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List of Contributors

DEUTICKE, BERNHARD, Prof. Dr., Abt. Physiologie der Med. Fakultät der RWTH, Medizin. Theoret. Institut, Aachen/ Federal Republic of Germany

CRANE, ROBERT K., Ph. D., College of Medicine and Dentistry of New Jersey, Rutgers Medical School, Dept. of Physiology, University Heights, Piscataway, NJ/USA

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Properties and Structural Basis of Simple Diffusion Pathways in the Erythrocyte Membrane

B. DEUTICKE

Contents

I. Introduction

The rapid development of new concepts and technical possibilities in the investigation of membrane phenomena, sustained by the growing interest of investigators from numerous disciplines, has provided in the past years a conspicuous body of information on the structure and function of cell membranes. Interest is focused either on the analysis of membrane composition and organization, or on the description of membrane functions. Among the latter, transport properties, responsible for the capability of membranes to serve as barriers or vectorial catalysts for downhill and uphill movements of matter, are a topic of major physiological and biomedical relevance.

Historically, the characterization of such transport processes has primarily been concerned with their kinetics and led to mathematical descriptions of transfer systems, oriented at general concepts and known facts on diffusion and enzyme catalysis. The pathways postulated on this basis are of necessity analog models, to which, in principle, a considerable number of structural equivalents can be assigned. For a proper assignment, intimate knowledge about membrane organization is required. Only the consideration of both kinetic and structural features will render possible physically correct descriptions of the movement of molecules via their pathway in a membrane.

Sufficient information for such an integrative description of transport pathways is presently at best available for a small number of objects. In case of eukaryotic, in particular mammalian cell membranes, the structural and functional analysis of erythrocyte membrane properties has reached a state, which may justify attempts to discuss transfer processes mainly in terms of their structural basis. The present review has been conceived under this aspect. To facilitate future work it shall furthermore serve the purpose to provide the "kineticist" with information on erythrocyte membrane constituents that may be relevant for the properties of pathways, and to outline to the "structural-

ist" the characteristics of some apparently simple transport processes that await interpretation in structural terms. The diffusional transfer of small nonelectrolytes and anions seemed a very suitable object for such a purpose.

The attempt of an integrative approach made it necessary to limit the review to the erythrocyte membrane, although inferential information on transfer pathways in this membrane might have been obtained by considering the relationship between structural parameters and transport properties in other types of membranes. However, even with this restriction the amount of information potentially valuable in composing a transport topology of the erythrocyte membrane proved to have reached an extent precluding a comprehensive treatment of the topic. The author is thus well aware that the pathways postulated and characterized in the subsequent sections are only first approximations of the physical reality that have to be subjected to a continuous remodelling in the future.

II. The Composition of the Erythrocyte Membrane

As a basis for discussing the nature of transfer pathways, the present state of knowledge about the composition of the mammalian erythrocyte membrane as well as the arrangement and the physicochemical state of its constituents shall be outlined. Comprehensive accounts of these subjects have recently been given in a number of reviews (NELSON, 1972; VAN DEENEN and DE GIER, 1974; ZWAAL et al., 1973; JULIANO, 1973; STECK, 1974; WEINSTEIN, 1974; CHAPMAN, 1973; LEE, 1975.

A. Gross Composition

The main components of the human erythrocyte membrane, lipids, proteins, and carbohydrates constitute approximately 41% ($\sim 5{,}3 \times 10^{-13}$ g/cell); 52% ($\sim 6{,}6 \times 10^{-13}$ g/cell), and 7% ($\sim 0{,}8 \times 10^{-13}$ g/cell) of the total membrane mass (adapted from JULIANO, 1973). Data on other mammalian species are available only to a limited extent. Some interspecific differences, in particular in the total membrane lipid (NELSON, 1967) could be due to the differences in surface area per cell (GRUBER and DEUTICKE, 1973). On the other hand, calculations of the lipid content per unit cell surface area do not cancel out the species differences and may indicate interesting variations in the surface density of lipids. Species differences of carbohydrate content are also likely in view of varying carbohydrate/protein ratios (HUDSON et al., 1975) and different glycolipid contents (NELSON, 1972). Comparative data for the protein contents of erythrocyte membranes are available as yet only for one species. LIVNE and KUIPER (1973) found about twice as much protein in the camel erythrocyte membrane as in human erythrocytes.

B. Lipid Constituents

Erythrocyte membrane lipids fall into three classes (NELSON, 1972; VAN DEENEN and DE GIER, 1974):

1. Neutral lipids. This fraction, almost exclusively unesterified cholesterol, contributes about 25–29% (on a gram weight basis) to the total lipids in all mammalian species studied

2. Phospholipids. They contribute between 50 and 65% to the total lipid mass and belong mainly to the following classes, characterized by the constitution of the polar head group:

Glycerophospholipids (Glycerol, 2 fatty acids, phosphate, head group): Phosphatidyl*choline* (PC), Phosphatidyl*ethanolamine* (PE), Phosphatidyl*serine* (PS), Phosphatidyl*inositol* (PI), Phosphatidic acid (PA).

Spingolipids (Sphingosine, 1 fatty acid, phosphate, choline): Spingomyelin (Sph).

Quantitatively, PC, Sph, PE, and PS dominate by far. The relative contributions of PC and Sph, but not those of PE or PS differ considerably depending on the animal species (Fig. 1). From a physicochemical point of view, the phospholipids can be divided into two classes, namely phospholipids devoid of net charge (PC+Sph) and phospholipids bearing a negative net charge (PE, PS, PA, PI).

The diversity of the phospholipids not only concerns the polar head groups, but even more so the apolar domain. The fatty acids in the erythrocyte membrane, which vary between 16 to 24 C-atoms in chain length and from 0 to 6 (usually cis-) double bonds per chain (NELSON, 1972), are not randomly distributed between the different classes of phospholipids (Fig. 2).

The acyl chains of PC and, in particular, sphingomyelin are more saturated than those of PE and PS. Sphingomyelines are characterized by a high content of long chain fatty acids (>20 C-atoms) (SWEELEY and DAWSON, 1969). The sphingosine moiety of the molecule also contributes to the apolar domain of the lipids. The length of the segment comparable in its localization to the fatty acid chains is probably 13 C-atoms.

An inhomogeneous mixture of apolar chains can be quantitated by its "mean chain length" and its "mean number of cis double bonds per chain" (the

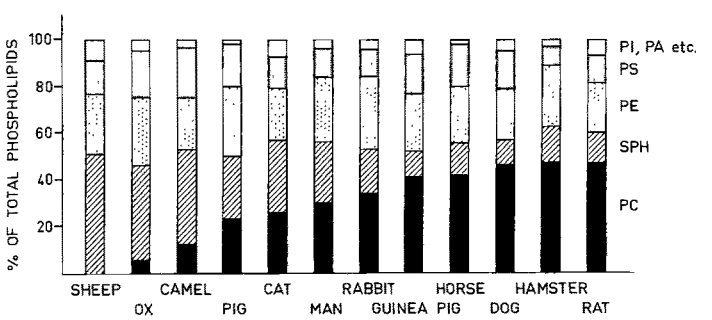

Fig. 1. Phospholipid patterns of the erythrocyte membrane in various mammalian species. (Data from NELSON, 1967; ROUSER et al., 1968, KUIPER et al., 1971, LIVNE and KUIPER, 1973)

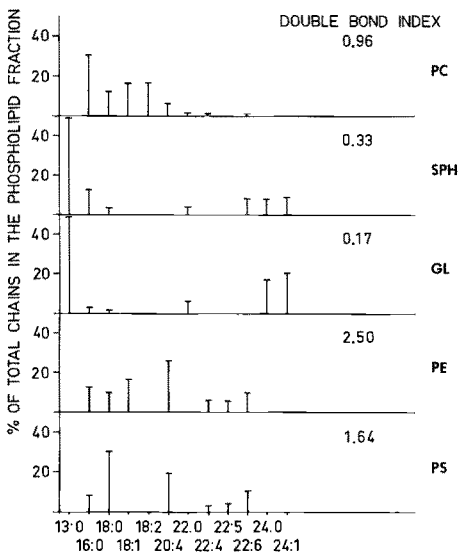

Fig. 2. Characteristics of the paraffin chains of major polar lipids of human erythrocyte membrane. Data for phospholipids from GERCKEN et al. (1972), data for glycolipids (*GL*) from ANDO and YAMAKAWA (1973). Notation indicates the number of carbon atoms and, behind the colon, the number of cis-double bonds in the chain. *13:O* in sphingomyelin and glycolipids refers to that part of the sphingosine chain equivalent in its position in the bilayer to the fatty acid chains

"double bond index"). Such values are obtained by the formula $\Sigma\, c_{n(m)} \cdot n(m)$, where $c_{n(m)}$ represents the fractional contribution of an acyl or alkyl chain with n C-atoms or m double bonds to the sum ($=1.0$) of chains in a lipid class or in the total lipids. These two parameters provide preliminary information on physicochemical properties of the membrane lipid domain (Sect. III C 2a).

3. Glycolipids. They contribute between 5 and 10% to the membrane lipids in different mammalian species and are sphingolipids, which consist of an apolar ceramide moiety (sphingosine+one fatty acid, usually long chain and highly saturated, ANDO and YAMAKAWA, 1973), coupled to an oligosaccharide varying in the number of monosaccharide units.

C. Protein Constituents

Human erythrocytes. The analysis of the red cell membrane proteins is still in its beginning. Solubilization of isolated membranes by sodium dodecylsulfate (SDS) and subsequent electrophoresis of the denatured proteins on SDS-polyacrylamide gels, however, has recently demonstrated, in the hands of numerous investigators, the presence of some 15–20 polypeptide fractions (LENARD, 1970a, b); FAIRBANKS et al., 1971; KNÜFERMANN et al., 1975; TRIPLET and CARRAWAY, 1972; KOBYLKA et al., 1972; ANSELSTETTER and HORSTMANN, 1975). A representative example is given in Figure 3. For a detailed survey the reader is referred to JULIANO (1973) and STECK (1974).

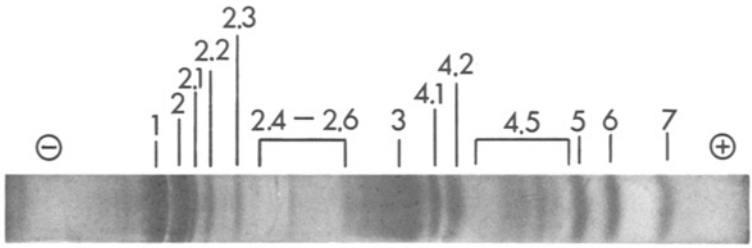

Fig. 3. SDS-gel electropherogram of the human erythrocyte membrane proteins. Technical procedure as described by FAIRBANKS et al. (1971). Gels stained with Coomassie brilliant blue (by courtesy of Dr. C.W.M. Haest). For explanation of the band numbers, see Table 1 and text

A generally accepted scheme of enumeration of the electrophoretic bands has not yet been agreed upon. The designation of FAIRBANKS et al. (1971) and STECK (1974) will be used in the following, since the classificiations of investigators using differing schemes can frequently be "translated" into this scheme.

Some of the polypeptide fractions contain a considerable amount (20–60%) of carbohydrate (WINZLER, 1969; GREFFRATH and REYNOLDS, 1974; FUJITA and CLEVE, 1975), which can be detected by staining with periodic acid and Schiff's reagent (PAS). Besides these major glycoproteins at least one of the other fractions (band 3, see below) also contains carbohydrate residues (GUIDOTTI, 1972; TANNER and BOXER, 1972; YU and STECK, 1975a).

Molecular weights have been assigned to the different polypeptide bands on the basis of their migration distances in SDS gels. This procedure, however, can be applied to membrane proteins, in particular to glycoproteins, only with reservations. If proper corrections are included, reliable molecular weights may be obtained from sedimentation equilibria (GREFFRATH and REYNOLDS, 1974).

Some of the most prominent polypeptide fractions are presently being isolated and subjected to an analysis of amino acid composition or even sequence in numerous laboratories. The state of progress is compiled in Table 1.

Many of these data concern proteins which probably span the membrane, i.e., connect the intra- with the extracellular compartment (band 3, PAS 1–4). This subject will be further considered in Section 3III D 1. With respect to the attachment of specific covalent labels or transport modifiers, to be discussed below, the presence of free NH_2 groups in the five proteins analyzed as yet and of -SH groups in four out of five is of importance.

Interestingly, all membrane-spanning proteins identified as yet carry carbohydrate residues. The composition of these constituents varies. The PAS-positive fractions are characterized by a high content of sialic acid, particularly in the glycophorin fraction, which is thus negatively charged. The chain lengths and sequences of the carbohydrates are partly known. Glycophorin, recently completely characterized by TOMITA and MARCHESI (1975), carries 16 oligosaccharide units per molecule (FURTHMAYR et al., 1975), bound to an NH_2-terminal chain segment of 50 amino acids. The oligosaccharide units are composed partly

of a tetrasaccharide and its degradation products, partly of more complex oligosaccharides (THOMAS and WINZLER, 1969, 1971; KORNFELD and KORNFELD, 1970; ADAIR and KORNFELD, 1974; TOMITA and MARCHESI, 1975). Band 3 appears to be free of sialic acid. It contains approximately 40 sugar residues, hexoses and hexosamine, per molecule (TANNER and BOXER, 1972). The involvement of the carbohydrate moiety of the glycoproteins in transfer processes is dubious. Nevertheless, they deserve interest in the present context since they act as surface receptors for antibodies and plant hemagglutinines, i.e., lectins. Although the receptors may not be fully specific with respect to their apoproteins (FINDLAY, 1974; TANNER and ANSTEE, 1976), lectin-glycoprotein interaction is a promising tool in the localization of particular membrane proteins. Recent evidence indicates that lectins from *Agaricus bisporus* and *Phaseolus vulgaris* as well as wheat germ agglutinin interact preferentially, although no exclusively, with glycophorin (PAS 1) (JACKSON et al., 1973; ADAIR and KORNFELD, 1974; TANNER and ANSTEE, 1976). Concanavalin A seems to be specific for Band 3 (FINDLAY, 1974; TANNER and ANSTEE, 1976), whereas *Ricinus communis* lectin and others bind to both of these and probably additional glycoproteins (TRICHE and TILLACK, 1975; TANNER and ANSTEE, 1976).

Erythrocytes from other species. The peptide patterns of erythrocytes from different species reveal conspicuous similarities between ox, sheep, pig, camel, dog, rabbit, guinea pig, and rat (LENARD, 1970b; KNÜFERMANN et al., 1971; KOBYLKA et al., 1972; HAMAGUCHI and CLEVE, 1972) and even nucleated goose erythrocytes (SHELTON, 1973). These similarities concern in particular the dominating polypeptide of 95,000 daltons (band 3), which is found in all species, although in varying amounts, and the "spectrin" fraction (bands 1 and 2) (LENARD, 1970b; KOBYLKA et al., 1972; TILLACK et al., 1970).

On the other hand, a certain variability is also clearly evident. For instance, a 120–160,000 dalton band in sheep and a 60,000 dalton band in pig erythrocytes seem to be specific for these animals (LENARD, 1970b; KOBYLKA et al., 1972). Rodent erythrocytes may contain a somewhat higher amount of low molecular weight polypeptides (HAMAGUCHI and CLEVE, 1972) although a varying extent of proteolytic degradation during the preparation of the samples has also to be considered (KOBYLKA et al., 1972).

The most obvious species differences concern the glycoproteins, which differ considerably in their carbohydrate contents (UHLENBRUCK, 1969; HUDSON et al., 1975). Molecular weights different from those for glycophorin, the main human glycoprotein, have been reported for the corresponding fraction in other species (CAPALDI, 1973a; HUNTER et al., 1974; HUDSON et al., 1975). To what extent these differences concern the peptide moiety is not yet known. Variations in amino acid composition were reported by HUDSON et al. (1975). Rabbit and guinea pig erythrocytes seem not to contain a "major glycoprotein" (HAMAGUCHI and CLEVE, 1972).

