studied. Other reported changes in plasma proteins have been previously reviewed (P9). Askanas (A3), using immunoelectrophoresis, found that serum from most Duchenne patients with specific rabbit anti-Duchenne serum displayed an additional arc in the β_1-globulin zone, thought to be hemopexin; it was suggested that the latter was present in greater amounts or in abnormal form in the Duchenne serum. In myotonic dystrophy a reduced amount of 7 S γ-globulin is often present (E8) and is due apparently to an increased breakdown of this fraction.

7.6. Amino Acid Excretion

Several studies on the pattern of urinary amino acids in muscular dystrophy and other muscle diseases have been carried out, partly motivated by the possibility that an inherited defect in the metabolism of an amino acid may be the cause of the disease. However, no specific change has been consistently found although there does appear to be a fairly general increase in the excretion of amino acids in muscular dystrophy. Thus Hurley and Williams (H18) found increased leucine, taurine, and possibly also threonine and valine, while Konieczny *et al.* (K11) could detect arginine, threonine, and proline in the urine of muscular dystrophy patients but not of normals. The hyperaminoaciduria might obviously be a consequence of the muscle wasting, although other factors may be involved. A recent attempt (G10) to correlate the hyperaminoaciduria with the clinical condition in Duchenne dystrophy showed that the highest amino acid excretion was during the middle course of the disease, while the patients were still active.

Hydroxyproline excretion, however, is decreased in Duchenne dystrophy at all stages (K4); in other muscle diseases the changes were less definite. As this amino acid occurs in collagen and elastin, its diminished excretion may be related to the formation of extra connective tissue in muscular dystrophy.

8. Metabolism of Nucleic Acids and Nucleotides

In common with other tissues, normal muscle contains several kinds of RNA; it is likely that ribosomal RNA forms the bulk of muscle RNA, with smaller quantities of messenger-RNA and transfer-RNA. In general, studies on RNA in diseased muscle have made no attempt to distinguish between these. When the significance of DNA changes in muscle is considered, it is pertinent to bear in mind that individual muscle fibers contain many hundreds of nuclei, which are the main site of the cell's DNA.

8.1. Changes in Nucleic Acid Content

An increased concentration of both RNA and DNA in muscle in nutritional muscular dystrophy is well established (K14). Similarly, McCaman and McCaman (M12) found a much higher DNA concentration than normal in dystrophic mouse muscle and in denervated muscle. However, as emphasized by these workers, the increased DNA concentration could be largely a reflection of a loss of protein without any corresponding destruction of nuclei and, in fact, when expressed per whole muscle, the DNA changes were relatively small. Also, Graff *et al.* (G12) reported that rat gastrocnemius muscle after denervation contained more RNA and DNA per unit weight of muscle but less in the whole muscle. However, other workers (S19) have concluded that in nutritional muscular dystrophy the loss of muscle protein is insufficient to account for the increased concentration of RNA and DNA. In a study of the phosphorus concentration in the fractions obtained by centrifuging homogenates of dystrophic mouse muscle, Oppenheimer *et al.* (O4) found an increase in total P in the low-speed sediment (myofibrils and nuclei), but a diminished amount in the supernatant.

8.2. Synthesis of Nucleic Acids

Changes in the rate of synthesis of nucleic acids as measured by the rate of incorporation of labeled precursors may be more informative than changes in nucleic acid concentrations in atrophying muscle. An enhanced incorporation of isotope into nucleic acids after glycine-^{14}C is administered to dystrophic mice (aged ½ to 2 months) was found by Coleman and Ashworth (C1); the free glycine pool was the same in normal and dystrophic muscle. More extended studies have been reported by Srivastava (S14), who used uridine-2-^{14}C as a precursor of RNA. At 30 days of age the incorporation in the dystrophic muscle was higher than normal, but it was the same at 60 days and lower at 90 days. These differences were confirmed by *in vitro* incorporation experiments

using muscle homogenates. Subsequently (S15) it was observed that this reversal at 90 days occurred in the incorporation of isotope into the RNA of the nuclei–myofibrils fraction; in mitochondria, microsomes, and supernatant fractions, incorporation was always higher in dystrophic muscle, except in microsomes at 90 days, when it was normal.