Table 1. Properties of main proteins present in human erythrocyte membrane

Band (2)	Synonyms	Refs.	Mol wt (Dalton $\times 10^{-3}$)	Refs.	Amino acid pattern	Refs.
1 } 2 }	Spectrin	1	200–250	2, 3	+	4
3	Fraction E, minor glycoprotein	5, 12	90–95	2, 12	+	12, 13, 14, 15
4	Fraction F 4.1+4.2	6, 12	72–78	2, 12	+	12
5	Actin	29	43	6	−	
6	Glyceraldehyde-3-phosphate dehydrogenase	30	36	2, 30	(+)	30
PAS 1	Glycophorin, Major sialoglycoprotein	32, 33	29	34	+	12, 32
PAS 2			18–56[a]	39	+	39
PAS 3			19–59[a]	39	+[b]	39
PAS 4	PAS II′	18				

+ = known; − = unknown, n.d. = not detected.
[a] Minimal values calculated from the amino acid and carbohydrate composition.

Comments		*Refs.*
Band 1 and 2	"Spectrin" may be present in the membrane as a dimer of 1 and 2	8
	Heterogeneity of the fraction has been claimed on the basis of multiple N-termini and immunologic diversity	9, 10, 11
Band 3	Claimed to be homogeneous	12, 20
	Claimed to be heterogeneous in composition	25, 26
	Probably consists of molecules with different carbohydrate moieties	15, 21, 22
	Probably present in the membrane as a dimer	27, 28
	Claimed to have *no* free N-termini	12, 14, 15, 20
	Claimed to have numerous different free N-termini	23, 34
Band 6	60% of the total amount of the tetrameric enzyme are reversibly bound to the cytoplasmic prospect of band 3	28, 31
	Can be dissociated by high ionic strength and NADH	28
PAS 1	N-terminus: serine and leucine	32
	The relationship to the other PAS-positive fractions is not quite clear. PAS 1 probably migrates in SDS gel electrophoresis as a dimer of monomer fractions migrating in position PAS 2	38

References

(1) MARCHESI et al. (1969a), (2) FAIRBANKS et al. (1971), (3) LENARD (1970a), (4) MARCHESI et al. (1969b), (5) GUIDOTTI (1972), (6) STECK (1974), (7) NICOLSON et al. (1971), (8) HULLA and GRATZER (1972), (9) FULLER et al. (1974), (10) BJERRUM et al. (1975), (11) DUNN et al. (1975), (12) TANNER and BOXER (1972), (13) ROSENBERG and GUIDOTTI (1968), (14) HO and GUIDOTTI (1975), (15) YU

Table 1 (continued)

Carbohydrate content	Refs.	Sequence	Refs.	% of total membrane protein	Refs.	Disposition in the membrane	Refs.
n.d.	1	–		25–30	2, 5, 6	Inner surface	7
7–9%	12	–		25	6	Spanning the membrane	16, 19
7%	12	–		9	6	Inner surface	6, 19
n.d.		–		4.5	6	Inner surface	6, 19
n.d.		–		5	6	Inner surface	17
55%	12, 34	+	35, 36	6[c]	6	Spanning the membrane	18, 43
19%	39	–					
24%	39	–				Spanning the membrane	17–19

[b] Different from PAS I.
[c] For the whole molecule including carbohydrate.

and STECK (1975a), (16) BRETSCHER (1971b), (17) STECK et al. (1971), (18) MUELLER and MORRISON (1974), (19) CABANTCHIK et al. (1975a), (20) JENKINS and TANNER (1974), (21) FINDLAY (1974), (22) ADAIR and KORNFELD (1974), (23) KNÜFERMANN et al. (1973), (24) KNÜFERMANN et al. (1975), (25) MUELLER and MORRISON (1975), (26) ANSELSTETTER and HORSTMANN (1975), (27) STECK (1972), (28) YU and STECK (1975b), (29) TILNEY and DETMERS (1975), (30) TANNER and GRAY (1971), (31) SCHRIER et al. (1975), (32) MARCHESI et al. (1972), (33) WINZLER (1969), (34) GREFFRATH and REYNOLDS (1974), (35) SEGREST et al. (1972), (36) TOMITA and MARCHESI (1975), (37) BRETSCHER (1971), (38) FURTHMAYR et al. (1975), (39) FUJITA and CLEVE (1975)

III. The Arrangement and the Physical State of the Membrane Constituents

A. General Concepts

A functional topography of the erythrocyte membrane requires information about the arrangement, the dynamic behavior, and the mutual interrelations of its elements. Numerous techniques have been applied during the last decade to biological membranes in order to obtain such data. Due to the relative ease of its experimental handling and its general availability, the erythrocyte has been one of the favorite study objects. In fact, earlier and present plasma membrane models rely to a considerable extent on evidence obtained for the

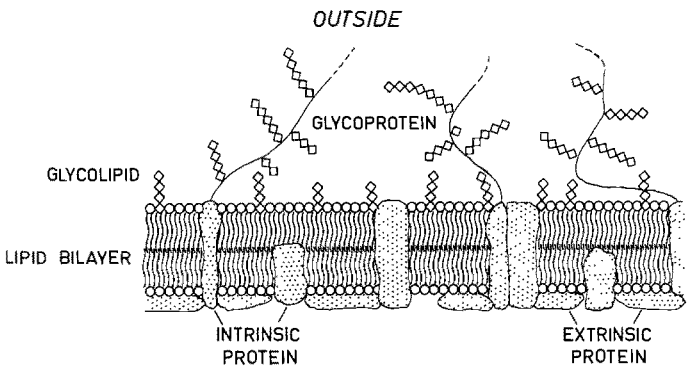

Fig. 4. Model of a lipid-protein mosaic membrane

erythrocyte membrane. This is also true for the concept of a lipid-protein mosaic membrane presently favored by many investigators. Already envisaged by early investigators (MOND, 1927), it has been elaborated in detail by WALLACH and ZAHLER (1966) and, notably, SINGER and NICOLSON (1972). Its most important features are the following (Fig. 4):

1. The amphipathic *lipids* of the plasma membrane are arranged as a bilayer, the hydrophobic domains buried in the interior. This basic concept originates from the studies of GORTER and GRENDEL (1925), which demonstrated that the lipids extracted from erythrocyte membranes suffice to prepare monolayers of approximately twice the surface area of the red cell. Reinvestigations by BAR et al. (1966) and calculations by ENGELMAN (1969) have confirmed this fundamental result.

Further evidence that the bilayer concept holds true for the erythrocyte membrane stems from spectroscopic data, demonstrating structural similarities between artificial phospholipid bilayers and the erythrocyte membrane. X-ray analyses indicating the presence of a lipid bilayer with a central hydrophobic core and a thickness of 45–55 Å have recently been reported by WILKINS et al. (1971) and STAMATOFF et al. (1975). Evidence for an ordered arrangement of parallel hydrocarbon chains perpendicular to the membrane surface comes from electron spin resonance (ESR) investigations using nitroxide-labelled fatty acids and sterols (HUBBELL and MCCONNELL, 1969; LANDSBERGER et al., 1971).

2. *Proteins* are arranged relative to this bimolecular lipid layer in two different ways:

a) *Intrinsic (integral)*, meaning that they are partly embedded in the hydrophobic membrane interior and kept in this position mainly by hydrophobic interactions with the lipids, while other segments are exposed to the membrane surface and the adjacent water phase (SINGER and NICOLSON, 1972; CAPALDI and GREEN, 1972). A special case of this type are "membrane-spanning" proteins.

b) *Extrinsic (peripheral)*, meaning that a protein is attached to the superficial domain of integral membrane proteins or to lipid head groups by all types

of interactions also characteristic for the binding of soluble proteins, i.e., polar, stereospecific, and apolar forces (SINGER and NICOLSON, 1972; SINGER, 1974).

This dualistic view, which does not exclude some overlapping of the two types of protein arrangement, is reflected in the observation that widely varying procedures are required for the solubilization of different membrane proteins. For the erythrocyte the essentials of such solubilization and extraction studies have recently been reviewed by JULIANO (1973) and STECK (1974). Accordingly, fractions 1, 2 (2.1, 2.2, 2.3), 4 (4.1, 4.2), 5, and 6 are regarded as extrinsic fractions, 3, 4.5, and 7, as well as PAS 1–4 as intrinsic proteins. Due to the presence of these proteins, the total thickness of the membrane may considerably exceed the thickness of the lipid bilayer, as indicated by recent x-ray studies of FINEAN et al. (1975).

B. Objects and Strategies of Investigation

For elucidating the disposition and physical state of membrane constituents, completely intact erythrocytes would conceptually be the only suitable object. However, localizing membrane constituents means to define their position relative to some permeability barrier. Usually, this necessitates the disruption of that very barrier at least temporarily. Whether or not after such a procedure, usually the preparation of a hemoglobin-depleted ghost, the membrane retains or regains its native state can in many cases only be inferred from a comparison of characteristic properties in the original cells and in the ghosts derived therefrom. Only very recently, agents of a new type have become available, which due to their particular permeability properties can be used in intact erythrocytes for a differential labelling of the external and the internal membrane surface (WHITELEY and BERG, 1974; STAROS and RICHARDS, 1974; STAROS et al., 1975).

The ghost preparations are usually obtained by osmotic hemolysis. The spectrum ranges from the white, hemoglobin-free ghost (DODGE et al., 1963), permanently leaky at low ionic strenth, to the "resealed ghost," elaborated in particular by BODEMANN and PASSOW (1972), presently the best approximation to an intact membrane. The characteristics of these two extremes have recently been compared by SCHWOCH and PASSOW (1973). "Intermediate" ghost types, i.e., partly resealed white ghosts have also been studied (JUNG, 1971; JOHNSON, 1975). For special purposes, normally oriented (right-side-out) and everted (inside-out) vesicle preparations of erythrocyte membranes are in use (STECK and KANT, 1974). In contrast to these low-tonicity preparations, a new technique, using the dielectric breakdown of the cell membrane (ZIMMERMANN et al., 1975) avoids the possible damage by temporary low ionic strength. The extent of perturbation induced by the high electric field strength remains to be elucidated.

The *transverse disposition* (sidedness) of membrane constituents has mainly been studied by treating intact cells and ghosts with agents assumed to have access only to the external membrane surface in intact cells and to both surfaces in leaky ghosts. This strategy resides basically on three assumptions, namely that:

1. In intact cells the agent is confined to the outer surface, i.e., is impermeable.

2. In ghosts the disposition of the membrane constituents is identical to that in the intact cell.

3. The differences between ghosts and intact cells are due to the sidedness or "accessibility" of a membrane constituent and not to differences in reactivity resulting from perturbations induced by the ghost preparation.

The justification of these assumptions, the conceptual and methodical basis of the approach in general, and its pitfalls have recently been discussed by CARRAWAY (1975).

The agents used for studying the transverse disposition fall into two classes:

1. Labels or markers, i.e., compounds which are bound covalently to reactive sites of membrane constituents, such as amino-, sulfhydryl- or carboxyl-groups etc.

2. Enzymes which cleave membrane constituents or catalyse their modification by certain agents

The *disposition in the plane of the membrane* has been investigated mainly by two techniques:

1. Crosslinking of membrane constituents by bifunctional reagents. This approach suffers from the drawback that the absence of crosslinking does not exclude nearest neighbor relationships, since unfavorable reaction conditions (e.g., steric exclusion or charge repulsion) or the exclusive formation of monosubstituted derivatives may prevent the crosslinking of nearest neighbors

2. Ultrastructural analysis by electron microscopy. Due to the limited resolution of the presently available techniques, this approach has been applied mainly to the localisation of proteins and protein complexes

The *physical state of membrane constituents* can be investigated by spectroscopic techniques, such as electron spin resonance (ESR) (KEITH et al., 1973), nuclear magnetic resonance (NMR) (LEE et al., 1974), x-ray (GULIK-KRZYWICKI, 1975), fluorescence (AZZI, 1975), and optical rotatory dispersion (ORD) (CHAPMAN, 1968; URRY, 1972) or by differential scanning calorimetry (DSC) (CHAPMAN, 1975). These methods monitor the behavior of either a natural membrane constituent or an external "probe" inserted into the membrane. In contrast to the enzyme and label techniques the results are not selfsustaining, but can only be interpreted in relation to data for artificial lipid bilayers or bulk lipid phases of known composition.

C. The Lipid Domain

1. Disposition

Transverse arrangement. The charged amino-phospholipids (PS, PE) of the erythrocyte membrane are supposed to be located preferentially at the cytoplasmic, the neutral ones (PC, Sph) at the outer surface (Fig. 5). This concept first emerged from labelling studies with the amino-reagent, formylmethionyl sulfone methylphosphate (BRETSCHER, 1972), which proved to react with PE in ghosts but not in intact cells. More quantitative studies by GORDESKY and MARINETTI (1973) using the very slowly penetrating amino reagent 2,4,6-trinitrobenzenesul-

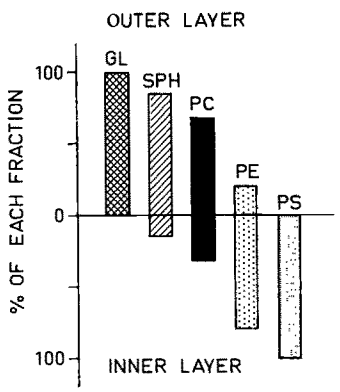

Fig. 5. Asymmetric disposition of polar lipids in the human erythrocyte membrane. Based on results of VERKLEIJ et al. (1973) and STECK and DAWSON (1974)

fonate (TNBS) demonstrated only 30% of the PE and none of the PS to react in intact cells, in contrast to approximately 95% of the PE and at least 50% of the PS in leaky ghosts. The straightforward interpretation of these results in terms of PE asymmetry (30% outer surface, 70% inner surface), however, remained inconclusive due to the finding of the same authors (GORDESKY et al., 1973) that a permeable (KRUPKA, 1972) amino reagent, 1-fluo-2,4-dinitrobenzene (FDNB) failed to label all the PE and PS in intact cells and ghosts. The conclusion, that factors other than sidedness, namely reactivity or local environment, contribute to the labelling pattern is also supported by the recent finding (HAEST and DEUTICKE, 1975) that TNBS labeling of PE in intact erythrocytes is enhanced by procedures disturbing energy metabolism and membrane protein integrity. An energy-dependent "shielding" of amino-phospholipids by membrane protein has been proposed on the basis of these results.

The label techniques available only detect the two amino-phospholipids PE and PS. The application of phospholipases offers wider possibilities. As is evident from Table 2, a number of such enzymes, when incubated with intact erythrocytes, cleave a certain amount of phospholipids without causing lysis. Since enzyme proteins will not penetrate the membrane prior to lysis, the phospholipids hydrolysed may be assigned to the outer membrane layer, provided that no measurable flip-flop of phospholipids (see below) occurs. Experiments with leaky ghosts demonstrate the principal susceptibility of the membrane phospholipids to the phospholipases.

Sequential application of phospholipase A_2 (*Naja naja*) and sphingomyelinase (VERKLEIJ et al., 1973) provides the most convincing evidence for a phospholipid asymmetry in human erythrocytes: A maximum of 48% of the total phospholipids, which could just constitute one leaf of a bilayer, were degraded, comprising 76% of the PC, 82% of the sphingomyelin, and 20% of the PE. Data on external phospholipid cleavage in sealed inside-out vesicles (KAHLENBERG and BANJO, 1972), internal phospholipid degradation in resealed ghosts (ZWAAL et al., 1975) from human erythrocytes, and results obtained on other mammalian erythrocytes (ZWAAL et al., 1973) qualitatively support the asymmetry concept. On the other hand, phospholipids assigned to te outer membrane layer are resistant to certain phospholipases in the intact cell, although readily attacked in ghosts

Table 2. Susceptibility of erythrocyte membrane phospholipids to various phospholipases

Phospholipase, source	Study object	Refs.	Phospholipids degraded (% of single fraction)	% of total phospholipids degraded	Comments
Phospholipase A_2 (*Naja naja*)	Intact cells (human) (1 h)	1	PC 68 S 0	20	
Phospholipid → Lysophospholipid + fatty acid	Dodge ghosts (human)	1, 2	PC 100 PE 100 PS 100 S 0	70–74	
	Sealed inside-out vesicles (human)	7	PC 50 PE 100 PS 100 S 0	~55	Vesicles prepared according to STECK (1972)
Phospholipase A_2 (pancreas)	Intact cells (human) (1 h)	3	none	< 5	
	Dodge ghosts	3	PC 100 PE 100 PS 100 S 0	~70	Rate of degradation PS > PE > PC
	Resealed ghosts (enzyme inside) (35 min)	10	PS 25 PE 50 PS 65	30–35	
Phospholipase A_2 (bee venom)	Intact cells (human)	3	PC 55 PE 9 PS 0 S 0	19	
Phospholipase C (*Bac. cereus*)	Intact cells human, bovine, porcine (1 h)	2, 5	none	0	
Phospholipid → Diglyceride + phosphorylcholine	Dodge ghosts	2, 3	PC 100 PE 100 PS 90–100 S 0	70	
	Reconstituted ghosts	4	not determined	17	
	Intact cells (rat)	11	PC 35 PE 26 PS 0 S 0	20	Lysis occurred during prolonged exposition to the enzyme
Phospholipase C (*Cl. perfringens*)	Dodge ghosts (human)	6	PC 100 PE 95 PS < 10 S	70	PS is no substrate for this phospholipase
	Intact cells (human)	9	PC 60 PE 0 PS 0 S 60	30	
	Sealed inside-out vesicles	7	PC 46 PE 85 S 50 PS 0	50	

Table 2 (continued)

Phospholipase, source	Study object	Refs.	Phospholipids degraded (% of single fraction)	% of total phospho-lipids degraded	Comments
Sphingo-myelinase C (*S. aureus*)	Intact cells (human)	1, 5	S 85	20	Other phospholipids are insensitive to this enzyme
Sphingomyelin →ceramide + phos-phorylcholine	(porcine) (bovine) (ovine)		S 79 S 50 S 58	20 18 30	
	Dodge ghosts (human)	1	S 100	25	
Phospholipase A_2 (*Naja naja*) + sphingomyelinase (added after 60 min)	Intact cells (human)		PC 76 PE 20 PS 0 S 82	48	Longer incubation does not produce further breakdown
	Dodge ghosts		PC 100 PE 100 PS 100 S 100	100	
Phospholipase D (cabbage)	Intact cells (human)	8	none	0	
	Dodge ghosts		PC 90 PE 52 PS 20 S 0	45	
Phospholipid →phosphatidate + choline	Resealed ghosts	8	PC 25 PE < 10 PS 0 S 0	13	Ghosts prepared according to BODEMANN and PASSOW (1972)

(1) VERKLEIJ et al. (1973), (2) KAHLENBERG and BANJO (1972), (3) ROELOFSEN et al. (1971), (4) LASTER et al. (1972), (5) COLLEY et al. (1973), (6) COLEMAN et al. (1970), (7) KAHLENBERG et al. (1974), (8) HAEST (1975) unpublished results, (9) ALLAN et al. (1975), (10) ZWAAL et al. (1975), (11) GAZITT et al. (1976).

(see Table 2). Such differences clearly show that, besides the disposition of a substrate, the specificity of a particular enzyme, and its accessibility to the substrate have to be considered in cleavage studies. In the case of interfacial reactions these factors may depend on steric properties (VERGER et al., 1973) as well as on the area per substrate molecule, which varies with the surface pressure (ZWAAL et al., 1974, 1975). The relevance of this parameter has been clearly demonstrated in model studies on the phospholipase sensitivity of phospholipid monolayers varying in composition and surface pressure (DEMEL et al., 1975). The results indicate that the lipids in the outer layer of the erythrocyte membrane are exposed to a lateral surface pressure between 31 and 35 dynes/cm.

The phospholipid asymmetry requires stabilisation. An asymmetry merely resulting from membrane biosynthesis could not last for the erythrocyte lifetime

due to the flip-flop of phospholipids, dissipating the asymmetry. Although in artifical lipid bilayers a flip-flop, i.e., a head-over-tail rotation of the phospholipid across the membrane occurs only very slowly ($T_{1/2} > 11$ days) (ROTHMAN and DAWIDOWICZ, 1975), its rate in the erythrocyte membrane has not yet been measured. Results of RENOOIJ et al. (1974) suggest that it is not a very rapid process. Recent studies (HAEST and DEUTICKE, 1976a, b) suggest that protein-lipid interactions, presumably at the inner membrane surface, stabilize the phospholipid asymmetry.

Glycolipids are also arranged highly asymmetric. In intact cells treated with galactose oxidase and ^{3}H-borohydride, STECK and DAWSON (1974) found all the labeled lipid carbohydrate on the outer membrane surface. Since erythrocyte glycolipids are sphingolipids, this finding may imply, in view of the asymmetric distribution of sphingomyelin (Fig. 5 and Table 2), that sphingolipids are largely confined to the outer membrane layer.

The transverse disposition of cholesterol is an open problem: Recent analyses on half layers of erythrocyte membranes produced by freeze-fracturing (FISHER, 1976) have provided some evidence that cholesterol may be localized preferentially in the outer layer.

Such an asymmetry would have to be stabilized, unless the flip-flop of cholesterol is an extremely slow process. Data on this subject are controversial as yet: A rather rapid flip-flop in sonicated egg lecithin/cholesterol vesicles ($T_{1/2} \sim 70$ min) has been inferred from results obtained with a fluorescent cholesterol analogue, sterophenol, which differs from cholesterol only in having an aromatic A ring (SMITH and GREEN, 1974). On the other hand, POZNANZKY and LANGE (1976) have obtained evidence that in sonicated dipalmitoyl lecithin/cholesterol vesicles the flip-flop of cholesterol between the membrane half layers is probably a very slow process ($T_{1/2} > 6$ days).

Lateral arrangement. In fluid lipid bilayers such as the erythrocyte membrane (see below) the constituents have a high lateral mobility. Diffusion coefficients in the range of 10^{-7}–10^{-8} cm^2/s (EDIDIN, 1974) should favor a random distribution of the phospholipids in the plane of the half layers. Experimental data, however, are not yet available. Attempts to study nearest-neighbor relationships by crosslinking of amino-phospholipids (MARINETTI et al., 1974; MARINETTI and LOVE, 1974; MARFEY and TSAI, 1975) have provided only inconclusive results.

The arrangement of cholesterol in the plane of the membrane is unknown. A preferential localisation around the largest circumference of the erythrocyte (MURPHY, 1965) as well as the formation of patches (HIGGINS et al., 1974) have been claimed on the basis of electron microscopic data. The high lateral mobility of cholesterol in hydrophobic phases (10^{-7} cm^2/s, STROEVE and MILLER, 1975) makes a random distribution more likely.

2. Physical State

a) General Considerations and Predictions

Phospholipids in the presence of excess water form ordered structures, e.g., bilayers (LUZZATI, 1968; TARDIEU et al., 1973) with temperature- and milieu-

dependent properties arising from their amphipathic nature and the flexibility inherent in the structure of their hydrocarbon moiety. In the range of biologically relevant temperatures such ordered phospholipid structures are present either in a "gel-state"—in which the molecules have a somewhat higher degree of freedom than in a truly solid crystal—, or in a "liquid-crystalline state," which combines a high degree of flexibility, rotational freedom and lateral mobility of the single molecules with the preservation of an ordered coherent structure of the whole lipid layer (TRÄUBLE, 1971a, 1972; CHAPMAN, 1975). The transitions between these two states occur at characteristic temperatures depending on the nature of the polar and the apolar part of the phospholipid. At the transition temperature, the "fluidity" of the bilayer, which is related to the flexibility of the alkyl chains, changes profoundly, in contrast to less pronounced changes of fluidity in other ranges of temperature. In the range of the transition, gel and liquid-crystalline phases coexist.

Fluidity is a parameter inversely related to viscosity. Thus, the fluidity of the bilayer interior will influence the intramembrane diffusion coefficient of permeating solutes, in some analogy to the STOKES-EINSTEIN relationship

$$D = \frac{kT}{6\pi r\eta} \tag{1}$$

(r = radius of diffusing molecule, η = viscosity, k = Boltzmann constant), although this relationship is probably not applicable in an anisotropic liquid-crystalline phase composed of "solvent" molecules large in relation to the diffusing solute.

The fluidity of the bilayer interior can be assessed by measuring "microviscosities." They are determined by comparing viscosity-dependent spectral parameters, such as the fluorescence depolarisation of probes, in bilayer systems and in bulk systems (usually oils) of known viscosity. Values between 50 and 400 cp have recently been compiled by AZZI (1975).

In view of the relationship between fluidity and diffusion coefficient, the characterization of the molecular parameters influencing membrane fluidity is of considerable interest in the present context. From a large number of investigations using spectroscopic, calorimetric, and monolayer techniques the following principles have emerged:

1. The flexibility of the hydrocarbon chains in a phospholipid bilayer decreases with increasing length of the hydrocarbon chains, due to a tighter packing (DEMEL et al., 1967; LEE et al., 1974). Membrane fluidity diminishes, as indicated by "microviscosity" measurements (compiled by AZZI, 1975). The gel to liquid-crystalline transition is shifted to higher temperatures (CHAPMAN, 1975).

2. The flexibility of the hydrocarbon chains increases with rising number of cis-double bonds per chain (LEE et al., 1974), since such cis-, in contrast to trans- double bonds, impose an irregularity on the packing of the chains. The fluidity rises (AZZI, 1975), the transition temperature decreases (CHAPMAN, 1975). Due to these relationships the "mean chain length" and the "double bond index" mentioned in Section IIB can be used as rough indicators of membrane fluidity.

3. The flexibility decreases from the glycerol backbone towards the terminal methyl group region of the phospholipid hydrocarbon chains. This creates gradients of flexibility from the surface to the core of bilayer membranes. In contrast to earlier measurements indicating a linear gradient (HUBBELL and MCCONNELL, 1971) it seems now to be clear (SEELIG and SEELIG, 1974), that the "order parameter," which quantifies the degree of flexibility, is constant over the first 9 C-atoms and only then decreases towards the core of the membrane.

A phenomenon related to the presence of such flexibility gradients is the formation of "kinks" in fluid lipid bilayers, transient structural defects (holes) between two hydrocarbon chains, which arise from rotational trans-gauche isomerizations around two C-C bonds separated by one trans bond (TRÄUBLE, 1971b, 1972; SEELIG and SEELIG, 1974).

4. The nature of the polar headgroups influences the fluidity of phospholipid bilayers as indicated, e.g., by some 40° C differences between the transition temperatures of PE's and PC's having identical acyl chains (CHAPMAN, 1975). The configurations of the different head groups, which influence charge and polarity and thereby the diffusional resistance at the membrane surface are still a matter of debate (LEE, 1975).

5. Complex mixtures of phospholipids differing in chain length, saturation and head group structure, as present in biomembranes, usually exhibit a very broad phase transition (LADBROOKE, 1968; BLAZYK and STEIM, 1972). This indicates the coexistence of gel and liquid-crystalline phases over an extended range of temperatures (CHAPMAN, 1975). The presence of two phases may go along with lateral phase separation, the gel phase coalescing in clusters, surrounded by liquid-crystalline areas (CHAPMAN, 1975; SHIMSHIK and MCCONNELL, 1973). The presence of cholesterol prevents these phenomena (see below).

6. Cholesterol considerably affects phospholipid bilayers (OLDFIELD and CHAPMAN, 1972b; JAIN, 1975). It increases the order of packing and reduces the mean acyl chain flexibility of phospholipids above the phase transition and thus lowers the bilayer fluidity (CHAPMAN and PENKETT, 1966; OLDFIELD and CHAPMAN, 1972b). This effect is related to the "condensing effect" observed in monolayers (DEMEL et al., 1967). Phospholipids below the temperature of the phase transition are fluidized by cholesterol (OLDFIELD and CHAPMAN, 1971). The transition between gel and liquid-crystalline state is suppressed progressively with increasing cholesterol content. As a result, phospholipid-cholesterol bilayers assume an "intermediate fluid condition" (LADBROOKE et al., 1968) between that of the gel and the liquid-crystalline state of the phospholipid. The same is true for glycolipids (OLDFIELD and CHAPMAN, 1972a). As an exception, highly unsaturated phospholipids are not affected by cholesterol (DEMEL et al., 1972c). This "equalizing" effect of cholesterol also prevents the above-mentioned lateral phase separations in complex phospholipid systems within the thermal phase transition, by fluidizing the gel and rigidifying the liquid-crystalline phase.

Concerning the molecular basis of the cholesterol-phospholipid interaction, three aspects deserve comment:

a) Stoichiometry. On the basis of NMR measurements, DARKE et al. (1972) have proposed 1:1 complexes between cholesterol and phospholipids giving rise to a phospholipid and a phospholipid/cholesterol phase in systems contain-

ing less than 50 mole-% cholesterol. In contrast, 2:1 complexes of phospholipid with cholesterol resulting from configurational properties of the two components were proposed on the basis of x-ray diffraction (ENGELMAN and ROTHMAN, 1972) and calorimetric (HINZ and STURTEVANT, 1972) data (cf. ROTHMAN and ENGELMAN, 1972).

b) Local differences of interaction. Cholesterol probably acts differently on different segments of liquid-crystalline acyl chains. The first 8–10 CH_2 groups are immobilized by the rigid sterol nucleus, the terminal CH_2 groups of the phospholipid and the adjacent aliphatic tail of cholesterol are highly mobile (DARKE et al., 1972; STOFFEL et al., 1974; KROON et al., 1975). The "intermediate fluid condition" in presence of cholesterol thus seems to be the statistical description of a gradient of fluidity.

c) Specificity. The molecular requirements for an effect on membrane phospholipids include the β-position of the OH group at position 3 as well as the planar nucleus of cholesterol (DEMEL et al., 1972a, b). The interaction with phospholipids may involve complex formation (DARKE et al., 1972) by hydrophobic interaction (DE KRUYFF et al., 1973) and/or hydrogen-bonding via the OH group of cholesterol (VERMA and HOELZL-WALLACH, 1973; BROCKERHOFF, 1974; HUANG, 1976). A steric accomodation of cholesterol by cavities of the phospholipid molecules has also been claimed (MARSH and SMITH, 1973; SHAH, 1973). From calorimetric measurements DE KRUYFF et al. (1974) have inferred that in complex systems cholesterol interacts preferentially with more unsaturated or short-chained species of phospholipids. This could imply inhomogeneities of cholesterol distribution in complex lipid membranes with a low cholesterol content.

In addition to its effects on membrane fluidity, cholesterol may also affect the dipole potential at the interface between phospholipid membranes and the adjacent water phase (SHAH, 1973; SZABO, 1974), the interior becoming more positive with increasing cholesterol content.

b) Experimental Results

The erythrocyte membrane contains polar lipids (phospholipids and glycolipids) differing widely in their head group and acyl chain composition, as well as cholesterol. The molar ratio cholesterol/polar lipids amounts to about 0.75[1] (DEUTICKE and RUSKA, 1976). The elucidation of the physical state of this complex system is still at its beginning.

HUBBEL and MCCONNELL (1969), using spin-labeled fatty acids demonstrated a fluidity gradient in dog erythrocyte membranes, analogous to that in soybean lecithin bilayers. The spin label was more strongly immobilized in the erythrocyte than in the artificial membrane probably due to the cholesterol content and the higher saturation of the erythrocyte lipid bilayer (NELSON, 1972) as compared to soybean lecithin.

[1] Although the ratio cholesterol/*phospholipid* is usually given in studies relating to the influence of cholesterol on biomembranes, the ratio cholesterol/*polar lipids* seems better suited for such considerations since glycolipids also interact with cholesterol (OLDFIELD and CHAPMAN, 1972a).