Weinstock and Dju (W9) have studied the phosphorylation of thymidine in developing breast muscle of the normal and dystrophic chicken; it has been suggested that the regulation of DNA synthesis may occur at this step. Over most of the period studied, phosphorylation was higher in the dystrophic muscle. There was, however, not always a direct correlation between the enzyme activity and DNA content of the muscle.

8.3. Nucleic Acid-Degrading Enzymes

Muscle homogenates are capable of breaking down RNA over a wide range of pH. The activity at a low pH is probably due to a lysosomal acid ribonuclease; it is increased by detergents such as Triton X-100 (T3). A ribonuclease with maximum activity at pH 8.5 has recently been purified from human skeletal muscle (G5). An increase in ribonuclease activity appears to be a characteristic feature of degenerating muscle. Tappel *et al.* (T3) observed increased acid ribonuclease in genetic muscular dystrophy of the mouse and chicken, and Epstein (E10) recorded increases of the peaks at pH 5.5 and 7.5 in skeletal muscle from rabbits deficient in vitamin E and in denervated pigeon breast muscle. After denervation there is increased activity in both red and white muscles (G8). In Duchenne muscular dystrophy, there is increased ribonuclease activity at several pH values (A1).

As in the case of the cathepsins, the significance of the existence of two or more ribonucleases with widely differing pH optima in muscle tissue is at present not clear. Until more is known of the normal and pathological functions of the muscle ribonucleases, it is difficult to assess the importance of the changes in disease.

An acid deoxyribonuclease also occurs in muscle, and its activity increases severalfold in hereditary dystrophies and in vitamin E deficiency (W10). Later, sequential studies during the progression of hereditary dystrophy in the chicken showed that the increase in deoxyribonuclease followed, rather than preceded, the fall in DNA, hence was not responsible for the latter (W11).

8.4. Ribonuclease Inhibitor

Many animal tissues contain a high-molecular weight inhibitor of ribonuclease (R9), and evidence was obtained for its existence in human muscle (A1). It exerts a potent effect in tissue homogenates and is presumed to control, in some manner, the action of one or more of the

ribonucleases in the living cell. In dystrophic mouse muscle the increased amount of ribonuclease is not paralleled by an increase in the ribonuclease inhibitor (G6, L5). Consequently, in dystrophic muscle, relatively more of the inhibitor appears to be bound to the ribonuclease. Little and Meyer (L5) have suggested that this relative deficiency of the free inhibitor may play a primary role in the etiology of the disease.

8.5. Metabolism of Nucleotides

Adenylate kinase, which is abundant in muscle as in many other tissues, decreases in dystrophic mouse and human muscle (H6, P7). This enzyme, by interconverting adenine nucleotides, probably functions in the control of glycolysis; it seems reasonable to suppose, therefore, that its activity may be governed by the same factors which influence glycolytic enzymes, as discussed above. A severe decline in the activity of AMP deaminase occurs in muscular dystrophy (P6, P7) and also in denervated muscle (M12) and in some cases of muscle affected by hypokalemic periodic paralysis (E6). Skeletal muscle normally contains a higher concentration of this enzyme than other tissues; in fact, it is almost absent from some, such as liver. Its physiological function, and hence the significance of the sharp decline in its activity in diseased muscle, is still a matter of speculation.

Early histochemical studies (G2) indicated an increase in human dystrophic muscle of a number of enzymes responsible for dephosphorylating certain nucleotides. These enzymes appeared to be largely located in the proliferated connective tissue, and the authors suggested that muscular dystrophy may be basically a connective tissue disease. There is little or no evidence, however, to substantiate this speculation. Quantitative studies (P6, R3) have confirmed an increase in the dephosphorylation of AMP by diseased muscle, but it is not clear to what extent, in muscle, this activity is due to a specific enzyme. An increase in the rate of hydrolysis of NAD in denervated muscle has been reported (T4).

Japanese workers (K10) have reported the occasional appearance of NAD in the urine of patients with progressive muscular dystrophy. Coenzyme A is said to be decreased in the muscle and increased in the serum of patients (R1). Such changes probably reflect increased leakage of these nucleotides from diseased muscle fibers rather than changes in their metabolism.