Proton NMR, a very successful tool in case of artificial membranes, has not contributed decisively to the characterization of the erythrocyte membrane lipid domain due to the interference of protein signals (KAMAT et al., 1970). ^{2}H, ^{13}C, ^{19}F, and ^{31}P-NMR may permit the successful application of this technique to intact membranes (METCALFE et al., 1971; LEE et al., 1974; STOCKTON et al., 1975; MCLAUGHLIN et al., 1975).

The fluidity of the hydrophobic domain of the erythrocyte membrane corresponds to that of phospho- and glycolipids in presence of cholesterol: Addition of cholesterol to a cholesterol-free dispersion of erythrocyte lipids reduces the motional freedom of the acyl chain domain as indicated by spin label studies (BERGER et al., 1971). Moreover, a phase transition which could not be detected in total lipid extracts and ghost membranes by calorimetric (LADBROOKE et al., 1968) and x-ray-techniques (GOTTLIEB and EANES, 1974) became evident after removal of cholesterol. The broad range of this transition (2–40° C) indicates that cholesterol is required to prevent the formation of gel and liquid-crystalline areas in the physiological temperature range (Sect. IIIC2a).

The microviscosity of the erythrocyte membrane lipid phase has been deduced from measurements of fluorescence depolarisation. Values of 132, 119, and 380 cp (ghost membrane) and 250 cp (intact erythrocytes) have been reported by RUDY and GITLER (1972), ALONI et al. (1974) and FEINSTEIN et al., (1975) in agreement with data for artificial lipid membranes and other biomembranes (AZZI, 1975). Although fluorescent probes may not always monitor the average viscosity of the hydrophobic domain (COGAN et al., 1973) the general agreement between data obtained with different probes seems to indicate that the intramembrane viscosity can be approximated by this technique.

Altogether, these data indicate that the physical state of the erythrocyte membrane lipid domain shares important features with artificial bilayers and may thus have in common with such model membranes characteristics of permeability. The heterogeneity of the membrane phospholipids, as well as the possible transverse asymmetry (Sect. IIIC1) suggest further characteristics not yet verified experimentally:

1. The average fluidity of the phospholipids in the two sheets of the lipid bilayer may differ considerably in view of their different double bond indices (see Table 3). These differences, however, could be compensated by two mechanisms:

a) The head groups of the charged amino-phospholipids on the inner layer could reduce the mobility of the acyl chains compared to PC acyl chains, as indicated by the higher transition temperatures of PE's (CHAPMAN, 1975). A more homogeneous fluidity would thus be obtained at the expense of differences in surface charge.

b) Different fluidities of the two membrane layers might theoretically be compensated by a asymmetric distribution of cholesterol. A fluidizing effect of an excess of the sterol in the, originally more rigid, outer layer would be in line with recent observations by FISHER (1976).

2. Differences in membrane fluidity may also be expected in erythrocytes of different species in the light of the phospholipid differences shown in Figure 1. These differences should arise particularly from the varying proportions of

Table 3. Approximate double bond indices for inner and outer leaves of phospholipid bilayers of erythrocyte membranes. (Values calculated from fatty acid patterns of individual phospholipids under the assumption that PC and Sph are located in the outer layer, PE and PS in the inner layer)

	Outer layer (PC, Sph)	Inner layer (PE, PS)	Refs.
Man	0.65	2.22	(1)
Rat	0.64	2.03	(2)
Rabbit	0.67	1.42	(3)
Camel	0.49	1.02	(4)
Pig	0.47	0.76	(2)
Sheep	0.32	0.41	(2)

Calculated from fatty acid determinations by: (1) GERCKEN et al. (1972), (2) NELSON (1972), (3) GERCKEN and BROCKMANN (1969), (4) LIVNE and KUIPER (1973).

PC and sphingomyelin. Bilayers of sphingomyelin have a 5 times higher microviscosity than bilayers of PC (SHINITZKY and BARENHOLZ, 1974). Moreover, PE in different animal species also varies in its fatty acid pattern (NELSON, 1972).

D. The Protein Domain

1. Disposition

Transverse arrangement. The disposition of membrane proteins relative to the lipid bilayer has been investigated by the use of labels differing in permeability and reactivity as well as by proteolytic or protein-modifying enzymes. Presently accepted patterns of membrane protein disposition in the erythrocyte membrane are summarized in Table 4. Although all data obtained by label and enzyme techniques rely on more or less well proven permeability properties and specificities of the modifiers, the agreement between the results makes a transmembrane arrangement of at least 5 protein fractions, namely PAS 1-4 and band 3, very likely. All these potential transport pathways contain carbohydrate. Additional polypeptides seem to be accessible from both sides of the membrane by recently developed labels. This concerns in particular bands 2.1–2.6 (mol wt 150–170,000 daltons (STAROS and RICHARDS, 1974). Thus, the number of membrane-spanning polypeptides may still increase.

Results obtained with labels and enzymes are species dependent. Band 3, e.g., is accessible to pronase in human but not in ox and sheep erythrocytes (TRIPLETT and CARRAWAY, 1972). In case of the major glycoprotein fraction, similar species variabilities may be due not to differences in the disposition of this protein but rather result from an inherent resistance to proteolytic enzymes or a shielding effect of the carbohydrate (CARRAWAY et al., 1975).

Table 4. Experimental evidence for asymmetric disposition of erythrocyte membrane proteins, as obtained by markers or enzymes

Marker, enzyme	Modified constituents	Attack from outside (I = intact cells, R = resealed ghosts, L = leaky ghosts, I O = inside-out vesicles)	Attack from inside or both sides	Side effects on permeability	Comments	Refs.
Diazobenzene sulfonate	NH_2, SH	Band 3 (I) PAS 1 (I)	n.t.	Enhances cation permeability, erythritol permeability probably unaltered		1, 2
Formylmethionyl methyl phosphate	NH_2 (phenyl-OH)	Band 3 (I) PAS 1 (I)	All detectable bands (L)	n.t.	Labeling at pH 10	3
Trinitrobenzene sulfonate	NH_2	Band 3 (I) PAS 1 (I)	All detectable bands (L)	Enhances cation permeability (6), reduces anion permeability (7), inhibits facilitated glucose fluxes (4)	Permeates through the membrane slowly (8)	5
4,4′-Diisothiocyano-2,2′-stilbene disulfonate	NH_2 (SH, imidazole, phenyl-OH)	PAS 1 (I, R) (weakly) Band 3 (I, R)	All detectable bands (L)	Inhibits anion permeability (11)	Probably binds to other components on the outside (13)	11,12
Pyridoxalphosphate + borohydride	Lysyl-ε-NH_2	Band 3 (I, R) PAS 1–4 (I, R)	All detectable bands (L, I[a])	Inhibits anion permeability. K^+ permeability and facilitated glucose fluxes not affected	Penetrates the membrane very slowly via the anion transfer system	12
Isethionylacetimidate (IAI) Ethylacetimidate (EAI)	NH_2	Band 2.3 (I) (170,000 daltons) Band 3 (I) Band 4 (I) (78,000 daltons) PAS 1 (I)	Bands 1–5 (L, I) PAS 1 (L, I)	K^+-permeability, anion permeability (15) and glucose fluxes unaffected	IAI is regarded as impermeable, EAI as a permeable label	14
N(-4-azido-2-nitrophenyl) 2-aminoethyl sulfonate (= NAP-taurine) + $h \cdot \nu$	↓ C–H	Band 2.1–2.6 (I, R)[b] Band 3 (I, R) PAS 1 (I, R) Band 4.2 (R, not I)	All detectable bands (L) All detectable bands except 2.6 and 4.1 (I)[c]	Inhibits anion permeability (18)	Photolysis of NAP-taurine produces a nitrene. The labeling pattern of resealed ghosts differed from that of intact cells depending on lysis conditions	16, 17

Lactoperoxidase, $H_2O_2-I_2$	Phenyl-OH, imidazole	Band 3 (I) PAS 1–4 (I)	Band 1 (R) Band 3 (R) PAS 1, 3, 4 (R)	Probably slight enhancement of K^+ permeability (19)	The labeling of PAS 1 from the inside has been disputed (21, 22), this could be explained by the absence of tyrosyl (histidine) residues in the cytoplasmic domain of PAS 1 (23)	19,20
Galactose oxidase, borohydride	Galactose, Galactosamine	Band 3 (I) PAS 1–4 (I)	None (I)	Unknown		24, 25
Galactose oxidase, methionine sulfone hydrazide-Mn^{++}	Galactose, Galactosamine	Band 3 (I) PAS 1–4 (I) Additional unidentified bands		Unknown		26
Pronase		Band 3 (I) PAS 1–4 (I)	All detectable proteins (cleavage of PAS 1 is disputed (24) (L)	Enhances cation permeability, inhibits anion permeability, slightly inhibits glucose fluxes	Band 3 (95,000 daltons) is cleaved into 2 fragments, one "stable" (65,000) firmly bound to the membrane, one labile (35,000)	27–29
Trypsin		PAS 1–4 (I)	All detectable proteins (L) Bands 2.1–2.6, 3, 4.1, 4.2, 7 (IO) PAS 1–4 not digested		Band 3 is cleaved by internal trypsin into 2 fragments of 58,000 and 48,000 daltons, firmly retained in the membrane (30)	28, 29
Chymotrypsin		Band 3 (I) PAS 1–4 (I)	All detectable proteins (L) Bands 2.1–2.6, 3, 4.1, 4.2, 7 (IO)		Split products as with pronase	28, 29

[a] Cells loaded with label for 15 h at pH 6.6, 37°.
[b] Photolysed at 0°.
[c] Cells loaded with label at 37°, washed and photolysed at 0°.

(1) BERG (1969), (2) CARRAWAY et al. (1971), (3) BRETSCHER (1971a, b), (4) JUNG and RAMPAL (1975), (5) STECK (1972), (6) KNAUF and ROTHSTEIN (1971a), (7) DEUTICKE (1975b), (8) GORDESKY et al. (1975), (9) SCHMIDT-ULLRICH et al. (1973), (10) CARRAWAY (1975), (11) CABANTCHIK and ROTHSTEIN (1974a), (12) CABANTCHIK et al. (1975a, b), (13) PASSOW (1976), (14) WHITELEY and BERG (1974), (15) CABANTCHIK (1976), (16) STAROS and RICHARDS (1974), (17) STAROS et al. (1974, 1975), (18) ROTHSTEIN et al. (1976), (19) PHILIPPS and MORRISON (1971), (20) MUELLER and MORRRISON (1974, 1975), (21) SHIN and CARRAWAY (1974), (22) REICHSTEIN and BLOSTEIN (1975), (23) TOMITA and MARCHESI (1975), (24) GAHMBERG and HAKOMORI (1973), (25) STECK and DAWSON (1974), (26) ITAYA et al. (1975), (27) BENDER et al. (1971), (28) TRIPLETT and CARRAWAY (1972), (29) CABANTCHIK and ROTHSTEIN (1974b), (30) PASSOW et al. (1974).

The arrangement of glycophorin (PAS 1) relative to the lipid domain has been established by SEGREST et al. (1973) using lactoperoxidase labeling as well as partial proteolytic degradation:

The N-terminal segment of the peptide chain, which carries the carbohydrate residues, seems to be located on the outside of the membrane, the C-terminal to protrude into the cytoplasmic space. The segment which anchors the molecule to the hydrophobic core of the membrane (34 amino acids, 37,000 daltons) contains a very high proportion of hydrophobic amino acids and mimics the lipid bilayer composition by a polar-apolar-polar series of its residues.

Preliminary information has become available concerning the arrangement of band 3. On the basis of iodination and proteolytic cleavage studies, JENKINS and TANNER (1975) have suggested an S-shaped arrangement of the polypeptide, which is assumed to transverse the hydrophobic core of the membrane at least twice. Thus, a stabilization of its tertiary structure by intramolecular interactions may well occur, giving rise to a structure suited for transport functions.

Lateral arrangement. The arrangement of the membrane-spanning proteins in the plane of the membrane and their interaction with extrinsic proteins have been studied by freeze-fracture electron microscopy, antibody and agglutinin-labeling, and crosslinking reagents.

Freeze-fracturing of erythrocytes or ghosts exposes the hydrophobic domain of the membrane (PINTO DA SILVA and BRANTON, 1970; WEINSTEIN et al., 1970). The preferential fracture plane is provided by the terminal methyl groups of the lipid alkyl chains. The two complementary fracture faces are characterized by a smooth or slightly granulated matrix, interrupted by randomly distributed particles (Fig. 6) having an average diameter—on the carbon-platinum replicas—of about 80 Å (WEINSTEIN, 1974).

The disappearance of these "membrane-intercalated particles" after treatment of membranes with proteolytic enzymes (ENGSTRÖM, 1970; YAMANAKA and DEAMER, 1976) has been taken as evidence that they are integral proteins. This interpretation is supported by the finding that glycophorin and its hydrophobic segment, incorporated into artificial bilayers, produce particles on the fracture faces (SEGREST et al., 1974). The particles are distributed unevenly between both fracture faces. 2,600/μ^2 on the inner fracture face (WEINSTEIN, 1974; KIRK and TOSTESON, 1973) and 1,200–1,400/μ^2 on the outer fracture face have been reported for human erythrocytes[2]. Values for other animal species differ with respect to the inner fracture face, but are suprisingly invariant for the outer fracture faces (KIRK and TOSTESON, 1973).

The polypeptides in the membrane-intercalated particles have been characterized making use of the fact that areas corresponding to these particles can be visualized on the *outer* membrane surface by the aid of the hemagglutinins mentioned in Section II C. Glycophorin is likely to be a constituent of the particles as indicated by binding studies with ferritin-labeled wheat germ agglutinin (TILLACK et al., 1972) and *Agaricus bisporus* lectin (TRICHE et al., 1975). The presence of the 95,000-dalton polypeptide (band 3) in the particles is suggested by the binding of concanavalin A to the particle region (PINTO DA SILVA and NICOLSON, 1974; BÄCHI and SCHNEBLI, 1975).

[2] Particles numbers obtained in different laboratories vary considerably. As suggested by LIMBRICK and KNUTTON (1975) such variations could arise from the use of different fracturing devices.

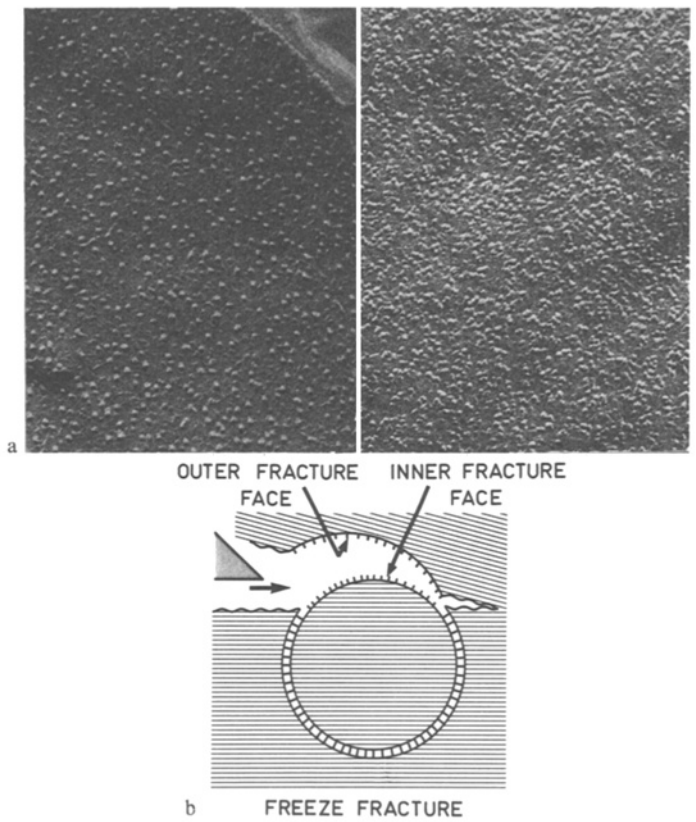

Fig. 6. Aspect of erythrocyte membrane interior as visualized by freeze-fracturing. Bovine erythrocytes, final magnification: × 150,000. For details, see DEUTICKE and RUSKA (1976)

The arrangement of the membrane proteins relative to each other has also been studied by the application of crosslinking reagents such as glutaraldehyde, bifunctional imidoesters, e.g., dimethyladipimate and dimethylmalonimidate, and mild oxidants, e.g., Cu^{++}/o-phenanthroline.

According to STECK (1972) and WANG and RICHARDS (1974) the 95,000-dalton protein may be present in the membrane as a dimer that can be linked covalently by disulfide bridges at the cytoplasmic surface in ghost membranes. Moreover, the 95,000-dalton protein seems to be in close contact with band 6 (GAPDH) and band 4.2 (WANG and RICHARDS, 1974; YU and STECK, 1975b). Polypeptides in band 2.1-2.3, for which there is some indication to span the membrane (STAROS and RICHARDS, 1974; ITAYA et al., 1975), may also be present as dimers (STECK, 1974).

In view of the lectin-labeling studies one might expect the appearance of crosslinking products of glycoproteins with each other and with the 95,000-dalton protein. Oligomers of glycoproteins (PAS 1 and PAS 2) have been described by JI (1973, 1974) but were not detected by STECK (1972), WANG and RICHARDS

(1974) and CAPALDI (1973b). Crosslinking between glycoproteins and 95,000-dalton proteins has not been observed. This may imply a spatial separation of these two most abundant membrane-spanning proteins, but seems more likely to be due to a lack of suitable reaction conditions.

2. Physical State

In contrast to the lipids, membrane proteins are very incompletely characterized with respect to their conformation. Results obtained by infrared spectroscopy, optical rotatory dispersion (ORD), circular dichroism (CD), and Laser-Raman spectroscopy on isolated membranes have been interpreted in terms of negligible amounts of β-conformation (MADDY and MALCOLM, 1966; LIPPERT et al., 1975; cf., however, SINGER and MORRISON, 1972), and some 25–50% of right-handed α-conformation (LENARD and SINGER, 1966; GLASER and SINGER, 1971; URRY et al., 1971; LIPPERT et al., 1975). The remaining protein may assume some sort of random conformation. The unequivocal interpretation of the results is complicated by a number of peculiarities in the spectra, discussed by HOELZL-WALLACH and CHAPMAN (1968) and by URRY (1972). Moreover, such an overall description does not provide insight into the conformation of individual proteins as would be desired under the aspects of permeability. The same reservation is true for attempts to monitor, by spectroscopic techniques, conformational changes in erythrocyte membrane proteins or the microenvironment of functional groups subsequent to membrane perturbation or changes of environmental parameters (SONENBERG, 1969; SANDBERG et al., 1969; KIRKPATRICK and SANDBERG, 1973; HOLMES and PIETTE, 1970; SCHNEIDER and SMITH, 1970; GRAHAM and WALLACH, 1971; RIGAUD et al., 1974).

E. Lipid-Protein Interactions

Intrinsic membrane proteins, by virtue of their localization in the lipid domain of the membrane, can be expected to interact with surrounding lipids. As a consequence, membrane lipids should modify the properties of membrane proteins and vice versa. From numerous studies it has become evident that the enzymatic or transport functions of membrane proteins frequently depend on the presence of phospholipids, with varying requirements concerning the head group as well as the apolar region of the molecule (COLEMAN, 1973; HALLINAN, 1974; FOX and TSUKAGOSHI, 1973). On the other hand, intercalated proteins may considerably affect the physical state of apposed lipids (CHAPMAN, 1975; VANDERKOOI, 1974). A systematic investigation by PAPAHADJOPOULOS et al. (1975b) on model membranes has revealed different types of such protein-induced changes.

Interaction with protein renders the boundary lipids unable to participate in thermotropic phase transitions. This effect has been used to obtain quantitative estimates of the amounts of lipid affected by hydrophobic interactions with integral protein. The values scatter around 20% of immobilized lipid for a number of biomembrane systems (TRÄUBLE and OVERATH, 1973). A somewhat

lower value (about 12%) has been reported by McLAUGHLIN et al. (1975) for erythrocyte membranes.

With respect to the erythrocyte membrane, a limited number of data demonstrate the relevance of lipid-protein interaction. Na^+-K^+-stimulated ATPase is activated by PS (ROELOFSEN and VAN DEENEN, 1973) and probably inhibited by cholesterol (PETER et al., 1973; FIEHN and SEILER, 1975). Data on passive transfer processes are discussed in Sections IV C 6 and IV F.

The influence of proteins on the arrangement of membrane lipids in the erythrocyte membrane is indicated by marked changes in the accessibility of membrane phospholipids to labels and phospholipases after perturbation of membrane proteins by alcohols and SH-reagents (HAEST and DEUTICKE, 1975). Pronounced increases in the phospholipase susceptibility of PS in cells treated with protein-pertubing SH-reagents suggest a preferential interaction of this phospholipid with membrane proteins (HAEST and DEUTICKE, 1976a).

Crosslinking studies with bifunctional amino-reactive agents, although not unequivocally interpretable, are also in line with such a concept (MARINETTI and LOVE, 1974; MARFEY and TSAI, 1975). In addition, associations between glycolipids and unidentified membrane proteins have been suggested by JI (1974) on the basis of crosslinking studies.

IV. Diffusional Transport Pathways of the Erythrocyte Membrane

A. General Considerations

The data on the nature, arrangement and physical state of the erythrocyte membrane constituents provide a framework into which data on transport characteristics can be inserted:

1. In order to assign permeating solutes to the *two possible transmembrane pathways,* namely the lipid bilayer and the intrinsic proteins
2. To establish structure-function relationhips of the respective pathway
3. To characterize the interaction between a permeant and its pathway.

Data obtained in transport studies differ in their relevance for the characterization of transfer pathways. Kinetic characteristics such as the type of concentration dependency or permeabilities, reflection coefficients, affinity constants, and transport maxima, may help to decide whether the number of transport sites is limited but can also provide information on structural aspects if transformed into molecular and physicochemical properties of the permeant (molecular volume and configuration, lipid solubility, hydrogen-bonding capacities etc.). The influence of pH, ionic strength, and ionic milieu informs about the presence of dissociable groups.

More direct information can be obtained by the use of reversible inhibitors and activators, and from the application of inhibitors covalently bound to membrane constituents. Even this approach, however, has its limitations since a modifier will only affect transfer if its binding alters the pathway in a region

and a way relevant to the binding or the translocation of the permeant. Moreover, crucial constituents may not be detected, if they are inaccessible to a modifier for electrostatic or steric reasons. A further restriction of the usefulness of covalent modifiers results from their low specificity. Most of the "classical" reagents interact with numerous side groups. This subject has recently been reviewed by MEANS and FEENEY (1971), HIRS and TIMASHEFF (1972) and GLAZER et al. (1975).[3] Agents which combine the high specificity of certain reversible inhibitors with the irreversible binding of a covalent modifier are therefore presently the most promising tools.

Furthermore, the comparison of transfer characteristics observed in red cell membranes with those of well-defined artificial lipid membrane systems can provide valuable information. The same is true for an other strategy, namely, the comparison of transport characteristics in erythrocytes of varying membrane composition, provided either by the experimental removal or addition of membrane components, or by biological variation, e.g., due to species differences.

B. The Lipid Pathway

As outlined above, some 80% of the erythrocyte membrane are probably arranged as a lipid bilayer. In principle, this domain is pervious to all solutes. In practice, energetic barriers will not permit a significant penetration of many hydrophilic and most charged solutes, and thus convert the pathway into a barrier. This difference, however, is merely operational when principal aspects of permeability are under consideration and the subsequent discussion will deal in numerous instances with solutes which by classical standards are not regarded as typical lipophilic permeants.

1. Relationship Between Permeant Properties and Permeability

In previous studies positive correlations between lipid solubilities of solutes (as reflected by partition coefficients organic solvent/water) and their permeabilities have been used as an indicator of penetration via the lipid bilayer domain of the membrane. Due to the high penetration rates involved these studies usually required optical measurements of net movements of solutes and water. The time course of swelling or shrinking of the cells or the rate of hemolysis served to calculate the permeability coefficient P or the reflection coefficient σ (STAVERMANN, 1952) which in a non-semipermeable membrane provides a measure of solute transfer relative to total volume flow.[4]

Various procedures, based on the formalism of nonequilibrium thermodynamics (KATCHALSKY and CURRAN, 1967; STEIN, 1967; FORSTER, 1971) or earlier formulations (JACOBS, 1934) have been used in order to extract permeability or reflection coefficients from measurements of optical density changes in red

[3] In order to reduce the number of references these compilations have been cited whenever a more general description of modifier properties was intended.

[4] The reflection coefficient equals 1 when a membrane is permeable only to the solvent. It decreases toward O—or negative values—with increasing solute permeability.

cell suspensions (GOLDSTEIN and SOLOMON, 1960; SHA'AFI et al., 1970; FARMER and MACEY, 1972; HILL and COHEN, 1972; OWEN et al., 1974).

For a number of reasons (SHA'AFI and GARY-BOBO, 1973) data obtained by the different techniques do not always agree and recent reinvestigations indicate that some of the earlier reported reflection coefficients may have to be revised (LEVITT, 1974; OWEN and EYRING, 1975). Nevertheless, the data obtained by SHA'AFI et al. (1971) and NACCACHE and SHA'AFI (1973) demonstrate a positive correlation between the lipid solubility of many small nonelectrolytes and their permeability through the human erythrocyte membrane (Fig. 7). Similar results were obtained by KLOCKE et al. (1972) for the transfer of aliphatic organic acids penetrating by nonionic diffusion (Fig. 7, *insert*). The partition coefficient ether/water (K_{ether}) served as a measure of lipid solubility because the largest number of values is available for this system. The choice of the organic phase, however, in this type of diagram is not very critical, since partition

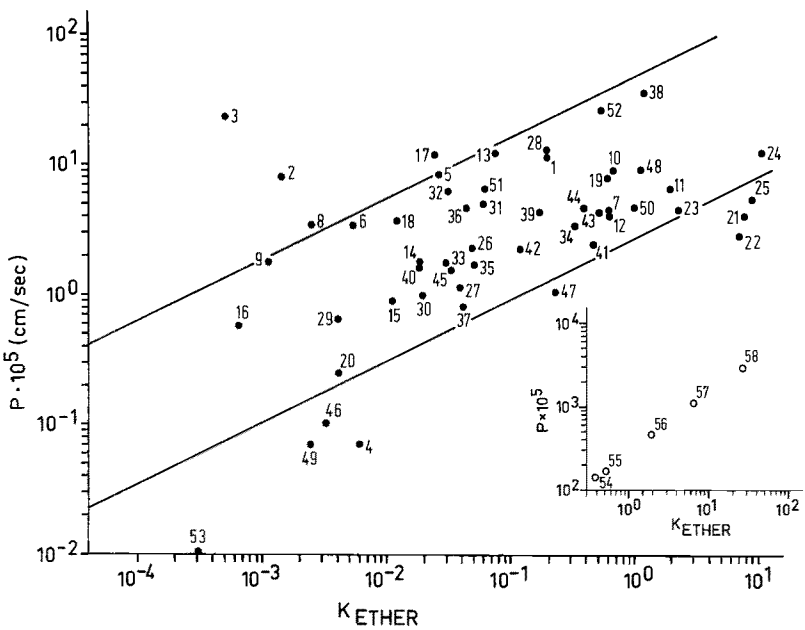

Fig. 7. Relationship between lipid solubility of non-electrolytes and their transfer across the human erythrocyte membrane. Diagram based on numbers given by NACCACHE and SHA'AFI (1973) and KLOCKE et al. (1972) *1* Methanol, *2* Formamide, *3* Urea, *4* Thiourea, *5* Ethanol, *6* Ethylene glycol, *7* Acetonitrile, *8* Acetamide, *9* Methylurea, *10*, Acetone, *11* n-Propanol, *12* Iso-propanol, *13* Ethylene glycol monomethylether, *14* 1,2-Propanediol, *15* 1,3-Propanediol, *16* Glycerol, *17* Dimethylformamide, *18* Propionamide, *19* Ethylcarbamate, *20* Ethylurea, *21* n-Butanol, *22* Iso-butanol, *23* Tert-butanol, *24* Diethylether, *25* Ethylacetate, *26* 1,3-Butanediol, *27* 1,4-Butanediol, *28* Ethylene glycol monoethylether, *29* Diethylene glycol, *30* 3-Methoxy-1,2-propanediol, *31* n-Butyramide, *32* Methylpropionamide, *33* Succinimide, *34* Succinonitrile, *35* 1,5-Pentanediol, *36* Diethylene glycol monomethylether, *37* Monoacetin, *38* Pyridine, *39* Isovaleramide, *40* Diethylurea, *41* 2,5-Hexanedione, *42* 1,6-Hexanediol, *43* 2-Methyl-2,4-pentanediol, *44* Pinacol, *45* Dipropylene glycol, *46* Triethylene glycol, *47* Diacetin, *48* Diethylene glycol monobutylether, *49* Tetraethylene glycol, *50* Triacetin, *51* Tetraethylene glycol dimethylether, *52* Triethylene glycol diacetate, *53* Malonamide, *54* Formate, *55* Acetate, *56* Propionate, *57* Butyrate, *58* Valerate

coefficients for various hydrophobic phases are to a first approximation linearly related to each other (HANSCH et al., 1968; SEILER, 1974; DIAMOND and KATZ, 1974).

In view of this dependency the diffusional flux J_n of a nonelectrolyte n should be describable by the equation:

$$J_n = P \cdot dC_n = K_n \cdot D_n \cdot dC_n/dx \tag{2}$$

where P = permeability coefficient, dC_n = the concentration difference between the water phases next to both faces of the membrane, K_n = partition coefficient (membrane lipid/medium), D_n = diffusion coefficient within the lipid pathway, dx = length of the pathway.

Although Figure 7 indicates a positive correlation of nonelectrolyte permeability to lipid solubility, a considerable scatter of the data is obvious, in particular towards permeabilities higher than expected for substances of low lipid solubility. These deviations might indicate that K_{ether} is not a valid index for the partition coefficient membrane lipid/water in case of more hydrophilic nonelectrolytes. Experimentally obtained partition coefficients for the erythrocyte membrane (compiled by SEEMAN, 1972), reveal a linear relationship between $K_{membrane}$ and K_{ether} but do not fully rule out this possibility since they were obtained only with rather hydrophobic compounds. On the other hand, the partition coefficients for a lecithin/water system (KATZ and DIAMOND, 1974) provide no indication for such deviations.

Moreover, the systematic evaluation of red cell permeabilities to nonelectrolytes differing in defined physicochemical properties (SHA'AFI et al., 1971; NACCACHE and SHA'AFI, 1973), as well as data on the dependency of lecithin/water partition coefficients on molecular properties of nonelectrolytes (DIAMOND and KATZ, 1974), indicate that the consideration of the following three parameters as determinants of penetration via the lipid phase will largely reduce the inconsistencies of Figure 7:

1. Molecular size as represented by molecular weight. Plotting $\ln P/K_{ether}$, which eliminates the influence of lipid solubility, versus the molecular weight provides a linear inverse relationship for each series of homologous nonelectrolytes, e.g., alcohols, ureas, amides etc.

2. Molecular shape, as indicated, e.g., by the cylindrical radius. Branching of nonelectrolytes reduces permeability to an extent unlikely to be accounted for by differences in partition coefficients. This points to differences in the intramembrane diffusion coefficient.

The dependency of permeability on these geometrical parameters indicates that molecules diffusing via the lipid pathway experience considerable frictional resistances within the membrane, which are related to intramembrane fluiditiy (Sect. III C 2a).

The precise mechanisms which enable molecules to move between the hydrocarbon chains are unknown. The transient formation of spaces of free volume between the chains, accessible to the diffusing molecule, seems a very appealing concept (LIEB and STEIN, 1971). One type of such temporary holes are the "kinks" (Sect. III C 2a) resulting from trans→gauche isomerisations of the hy-

drocarbon chains (TRÄUBLE, 1971b, 1972). The concentration and the mobility of such kinks, which move up and down the alkyl chains, will vary in relation to membrane fluidity.