Changes in the enzyme inorganic pyrophosphatase may be mentioned here, also. Although not directly involved in the transformations of nucleotides, it is probably important in relation to biosynthetic reactions involving the splitting of the inner pyrophosphate bond of ATP or other nucleoside triphosphates; by destroying the pyrophosphate formed it may

serve to "drive" such reactions in the one direction. McArdle (M9) has made an extensive study of inorganic pyrophosphatase levels in normal and diseased muscle. Widely varying levels were found in muscle disease, and the author tentatively concluded that increased pyrophosphatase activity may be associated with muscle regeneration.

9. Possible Changes in Myoglobin

Myoglobin, the intracellular oxygen carrier of muscle tissue, has received the attention of a number of workers in the field of muscle disease. By analogy with the well-known genetic variations in hemoglobin it has been suggested that muscular dystrophy may be a result of an inherited abnormality in myoglobin. Unfortunately, although changes in myoglobin in dystrophic muscle have been reported, no consistent picture has emerged, and indeed, some investigators have been unable to find any abnormality. Whorton *et al.* (W15) claimed to have found differences in the absorption spectra of both skeletal muscle and cardiac myoglobin in Duchenne dystrophy. In a number of studies, Perkoff (e.g., P15) observed that the major myoglobin fraction in adult muscle was absent or greatly decreased in cases of childhood muscular dystrophy, dermatomyositis, and myoglobinuria, but was normal in myotonic and fascioscapulohumeral muscular dystrophy. Moreover, this fraction was diminished in normal fetal muscle, thus recalling other resemblances between fetal and dystrophic muscle (see above). Others, however, have questioned the differences between adult and fetal myoglobin, and the significance of the appearance of a number of myoglobin fractions on chromatography is far from clear. More recently, a Japanese group (M16) have found four zones after electrophoresis of myoglobin and a marked reduction in one of these zones in Duchenne dystrophy, which was not seen in other myopathies examined. On the other hand, Rowland and co-workers (R11) were unable to detect any electrophoretic or immunochemical differences in myoglobin from human dystrophic muscle. Further studies are necessary to clarify these questions.

A fall in total myoglobin content of dystrophic muscle has been recorded by several investigators (e.g., H14).

10. Creatine Metabolism

10.1. Creatine

The association of muscle disease with a disturbance of creatine metabolism is a long-established contribution of biochemistry to this field, and the subject of much investigation in the past. It has been recognized for over half a century that, in contrast with normal adults, patients with nearly all types of muscle disease exhibit a creatinuria. Comprehensive

data are provided, for instance, by Van Pilsum and Wolin (V1). A decrease in urinary creatinine and an increase in blood creatine level usually accompanies the creatinuria, and there is a decrease in creatine tolerance in muscular dystrophy patients (D1). Although at one time considered to be a primary defect and the inspiration for attempted therapy, it is now clear that the creatinuria can be largely explained as merely the consequence of the reduction in the mass of muscle resulting from wasting. Creatine is synthesized by methylation of guanidoacetic acid, which takes place largely in the liver and is carried to the muscles, which normally contain about 95% of the body's creatine. Here it is reversibly phosphorylated by ATP to phosphocreatine which functions as a reservoir of high-energy phosphate. Creatine is formed steadily and probably spontaneously from either creatine or phosphocreatine, diffuses into the body fluids and is excreted in the urine. If the total amount of muscle is reduced there is insufficient to take up all the creatine which is formed, the blood level of creatine increases, and its tubular reabsorption becomes incomplete.

Myotonic dystrophy appears as a rather special case, since creatinuria is small or absent. In this disease it is thought that there is also a decrease in creatine synthesis, possibly due to an endocrine disturbance (see Z1). There is some indication that the same may be true of the hereditary myopathy in mice (P16).

In addition, however, to the expected and nonspecific effect of the reduced muscle mass, there may be, in muscular dystrophy, an inability of the remaining muscles to hold creatine to a normal degree (F4); in Duchenne or fascioscapulohumeral dystrophy, but not in amyotrophic lateral sclerosis, there was a more rapid fall in the specific activity of urinary creatinine after the administration of creatine-^{14}C. Dystrophic mouse muscle contains a reduced amount of creatine (F5), and this animal provides a convenient tool for investigating any abnormalities in the handing of creatine by dystrophic muscle. Recent studies by Fitch and Rahmanian (F3) showed that entry of creatine-^{14}C into isolated skeletal muscle preparations was accelerated in the dystrophic mouse, thus implying that the low creatine content of the dystrophic muscle is due to a more rapid loss of this compound. From further work (F2), the authors concluded that this is due not to an increased membrane permeability to creatine, but to an impaired exchange between creatine with access to the membrane and creatine in a relatively inaccessible form.

10.2. Creatine Kinase

Creatine kinase (ATP:creatine phosphotransferase) is responsible for the phosphorylation of creatine in tissues, and this process is of particular importance in skeletal muscle, where this enzyme is abundant. Consid-

erable interest was aroused by the report (H8) that the creatine kinase in dystrophic mouse muscle differed from the normal enzyme in possibly one amino acid, involving the loss of one essential thiol group; this suggested the possibility of a mutation in the structural gene. Further work by the same authors (H9), however, pointed to the possibility that the change was due to oxidation of the thiol group, since they obtained evidence that the soluble proteins of dystrophic mouse muscle are more oxidized than normal. In addition, the ratio of reduced to oxidized glutathione in dystrophic mouse muscle was slightly decreased. More detailed data on glutathione levels in dystrophic muscle, at various ages, has been reported (S5). More recently Jacobs *et al.* (J1) were unable to find any differences between creatine kinase from normal human muscle and from the muscle of a patient with Duchenne dystrophy. Similar negative findings have been reported for the dystrophic chicken (R12).

The isoenzyme pattern of creatine kinase in normal and diseased muscle has been investigated by several workers. Two subunits are recognized, usually denoted M and B; the enzyme is a dimer, hence three forms are possible: MM, BB, and the hybrid MB. Most muscles contain largely MM with sometimes a proportion of BB (the latter is characteristic of brain). Normal fetal muscle, however, contains a preponderance of BB (G11, S3). After denervation, there is a relative decrease in MM (S3). In muscle from patients with fascioscapulohumeral dystrophy and polymyositis there was an increase in MB relative to MM, but a normal ratio was found in Duchenne and limb-girdle dystrophies and in most cases of neurogenic atrophy; BB occurred in some cases of myotonic dystrophy (G11).

11. Calcium Uptake by Sarcoplasmic Reticulum

It is now generally accepted that muscle contraction is initiated by the release of calcium ions from the sarcoplasmic reticulum, following depolarization of the muscle membrane and the inward spread of depolarization through the transverse tubules. Relaxation is caused by the reaccumulation of calcium by the sarcoplasmic reticulum. This process can be studied *in vitro* using fragmented sarcoplasmic reticulum obtained by differential centrifuging of muscle homogenates; calcium uptake is measured by using radioactive calcium. Energy is required in the form of ATP, and it is presumed that the calcium-activated ATPase, which is found in this fraction, is involved in the process. Martonosi (M4) showed that the uptake of calcium by sarcoplasmic reticulum of dystrophic mouse muscle was significantly lower than normal. Studies on humans (S24) indicated a similar defect in progressive muscular dystrophy, polymyositis and some cases of neurogenic atrophy. The abnormality in

progressive muscular dystrophy was confirmed by Samaha and Gergely (S1); these workers could find little correlation of the degree of change with the duration and severity of the disease, the serum enzyme level or the extent of histopathological change, and concluded that this represented one of the earlier changes in this type of dystrophy. No such abnormality, however, was seen in myotonic dystrophy, although a delay in muscle relaxation is characteristic of this condition. It has recently been reported (M3) that, following denervation, there is an increase in calcium accumulation of both red and white muscle in the rat. Finally, a new type of approach has been explored by Hsu and Kaldor (H11); they found that the calcium-accumulating ability of sarcoplasmic reticulum of dystrophic chicks was impaired to a greater degree by the action of phospholipase C than that of normal muscle.