3. As a further parameter, the *hydrogen-bonding capacity* of a nonelectrolyte influences its permeability. This parameter can be quantitated rougly by the sum of H-accepting and H-donating functions in a molecule. As was first pointed out by STEIN (1967), the penetration rates of small nonelectrolytes across the erythrocyte membrane are inversely related to their H-bonding capacity. This indicates a retarding effect of H-bonds between the permeant and bulk water in the medium or hydrogen-bonding groups in the polar head group region of the phospholipid domain. Such interactions are probably a main determinant of the "interfacial resistance"—as opposed to the "intramembrane resistance"—experienced by permeants on their way across the lipid bilayer.

In the studies summarized so far, the lipid domain of the erythrocyte membrane was treated, except for its hydrophobic nature, as a phase of unknown properties. Increasing knowledge on the organization of lipid bilayers has rendered possible, as a promising alternative, the characterization of the lipid pathway in its *dependence on environmental parameters* and the *nature of its constituents.* This approach also profits by the comparison with transport data obtained on artificial bilayers, in particular "liposomes," vesicular and spherical systems (BANGHAM et al., 1974; TOYOSHIMA and THOMPSON, 1975a).

In order to be readily interpretable, such studies require data on fluxes under steady state conditions. This prerequisite can be met by tracer techniques, which have so far only been used rarely in the field of simple nonelectrolyte diffusion (SAVITZ and SOLOMON, 1971). Unless combined with rapid sampling methods, tracer studies underly certain restrictions with respect to the transfer rates that can be measured. To characterize the lipid pathway in this alternative way, however, only few test solutes are required. Glycerol and erythritol (K_{ether} = 0.00066, 0.00011) (COLLANDER, 1949) can be used as hydrophilic, acetic acid (K_{ether} = 0.5) as a lipophilic test solute. As will become evident in the further discussion, all these compounds are suitable probes of the lipid pathway, although in certain types of erythrocytes they have also access to parallel protein pathways. In order to study the lipid pathway one either has to use erythrocytes devoid of such parallel protein pathways to a permeant, or to measure the transfer after specific blockade of the parallel pathway.

2. Influence of Environmental Parameters

Concentration of permeant. Osmotic studies on nonelectrolyte permeability suggested that nonelectrolyte fluxes increase linearly with concentration (SHA'AFI et al., 1971). Measurements of tracer fluxes of hydrophilic and lipophilic nonelectrolytes have demonstrated that this is actually true over a large range of concentrations of glycerol (Fig. 8A), erythritol and acetate in erythrocytes of various mammalian species (LA CELLE and PASSOW, 1971; WIETH, 1971; DEUTICKE, 1975). Although a linear dependency is expected for simple diffusion, it might be obscured by effects of the permeating solutes on membrane permeability: Lipophilic nonelectrolytes (e.g., alcohols, acetone, dioxane) induce membrane

perturbations (METCALFE et al., 1968; PATERSON et al., 1972) as indicated by a non-linear concentration dependency of their partition coefficients (METCALFE et al., 1968) and an enhancement of nonelectrolyte permeability (Sect. IVB3). Such effects could cause an apparent overproportional increase of diffusional fluxes with concentration for membrane-perturbing nonelectrolytes[5].

Ion milieu and pH. Variations of pH, ionic strength and anion composition do not affect the transfer of nonelectrolytes via the lipid pathway (Fig. 8B-D). Primarily this is what one would expect for an uncharged permeant. On the other hand, it has been shown (TRÄUBLE and EIBL, 1974; JACOBSON and PAPAHADJOPOULOS, 1975) that in artificial membranes prepared from charged phospholipids, pH and ionic strength influence the fluiditiy of the hydrophobic core via changes of the surface charge. Membrane fluidity, i.e., viscosity, on the other hand, profoundly affects permeability, due to changes of partition and intramembrane diffusion coefficients of permeants (Eq. 1). Surface charge dependent variations of the nonelectrolyte permeability of phospholipid vesicles have in fact been reported by LELIEVRE and RICH (1972).

The opposite observations for the erythrocyte membrane indicate that either the head group charge remains unchanged under the conditions studied or changes of head group charge are not accompanied by changes of fluidity. This conclusion is also borne out by the finding of RIGAUD et al. (1974), that the flexibility of fatty acid acyl chains in intact human erythrocytes, monitored by ESR, is independent of pH and ionic strength. The difference between phospholipid systems and the erythrocyte membrane may be due either to the presence of cholesterol, which levels out perturbations arising from the head group region (Sect. IIIC2) or to an interaction of the charged phospholipids with protein (Sect. IIIE).

Temperature. The rates of simple diffusion of glycerol, erythritol, and acetic acid via the lipid phase increase considerably with temperature in erythrocytes of various species including human erythrocytes. Linear Arrhenius diagrams are obtained in all temperature ranges studied (Fig. 8E). Linearity can be expected if there is a single rate limiting barrier to the transport. The Arrhenius activation energies (E_a) for a given solute are rather constant in different species in spite of differences in the absolute permeabilities (Table 5a).

In terms of the Absolute Rate Theory, applied to diffusion rates (ZWOLINSKI et al., 1949; JOHNSON and BANGHAM, 1969) it can be defined (simplified) that

$$K = \frac{kT}{h} \cdot e^{-\frac{\Delta G_+^+}{RT}} \tag{3}$$

[5] This phenomenon may affect to some extent the permeability coefficients derived from osmotic measurements (NACCACHE and SHA'AFI, 1973) at high (0.3 M) concentrations of lipophilic permeants.

Fig. 8A–E. Characteristics of glycerol transfer via the lipid pathway of the erythrocyte membrane. (A) Linear concentration dependency. (B) pH-independency. (C) Independence of ionic strength. (D) Independence on anion milieu. (E) High temperature-dependency (Arrhenius plot) (DEUTICKE, 1975). ^{14}C-glycerol and ^{14}C-acetate efflux (at concentration equilibrium) measured as described by DEUTICKE et al. (1973). Permeant concentration 10 mM, pH 7.35, unless otherwise specified. Modification of ionic strength by addition of NH_4Cl. K= rate coefficient of tracer efflux. Data for human erythrocytes obtained in the presence of Cu^{++} or EDC, inhibitors of the protein pathway of glycerol transfer (Sect. IVC5b)

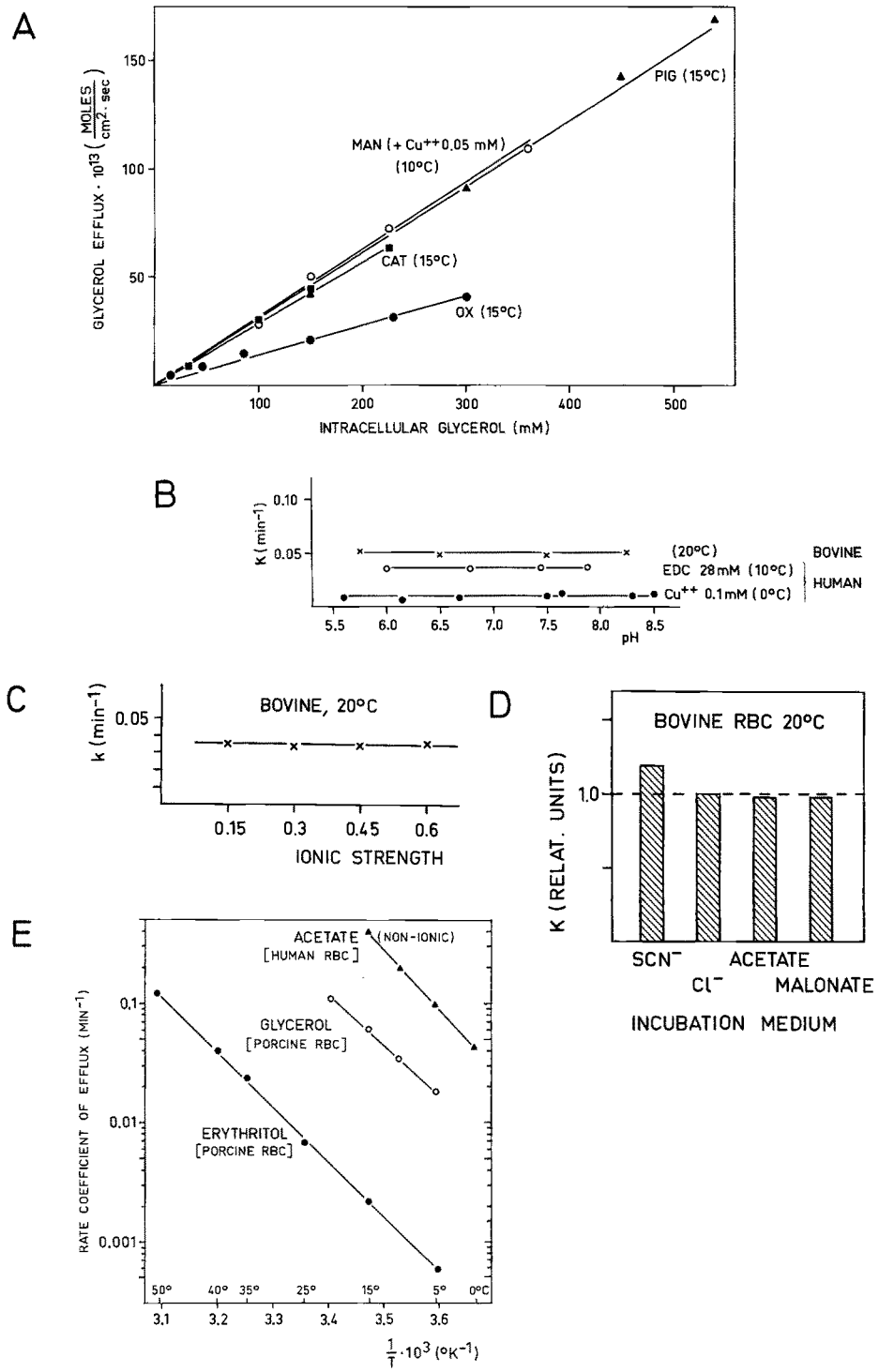
A
GLYCEROL EFFLUX · 10^13 (MOLES / cm² · sec)
150
100
50
PIG (15°C)
MAN (+ Cu++0.05 mM)
(10°C)
CAT (15°C)
OX (15°C)
100
200
300
400
500
INTRACELLULAR GLYCEROL (mM)
B
K (min⁻¹)
0.10
0.05
(20°C)
BOVINE
EDC 28mM (10°C)
HUMAN
Cu++ 0.1mM (0°C)
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH
C
k (min⁻¹)
0.05
BOVINE, 20°C
0.15
0.3
0.45
0.6
IONIC STRENGTH
D
BOVINE RBC 20°C
K (RELAT. UNITS)
1.0
SCN⁻
Cl⁻
ACETATE
MALONATE
INCUBATION MEDIUM
E
RATE COEFFICIENT OF EFFLUX (MIN⁻¹)
0.1
0.01
0.001
ACETATE (NON-IONIC)
[HUMAN RBC]
GLYCEROL
[PORCINE RBC]
ERYTHRITOL
[PORCINE RBC]
50°
40°
35°
25°
15°
5°
0°C
3.1
3.2
3.3
3.4
3.5
3.6
1/T · 10³ (°K⁻¹)

Fig. 8

Table 5. Temperature-dependency of permeation via the lipid pathway (E_a = Arrhenius activation energy (kcal/mole))

a) Species variations

	Glycerol (1)		Erythritol (1)	
	$P \cdot 10^8$ (5° C) ($cm \cdot s^{-1}$)	E_a	$P \cdot 10^8$ (35° C) ($cm \cdot s^{-1}$)	E_a
Man	1.21[a]	19.9[a]	2.44[b]	23.5[b]
Cat	0.64	20.8	1.51	—
Pig	0.70	20.2	1.42	25.1
Ox	0.34	21.0	0.58	27.1

Measured in the presence of 10^{-4} M Cu^{++} [a] and 300 mM glucose [b] which inhibit the protein pathways of these nonelectrolytes (Sec. IV C 5b)

b) Comparison between erythrocytes and artificial lipid membranes

	Apparent activation energies (kcal/mole)			
	Erythrocytes		Artificial lipid membranes	
Ethylene glycol	—	19 (4)	14.3 (5)	6.2 (6)
Glycerol	20 (1)	24	18.4	11.9
Erythritol	23–27	—	20.8	15.8
n-Propionamide	19 (2)		10.2 (3)	
n-Butyramide	26		12.7	
n-Valeramide	38		10.7	

(1) DEUTICKE (1975) and unpublished results: Tracer techniques
(2) GALEY et al. (1973): Human erythrocytes
(3) POZNANSKY et al. (1976): Red cell total lipids
(4) JACOBS et al. (1935): Ox erythrocytes
(5) DE GIER et al. (1971): Unsaturated lecithins with or without 30 mole % cholesterol
(6) COHEN (1975a): Egg lecithin

} Osmotic measurements

where K = rate coefficient of transfer, k = Boltzmann's constant, h = Planck's constant, T = absolute temperature, ΔG_+^+ = free energy of activation. ΔG_+^+ can be separated into an activation enthalpy (ΔH_+^+) and an activation entropy (ΔS_+^+) term by $\Delta G_+^+ = \Delta H_+^+ - T \cdot \Delta S_+^+$.

Consequently $K = \frac{kT}{h} \cdot e^{-\frac{\Delta H_+^+}{RT}} \cdot e^{\frac{\Delta S_+^+}{R}}$

ΔH_+^+ is almost equal to the Arrhenius activation energy at high values, since $\Delta H_+^+ = E_a - RT$ and $RT \sim 0{,}5$ at $T = 273$ to 323° K.

In the framework of the Absolute Rate Theory, similar ΔH_+^+ values for solutes with different permeabilities indicate differences in the entropy of activation, ΔS_+^+, which in turn may be related to variations in the motional freedom, i.e., the fluidity of the pathway. Observations which agree with this concept

have been reported by DE GIER et al. (1971). In their study, identical activation enthalpies were obtained for the penetration of the same solute in artifical phospholipid membranes differing in chain length, saturation and cholesterol content, i.e., varying in fluidity.

The activation energies for the transfer of *polyols* increase with the number of OH-groups (Table 5b). The same is true for the penetration of these compounds across artificial lipid membranes (DE GIER et al., 1971, COHEN, 1975a). Although the absolute values of E_a, obtained in different systems by different investigators, vary to some extent, the differences between the E_a values for the three homologues are fairly constant ($\sim$ 4–5 kcal/mol per additional CH_2OH group). As was first pointed out by STEIN (1967) and later elaborated in detail by DIAMOND and WRIGHT (1969), these differences may reflect the rising number of H-bonds (N_H) which have to be broken in the course of the transfer of these H-bonding solutes from the aqueous phase into the hydrophobic membrane. Evidence for the validity of this concept has recently been provided by COHEN (1975a) demonstrating a linear increase of E_a with N_H for the transfer of 15 solutes (N_H= 2–8) across egg lecithin bilayers.

Activation energies for *other permeants* using the lipid pathway of the erythrocyte are available only to a limited extent. Activation enthalpies of 13 and 22 kcal were determined for the nonionic diffusion of salicylic and acetic acid (DALMARK and WIETH, 1972; DEUTICKE, 1975b). GALEY et al. (1973) reported temperature coefficients, obtained by swelling studies, of 19, 26, and 38 for propionamide, butyramide, and valeramide, respectively. Unfortunately, the statistical deviations of these data are considerable. From the mean permeabilities a value of approximately 22 kcal/mole for valeramide can also be computed. Thus, it is not yet clear, whether or not the activation energies for amide transfer across the erythrocyte lipid domain, vary with the chain length or are essentially chain length-independent as in the case of the artificial lipid membranes (Table 5b). A constant activation enthalpy would be consistent with the constant number of H-bonding groups in this homologous series (see above).

3. Influence of Alcohols and Other Perturbants

Aliphatic alcohols enhance the permeability of artificial lipid membranes (BANGHAM et al., 1965; JOHNSON and BANGHAM, 1969; GUTKNECHT and TOSTESON, 1970; LELIEVRE and RICH, 1972; JAIN et al., 1973). Analogous observations have been reported for the transfer of many small nonelectrolytes and the nonionic diffusion of organic acids across the erythrocyte membrane in various species (JACOBS and PARPART, 1937; HUNTER et al., 1965; DEUTICKE, 1975a, b). In other species, however, the transfer of the same nonelectrolytes was found to be inhibited.

The transfer-accelerating action of the aliphatic alcohols increases with their chain length, a two-fold enhancement of glycerol permeability requiring 110 mM butanol in contrast to 1 mM octanol. The effect is fully reversible. It is not restricted to aliphatic alcohols but can also be produced with aromatic alcohols and, although to a lesser extent, with other organic solvents (acetone, dioxane) or even organic electrolytes (salicylate, caprylate) (DEUTICKE, to be published).

It was proposed by HUNTER (1965), that alcohol enhancement indicates "simple" diffusion. In our opinion the enhancement indicates diffusion via the lipid pathway. Spectroscopic and binding studies on artificial systems offer a structural explanation for this assumption: alcohols and other perturbants bind to hydrophobic membrane domains (SEEMAN, 1972) and increase the fluidity of lipid bilayers (HUBBELL et al., 1970; PATERSON et al., 1971) by disorganizing the acyl chain and/or the head group region. A rise of membrane fluidity, in turn, will enhance the intramembrane diffusion coefficient of permeants and affect the partition coefficient (LANGE et al., 1974). The relative contributions of both parameters have not yet been separated experimentally.

Besides these simple perturbants, phloretin, a well-known inhibitor of catalyzed transport processes in the erythrocyte membrane (WILBRANDT, 1950; DEUTICKE, 1970) has recently been shown to enhance the transfer of lipophilic ($K_{ether} > 0.003$) nonelectrolytes (OWEN and SOLOMON, 1972; OWEN et al., 1974) and to inhibit the permeation of certain small hydrophilic nonelectrolytes. Since phloretin binds to lipid membranes (EHRENSPECK, 1975) and enhances their nonelectrolyte permeability (POZNANZKY et al., 1976), the enhancement may be due to membrane perturbations comparable to those induced by the alcohols. Considering the large number of H-bonding OH-groups distributed over the phloretin molecule, a perturbation of the head group region of the phospholipids may be involved.

4. Influence of Physiological Constituents

a) Cholesterol

According to the structural investigations outlined in Section III C 2, cholesterol, a major constituent of the erythrocyte membrane, modifies the flexibility of the alkyl chains and thus alters the fluidity of the hydrophobic domain of artificial phospholipid membranes. In view of the relationship between membrane fluidity and intramembrane diffusion coefficients cholesterol should thus affect the lipid pathway. Numerous investigations have confirmed this concept for the permeability of artificial membrane systems toward water (FINKELSTEIN and CASS, 1967; JAIN et al., 1973); nonelectrolytes (DE GIER et al., 1968; DEMEL et al., 1972a; LELIEVRE and RICH, 1972) and ions (PAPAHADJOPOULOS et al., 1971; VANDERKOOI and MARTONOSI, 1971; DE GIER et al., 1970).

The influence of cholesterol on erythrocyte permeability was first studied by BRUCKDORFER et al. (1969). By incubating human and porcine red cells with egg lecithin suspensions some 30% of the membrane cholesterol was removed. Glycerol permeability, measured by osmotic swelling, was reported to be greatly enhanced. Cholesterol depletion, however, also increases the osmotic fragility of erythrocytes due to a loss of surface area (BRUCKDORFER et al., 1969; COOPER and JANDL, 1968). Consequently, osmotic techniques do not allow unequivocal conclusions on permeability changes of cholesterol-depleted cells. Tracer equilibrium exchange of glycerol, erythritol and acetic acid in cholesterol-depleted cells of different species was measured by DEUTICKE and ZÖLLNER (1972) and GRUNZE and DEUTICKE (1974). In contrast to the data of BRUCKDORFER (1969),

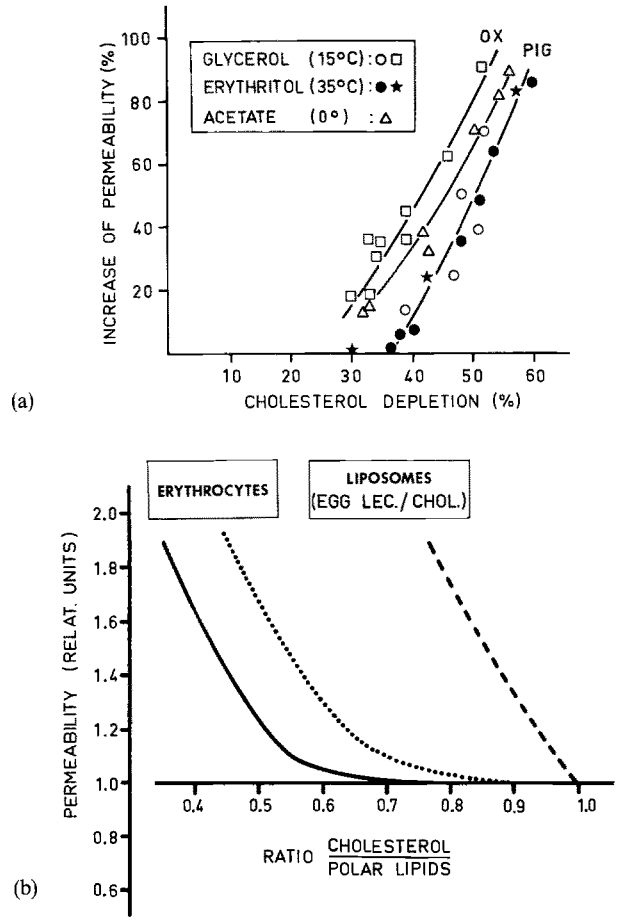

Fig. 9a and b. Influence of cholesterol depletion of the erythrocyte membrane on transfer processes involving the lipid pathway. (a) Experimental data, mainly from GRUNZE and DEUTICKE (1974). (b) Schematized diagram relating permeability to cholesterol/polar lipid ratio: (1) in the erythrocyte membrane, assuming either that all polar lipids interact with cholesterol (*full line*) or that 20% of the polar lipids are not available for cholesterol (*dotted line*), (2) in an artificial lipid membrane (*hatched line*) as observed by DE GIER et al. (1968)

cholesterol depletion up to 25–35% (depending on the species and the permeant) did not affect permeability. More extensive depletion produced marked increases (Fig. 9a) in qualitative agreement with results obtained on cholesterol/lecithin liposomes (DE GIER et al., 1968). This parallelism constitutes further evidence for the lipid nature of the diffusion pathway of the solutes studied, since protein pathways react differently, as will be discussed in Sections IVC6 and IVF.

The quantitative relationship between fractional cholesterol content and permeability in the complex erythrocyte membrane differs from that in simple phospholipid/cholesterol bilayers (DE GIER et al., 1968; DEMEL et al., 1968, 1972a). In the artificial system any lowering of the ratio cholesterol/phospholipid below 1.0 enhances permeability according to the schematized diagram in Fig-

ure 9b. In the erythrocyte membrane, the corresponding ratio cholesterol/polar lipids (Footnote 1) can be lowered from its normal value of 0.75 to a value of 0.55 without changes of permeability. Thirty percent of the membrane cholesterol apparently do not affect permeability. Possible reasons for this unexpected discrepancy are the following:

1. Some of the polar lipids interact with proteins as do polar lipids in other plasma membranes (TRÄUBLE and OVERATH, 1973) and thus are not available for an interaction with cholesterol. If this fraction comprises some 20%, the physiological ratio would rise to 0.95. The different response of erythrocyte and artificial membrane to cholesterol depletion, however, would still be present.

2. A certain fraction of the erythrocyte membrane phospholipids, although available for an interaction with cholesterol, does not interact with the sterol in the same "specific"[6] way, as do egg lecithin and other simple phospholipids. This noninteracting fraction may comprise phospholipids with highly unsaturated or fully saturated acyl chains which are not affected or rendered more flexible by cholesterol (DEMEL et al., 1972c; OLDFIELD and CHAPMAN, 1971). The presence of such phospholipids in the erythrocyte membrane has been demonstrated by VAN GOLDE et al. (1967).

The possible occurrence in the erythrocyte membrane of cholesterol without an effect on permeability is also supported by the observation that insertion in vitro of additional cholesterol into the membrane reduces transfer via the lipid pathway only to a very limited extent (DEUTICKE and RUSKA, 1976).

The increase of permeability at ratios cholesterol/polar lipids below 0.55 may be explained by a decrease in microviscosity, demonstrated by VANDERKOOI and COOPER (1974). This would enhance the intramembrane diffusion coefficient D. On the other hand, a rise of the partition coefficient K (membrane/medium) should also be considered. Such a phenomenon was demonstrated by COLLEY and METCALFE (1972) in egg lecithin/cholesterol mixtures.

An interesting alternative explanation for the permeability effects of cholesterol on phospholipid bilayers has recently been proposed by BROCKERHOFF (1974). According to his concept the presence of cholesterol stabilizes a region of strong hydrogen bonds between phospholipids, cholesterol OH-groups, and water near the head group region of the phospholipids. This "hydrogen belt" and not the hydrophobic membrane interior is supposed to constitute the rate-limiting barrier to nonelectrolyte transfer.

b) Phospholipids

The establishment of an "intermediate state of fluidity" by cholesterol in phospholipid membranes (Sect. IIIC2) does not imply necessarily that cholesterol-containing bilayers of different phospholipid composition, have the same fluidity. Even in the presence of cholesterol an influence of chain length and unsaturation on the fluidity of the hydrophobic region can be demonstrated (OLDFIELD and CHAPMAN, 1972b). Consequently, erythrocytes of varying fatty acid composition should differ in the permeability of the lipid domain. For artificial phospholipid

[6] A "specific" interaction is defined as one going along with a decrease of the mean flexibility of the acyl chains and a consecutive diminution of permeability.

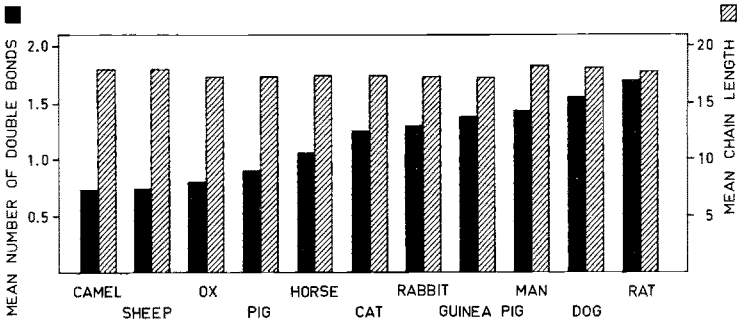

Fig. 10. Double bond indices as a measure of unsaturation of the erythrocyte membrane lipids of various mammalian species. These numbers provide a rough indicator of the differences in intramembrane fluidity to be expected. Calculated from data given by WESSELS and VEERKAMP (1973), except for camel (ALONI and LIVNE, 1973), cat (HAEST, unpublished data) and guinea pig (OSTWALD, 1970)

membranes a considerable increase of nonelectrolyte permeability with rising unsaturation (i.e., increasing double bond index) and decreasing chain length is well documented (DE GIER et al., 1968; DEMEL et al., 1968; CHEN et al., 1971; KLEIN et al., 1971).

Erythrocytes from various species differ considerably in their fatty acid composition, as indicated by the double bond indices (Fig. 10). The permeabilities for erythritol, glycerol and acetic acid also differ considerably from species to species, following the same systematic pattern for all three permeants (Fig. 11a) (DEUTICKE, 1974, 1975a, b). The permeability rises with the double bond index. This correlation agrees with the concept that in the erythrocyte too, membrane fluidity influences permeability either via the intramembrane diffusion coefficient or due to variations of the partition coefficient membrane/medium. The latter should increase with the double bond index of the lipid phase as indicated by partition studies on artificial lipid systems by LANGE et al. (1974).

Other results, however, do not fully bear out this conclusion: The fatty acid pattern of the erythrocyte membrane is sensitive to dietary alterations (VAN DEENEN et al., 1962; WALKER and KUMMEROW, 1964; BLOJ et al., 1973). Taking advantage of this possibility, the double bond index of rat erythrocytes was reduced from 1.6 to 1.25 by feeding hydrogenated arachis oil (DEUTICKE and HAEST, unpublished results). According to Figure 11a, this should lower the permeability by 30%. The observed diminutions of acetic acid and erythritol permeability did not exceed 5 to 10%. Thus, other parameters may contribute to the species' differences in permeability. As shown in Figure 11b, a positive correlation between nonelectrolyte permeability and membrane phosphatidylcholine content and a negative one with the sphingomyelin content can also be demonstrated. Such correlations support a concept put forward by BROCKERHOFF (1974): sphingomyelin, due to a high tendency to form H-bonds, may stabilize the "hydrogen belt" at the membrane surface like cholesterol (Sect. IV-B 4a). In view of the preferential localization of phosphatidylcholine and sphingomyelin in the outer layer (cf. Sect. III C 1), *a region near the external membrane*

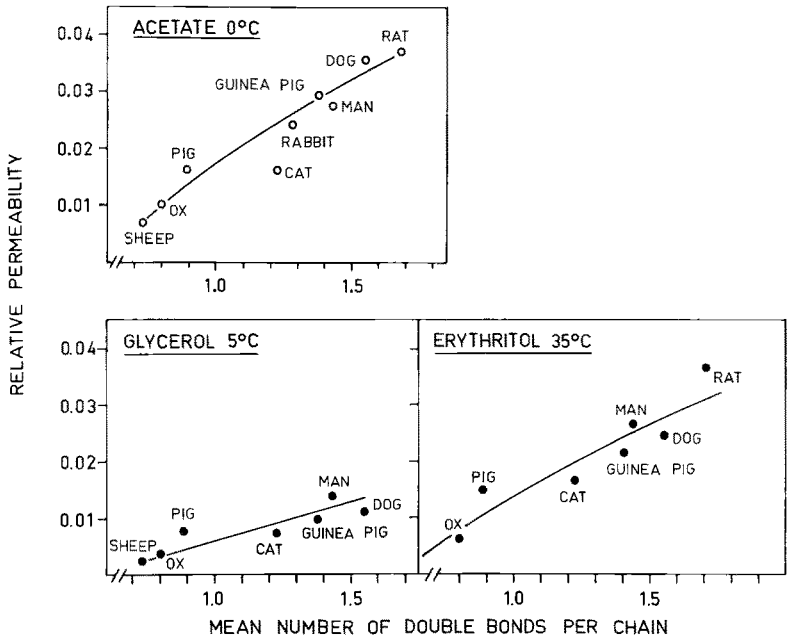

Fig. 11a and b. Correlation between the composition of the erythrocyte membrane lipid phase and its permeability to nonelectrolytes and acetic acid. (a) Correlation with the double bond index. (b) Correlation with the fractional concentration of the phospholipids assumed to constitute the outer leaf of the lipid bilayer (Fig. 1 and Sect. IIB. and III.C.1). Relative permeabilities calculated from rate coefficients of tracer efflux corrected for the ratio cell volume/cell surface area. Nonionic acetate fluxes measured after inhibition of the ionic pathway by phenopyrazone (DEUTICKE, 1974). Glycerol and erythritol fluxes in human cells measured in presence of Cu^{++} or glucose (Sect. IV.C.5.)

surface would thus act as rate-limiting barrier for nonelectrolytes. This statement, however, cannot be generalized. As pointed out by GALEY et al. (1973) the main barrier may differ for different solutes. Nevertheless, the barrier properties of the lipid pathway must be expected to depend on parameters other than the fluidity of the acyl chain region. The modification of phospholipid head groups by enzymes or covalent agents should provide a means to test this assumption.