12. Plasma Enzymes in Muscle Diseases

Stemming from the observation of Sibley and Lehninger (S8) in 1949 of a high level of aldolase in serum from two cases of muscular dystrophy, the elevation of plasma enzymes in muscle disease has been extensively studied. Although the nature of the changes in diseased muscle which are responsible for the leakage of enzymes into the circulation are not clear, the phenomenon has provided the most useful biochemical tool in the diagnosis of muscle disorders. Creatine kinase (E2) is the preferred enzyme for this purpose, on grounds of both sensitivity and specificity. The use of creatine kinase and other enzymes in the diagnosis of muscle disorders was adequately reviewed by Thomson (T5) in a previous volume, and what is said here will be largely confined to more recent developments.

12.1. Duchenne Muscular Dystrophy

The largest increases in serum creatine kinase occur in this disease, and it has long been recognized that high levels precede the clinical signs of the disease. A close study of the time course of the early changes in several serum enzymes in a single case (H5) revealed an elevation directly after birth, peak levels at 14–22 months, then a slow decline.

The determination of serum creatine kinase still represents the most valuable method for the detection of the female carriers of Duchenne dystrophy (W7), although a minor proportion of carriers have normal levels of the enzyme. Why this is so is still not clear. Roy and Dubowitz (R13) could find no consistent correlation between serum creatine kinase and histopathological changes in carriers. Possibly some carriers with normal values have a higher rate of inactivation of the enzyme in the blood or of elimination from the circulation; much is yet to be learned

of the factors involved in the turnover of circulating enzymes (B4, P22). Some attention has been paid to the effect of physical exercise upon serum creatine kinase levels, in an attempt to refine the test and detect a higher proportion of carriers. Severe and prolonged exercise may substantially raise serum creatine kinase in normal individuals (G14, V2). The possibility that the enzyme level in carriers may be unduly sensitive to the effect of exercise would offer the hope of improved diagnosis and has been investigated, but conflicting results have been obtained. Stephens and Lewin (S23) found that three out of four carriers which were tested had higher than normal levels when sampled at random but, after resting, none of the four were elevated. Emery (E4) reported that a standardized amount of moderate exercise had little effect on the serum creatine kinase of normal women but produced a significant rise in some carriers, and he tentatively concluded that such a stress test may be useful in detecting carriers. Results obtained by Hudgson *et al.* (H12), however, have been less encouraging; in most subjects, the creatine kinase after normal everyday activity was slightly higher than after bed rest, but the change was on average no greater in carriers than in controls. Similarly, after a three-mile walk, most subjects showed an increased enzyme level, but this effect was not significantly larger in carriers.

Enzymes other than creatine kinase have found little application in the detection of Duchenne carriers. Recently, however, the Berlin group have claimed that measurement of alanine aminotransferase, lactate dehydrogenase and aldolase and application of the formula, 108 — 4.9 GPT — 0.2 LDH — 19.6 ALD (where GPT, LDH, and ALD represent the levels of these three enzymes) is a useful discriminative test; a negative result suggests that the individual is a carrier (B18).

12.2 Other Muscle Disorders

Although earlier reports indicated that there was little or no increase in serum creatine kinase in muscle atrophy of recognized neurogenic origin, it is now clear that a rise does occur in some cases, although this is not as high as that observed in Duchenne dystrophy. Increased levels have been reported in Kugelberg-Welander disease and spastic spinal paralysis (I3); the author and his colleagues have observed elevations in the former case. In a study of forty-six cases of motor neuron disease, many had raised serum creatine kinase but there was no relation between creatine kinase level and the activity of the disease (W17). Elevated serum creatine kinase has been found also in hyperkalemic and hypokalemic myopathies (H10, M9).

An increase in serum creatine kinase is encountered following head

injuries and many cases of stroke, and it is of interest that isoenzyme studies have indicated that the blood enzyme in such conditions originates from the muscles (D12). In tetanus, also, there appears to be an increased release of muscle enzymes (B11).

12.3. Problems Concerning Release of Muscle Enzymes

Although there is no reasonable doubt that the elevated plasma enzymes in muscle diseases result from leakage from the affected muscles, many questions remain unanswered; for example, the relatively massive leakage in Duchenne dystrophy compared with muscle atrophy which is recognizably of neurogenic origin. *In vitro* studies (D2, P3) have demonstrated a greatly increased rate of leakage of enzymes from denervated muscle; such experiments, however, must be interpreted tentatively, since rates of enzyme leakage from isolated muscles are probably much higher than occur *in vivo*.

It is also not entirely clear why the blood levels of individual enzymes in muscular dystrophy differ so widely. In some instances, even, there appears to be no increase. Thus blood AMP deaminase levels are barely raised (P8), and Rowland and co-workers (R10) were unable to detect phosphofructokinase or myoglobin by enzymological or immunochemical techniques. The relative abundance of the enzyme in muscle is obviously important; indeed, Dawson (D2) found that *in vitro* there was a good correlation between the amounts in muscle and the amounts leaking out. In Duchenne dystrophy, however, there is a poor parallel between the concentrations in muscle and blood (P8). The size and shape of an enzyme molecule would be expected to influence its rate of leakage, and a further factor is the attachment of an enzyme to intracellular structures; there is some evidence that even the "soluble" enzymes of muscle may be, in some manner bound within the fibers (A2, H17). Probably other factors also are involved.

13. Involvement of Other Tissues in Muscular Dystrophy

The human muscular dystrophies are characterized above all by weakness and wasting of skeletal muscles, and practically all biochemical studies, other than those concerned with body fluids, have been carried out on the muscle tissue. From time to time, however, reports have appeared of abnormalities in other tissues. Insofar as these can be confirmed, they may represent indirect changes resulting in some way from the presence in the body of the mass of diseased muscle; on the other hand, the genetic defect could be manifested directly in many types of

tissue. At present it is generally not possible to decide between these alternatives.

13.1. Nervous System

Changes in the nervous system are of particular interest since, as discussed above, the muscle damage could, itself, result from disease of the nerves. Many workers believe that intellectual impairment is associated with Duchenne dystrophy (D13). Rosman and Kakulas (R7) observed abnormalities in the brains of some Duchenne patients at autopsy, but Dubowitz and Crome (D14) could find no consistent pathological changes. Recently, McComas and his colleagues (M13) have presented electrophysiological evidence for the loss of motor neuron activity in human muscular dystrophy; similar findings have been reported in the mouse from the same unit (H3). Evidence for a defect in the nerves does not, of course, exclude the presence of the genetic defect in the muscle also, but it seems likely that, in future, more attention may be given to the possibility of biochemical changes in the nervous system.

13.2. Other Tissues

It has been reported (C2), but could not be confirmed (P11), that the rate of utilization of glucose by erythrocytes from muscular dystrophy patients was less than normal. More recently (B9), in a detailed study of glycolytic intermediates in red cells from Duchenne patients, changes were found which were taken to indicate increased activity of hexokinase and perhaps certain other enzymes of glycolysis. It was thought that these changes may be related to the abnormality in the ATPase of erythrocyte ghosts obtained from Duchenne patients reported by Brown and colleagues (B13); this enzyme was stimulated by ouabain, whereas that derived from normal individuals is inhibited. The same workers found a similar abnormality in erythrocytes from myopathic ducks (B14). Peter *et al.* (P18), however, stated that the abnormal response to ouabain can be induced in normal red cells by incubating them with serum from Duchenne patients, but others (K5) have been unable to demonstrate any such abnormality. It is notoriously difficult to obtain a standardized preparation of red cell ghosts. The existence of a genetic defect in the erythrocyte would provide a very convenient tool in muscular dystrophy research, but the present evidence for this is not very convincing.

Measurement of a wide range of serum enzymes in Duchenne dystrophy and comparison with the changes seen in other diseases has led Kleine (K7) to postulate that in this disease enzymes are released not only from skeletal muscle, but also from heart, liver, and possibly erythrocytes.

14. Conclusion

In conclusion it may be seen that up to the present time a large number of diverse biochemical changes have been recorded in the muscular dystrophies. The interrelationships of many of these is not clear, and much more fundamental work is likely to be needed before they can be fully understood and perhaps controlled.

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AUTHOR INDEX

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