5. The Structural Basis of Selectivity

The *selectivity* of the lipid pathway is probably also related to its composition. For a discussion of this topic it may be useful to distinguish "partitional selectivity" from "diffusional selectivity". The selectivity of partition, i.e., the ratio of the partition coefficients for two different solutes, decreases with rising polarity of the lipid phase (COLLANDER, 1951; DIAMOND and KATZ, 1974). In view of this relationship, illustrated in Table 6 for partitions phospholipid/water and ether/water polarities can be assigned to membrane systems relative to bulk solvents or other membrane systems by computing the correlation between

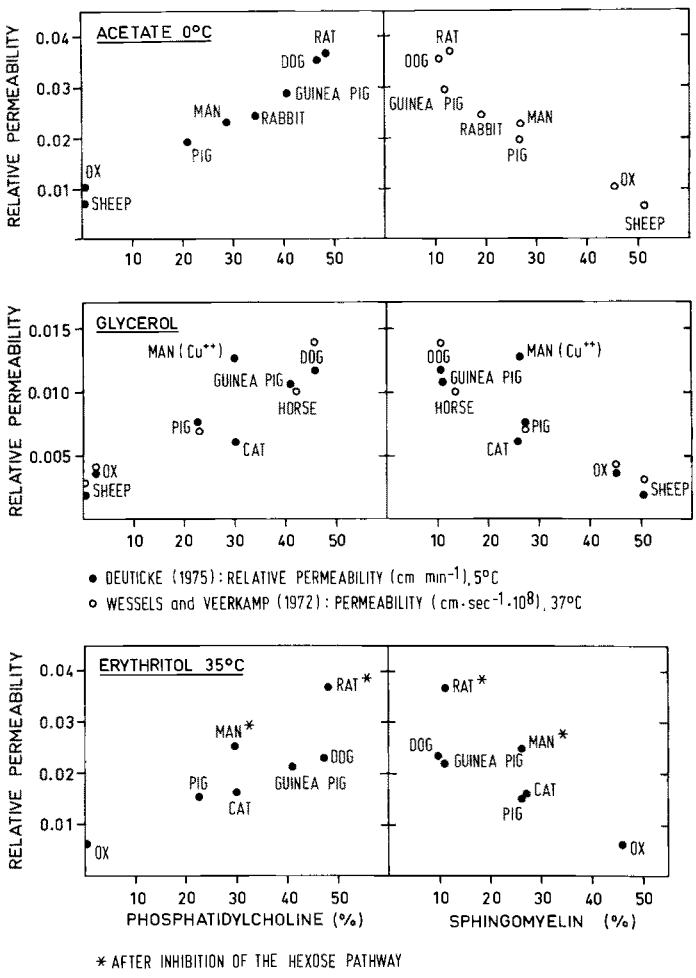

Fig. 11b

the partition coefficients (K_1, K_2) obtained for numerous solutes in two lipid/water systems 1 and 2, i.e., by calculating the slope a for the equation:

$$\log K_1 = a \cdot \log K_2 + b \tag{4}$$

$a = 1$ indicates equiselectivity of two systems. According to calculations by SEEMAN (1972) and KATZ and DIAMOND (1974) the mean polarity of the erythrocyte membrane is comparable to that of octanol and slightly higher than that of dimyristoyl lecithin above the phase transition.

Diffusional selectivity, i.e., the ratio of diffusion coefficients for two solutes, is not likely to vary in isotropic systems obeying the Stokes-Einstein relationship. Diffusion in anisotropic systems involving "kinks" (Sect. IIIC2a) may well exhibit this type of selectivity (CRANK and PARK, 1968).

One possible way of defining the structural basis of the permselectivity of the erythrocyte membrane lipid domain is to compare it with that of artificial

Table 6. Nonelectrolyte selectivity of erythrocyte membrane lipid pathway and artificial lipid membranes

	Permeabilities				Partition coefficients	
	Artificial phospholipid membranes		Erythrocytes		DML[c]/ water (5) (40° C)	Ether/ water (6) (20° C)
			Human (3)	Dog (3)		
	Egg lecithin/ cholesterol/ PA (48/48/4), (1) P (10° C) (relat. units)	Erythrocyte total lipids (2) P (10°C) (relat. units)	$P \cdot 10^5$ (22–25° C) ($cm \cdot s^{-1}$)			
n-Propionamide	0.45	1.05	7.8	17.1	0.45[d]	0.013
n-Butyramide	0.59	n.t.	24.4	52.2	0.64	0.058
n-Valeramide	0.75	1.51	58.0	153.0	0.89[d]	0.170
	Egg lecithin/ cholesterol/ PA (1) P (10° C) (relat. units)	Phosphatidylglycerol (7) P (25° C) (relat. units)	Human (8)	Ox (8)		
			$P \cdot 10^8$ (0° C) ($cm \cdot s^{-1}$)			
Ethylene glycol	0.35	3.6	68.3	27.4	0.161	0.0053
Glycerol	0.015	0.42	0.74[a]	0.22	0.091	0.00066
Erythritol	n.t.	0.015	0.0027[b]	0.0033	0.058	0.00011

(1) COHEN (1975b), (2) POZNANSKY et al. (1976), (3) GALEY et al. (1973), (4) SHA'AFI et al. (1971), (5) KATZ and DIAMOND (1974), (6) COLLANDER (1949), (7) HAEST et al. (1972), (8) DEUTICKE (in preparation).

[a] Measured in the presence of 10^{-4} M Cu^{++}, which inhibits the protein pathway of glycerol (Sec. IV C 5b).

[b] Measured in the presence of 0.3 M glucose, which inhibits the protein pathway of erythritol (Sec. IV C 5c).

[c] DML = Dimyristoyllecithin.

[d] The data for propionamide and valeramide were calculated from the measured value for butyramide and the K_{oil} values of the amides (COLLANDER, 1954) using the equation given by DIAMOND and KATZ (1974).

lipid membranes. As yet, data are available only for amides and polyols. The comparison is greatly hampered by the fact that, due to different activation enthalpies, these selectivities vary with temperature (DIAMOND and KATZ, 1974). From the data compiled in Table 6, it can be concluded that the erythrocyte lipid pathway discriminates much more sharply between different amides and polyols than artificial lipid systems. In terms of Equation (4), which can also be applied to permselectivities, the slopes, a, are about 12 and 3, respectively, for amides and polyols (in a plot human erythrocyte versus liposome permeability).

This difference probably results only to a minor extent from a lower polarity of the erythrocyte lipid domain, i.e., variations in partitional selectivity. As a main reason, differences in the diffusional selectivity have to be assumed,

which could either be located in the hydrophobic membrane interior or at the interface between aqueous bulk phase and membrane. The further analysis of this problem is complicated by the fact that artificial and erythrocyte membrane selectivities respond differently toward changes of lipid composition: Cholesterol depletion *decreases* the polyol selectivity of liposomes (COHEN, 1975b) but increases that of human erythrocytes (GRUNZE and DEUTICKE, preliminary results).

6. The Influence of Membrane Anisotropy

In the above treatment of permeability, the lipid pathway was treated as a homogeneous structure. However, a head group and acyl chain asymmetry and a fluidity gradient within the hydrophobic domain of the membrane are very likely (Sect. III C). Consequently, two parameters relevant to permeability, namely polarity and microviscosity, most likely depend on the position within the membrane. The possible profiles of the two parameters have been studied and discussed by DIAMOND and KATZ (1974) and GRIFFITH et al. (1974) for symmetric artificial bilayers. The more complex situation in a biomembrane has recently been investigated by MORSE et al. (1975) for sarcoplasmic reticulum.

C. Protein Pathways for Small Nonelectrolytes

1. General Aspects

From the concept of a mosaic membrane it follows that, in addition to penetrating via the lipid, a solute may take its way via intercalated protein structures. The characteristics of such a pathway will depend on the protein's properties. Even a single polypeptide chain may already alter the surface charge at the lipid-water interface or perturb the relative order of the membrane interior and thereby create permeabilities different from those of the pure lipid phase. This type of an operationally defined "protein pathway" would correspond to the frequently observed rises of permeability in artificial lipid membranes treated with various membrane-related (LEA et al., 1975; TOSTESON et al., 1973) and other (CALISSANO et al., 1972; KAPLAN, 1973) proteins.

As concerns the erythrocyte membrane, the evidence that a number of its proteins span the membrane and even form aggregates visible as particles (Sect. III D 1) suggests that, in addition to single proteins, organized complexes of closely apposed proteins may constitute protein pathways.

Protein pathways for small nonelectrolytes providing an aqueous continuum through the membrane have originally been postulated on the basis of the following kinetic data:

1. The diffusional permeability P_d of the erythrocyte to water, as measured by tracers at osmotic equilibrium, is 3–5 times lower than its hydraulic (convective) water permeability L_P, measured under gradients of osmotic pressure. Data obtained by various authors have been compiled by FORSTER (1971) and SHA'AFI and GARY-BOBO (1973). In human erythrocytes, e.g., P_d (H_2O) = 3–

5×10^{-3} cm/s, $L_p = 12\text{–}19\times 10^{-3}$ cm/s. For simple lipid bilayer membranes the two coefficients are equal (CASS and FINKELSTEIN, 1967).

2. Below a molecular weight of 60–90 Dalton the permeability of nonelectrolytes is inversely related to their lipid solubility:

$P_{\text{formamide}} > P_{\text{acetamide}}$; $P_{\text{urea}} > P_{\text{methylurea}}$; $P_{\text{methanol}} > P_{\text{ethanol}}$ (NACCACHE and SHA'AFI, 1973). Similar inversions have recently also been observed in artificial lipid membranes by COHEN (1974b) and POZNANSKY et al. (1976) and were taken to reflect the relevance of molecular size for the penetration across lipid bilayers. This aspect, however, cannot account for all inverse relationships observed in the erythrocyte membrane. The permeability to ureas, e.g., decreases with their K_{ether} in this membrane, but increases with K_{ether} in lipid membranes (COHEN and BANGHAM, 1972).

3. For small nonelectrolytes, the diffusional permeability P_d is much lower than would be anticipated from the reflection coefficient σ[7] (GOLDSTEIN and SOLOMON, 1960; OWEN et al., 1974; FORSTER, 1971; SHA'AFI and GARY-BOBO, 1973). This discrepancy is taken to imply that small nonelectrolytes penetrate via a pathway in which they experience frictional interaction with water (KEDEM and KATCHALSKY, 1961; SOLOMON, 1968).

The sum of these observations has been rationalized in terms of aqueous channels (pores) for water and small solutes with "equivalent radii" of approximately 4–6 Å (SOLOMON, 1968). Solutes are dragged through these "pores" by volume flow in excess of their diffusion rates.

The justification for detailed calculations of pore geometry and the pore concept in general have recently been criticized on conceptual and methodological grounds (LIEB and STEIN, 1971; FORSTER, 1971; SHA-AFI and GARY-BOBO, 1973). Nevertheless, there can be little doubt, already on the basis of these kinetic peculiarities, that water and small nonelectrolytes have access to low-resistance pathways different from the lipid pathway. This concept is further borne out by certain characteristics of permeation of small nonelectrolytes and in particular their sensitivity to covalent inhibitors.

2. Water Transfer

Properties of the pathway. Artificial lipid bilayers are highly permeable to water: Values between 1 and 5×10^{-3} cm/s have been reported for various phospholipids (REEVES and DOWBEN, 1970; ANDRASKO and FORSÈN, 1974; CASS and FINKELSTEIN, 1967). These values are in the same range with the diffusional water permeability and only 3–4 times lower than the hydraulic permeability of the erythrocyte membrane (see above). Water transfer across the erythrocyte membrane is thus only moderately enhanced by the presence of the low-resistance pathway.

[7] The expected relationship between both parameters for a lipid membrane, in which solvent and solute diffuse independently of each other, would be (at zero volume transfer):

$$1-\sigma = \frac{P_d \cdot \bar{V}_s}{L_p \cdot RT}$$

where $\bar{V}_s$ is the partial molar volume of the solute.

Convincing evidence that this pathway involves peptides was first obtained by MACEY and FARMER (1970) and MACEY et al. (1972). They demonstrated partial inhibition of L_P and P_d by the organomercurials, PCMB and PCMBS, which bind to sulfhydryl groups with high selectivity. The inhibition was reserved by cysteine. In the presence of maximally inhibitory concentrations of PCMBS, the ratio L_P/P_d for the residual water transfer was unity, as in lipid membranes.

These findings can be interpreted in terms of two parallel routes of water transfer: A "high-resistance pathway" via the lipid domain and a "low-resistance pathway" involving proteins containing SH-groups. This dualistic concept is further supported by the observation (MACEY et al., 1972) that the activation energy of the residual water transfer in presence of PCMBS amounts to 11.4 kcal/mole, a value characteristic for pure lipid systems (REEVES and DOWBEN, 1970; REDWOOD and HAYDON, 1969; PRICE and THOMPSON, 1969). In the unmodified erythrocyte membrane the activation energy is only 4.8 kcal/mole, in agreement with the presence of a low-energy barrier to water transfer. Interestingly, in chicken erythrocytes the activation energy of water transfer is 11.4 kcal even in the unmodified membrane (FARMER and MACEY, 1970) indicating the absence of a protein pathway of water transfer, a notion also borne out by identical values for L_P and P_d (WIETH, personal communication).

In view of protein nature of the "low-resistance pathway" for water, one might expect the ion environment to affect P_d or L_P. As shown by RICH et al. (1968), L_P in human and dog erythrocytes is insensitive to changes of pH between 6 and 8, of ionic strength between 0.15 and 0.24, and to replacement of Cl^- by Br^-. This makes a significant contribution of charged groups dissociating in this pH range, in particular imidazole residues, rather unlikely. Increases of the extracellular but not the intracellular osmolarity (from 220 to 420 mosm) seem to lower L_P by a factor of 2 (RICH et al., 1968; SHA'AFI et al., 1970), while P_d is probably not affected (VEATCH, cited in SHA'AFI et al., 1970). The authors have interpreted their findings in terms of a superficial membrane layer responsive to osmolarity.

Structural features. As shown by NACCACHE and SHA'AFI (1974), the SH-specific agent DTNB inhibits water transfer like PCMBS, while iodoacetamide and NEM are ineffective. The two inhibitory SH-reagents are anions which penetrate the membrane at best very slowly (CHAN and ROSENBLUM, 1969; KNAUF and ROTHSTEIN, 1971 b; SMITH and ELLMAN, 1973). A specific sensitivity of water transfer to anionic SH-reagents deserves particular interest in the light of a recent report of BROWN et al. (1975) that DTNB binds selectively to band 3 proteins (Sect. II C). This major group of membrane-spanning proteins, which contains 13 SH groups per 1,000 amino acids, probably mediates the transfer of anions including that of PCMBS (KNAUF and ROTHSTEIN, 1971 b). Consequently, the SH groups involved in water transfer may be located in the vicinity of the anion pathway. Since the inhibition by PCMBS and DTNB develops slowly with time (NACCACHE and SHA'AFI, 1974), the SH-groups are not to be expected at the membrane surface. On the other hand, they are probably located outside of the barrier limiting the rate of anion transfer, since the development of inhibition is less temperature-dependent than the transfer of anions (NACCACHE and SHA'AFI, 1974 and Sect. IV D 2). It seems also likely

that only a distinct population of SH groups in band 3 is related to water transfer, since NEM, which is not an inhibitor, has been shown to interact readily with SH groups of this protein (STECK, 1974; CARRAWAY and SHIN, 1972).

The organization of the proteinaceous water pathway and the physical state of water in its interior are only a field of speculation. Since structures as different as gramicidin A helices and circular complexes of planar macrocyclic polyene antibiotics (nystatin, amphotericin B) form aqueous channels across lipid membranes (COHEN, 1975; MYERS and HAYDON, 1972; HOLZ and FINKELSTEIN, 1970; ANDREOLI, 1974), the number of possible arrangements is certainly large. In view of the dimensions of the "free space" to be expected within a protein or a protein assembly, water is most likely highly immobilized inside the channel. Restricted mobility of water bound to the erythrocyte membrane has recently been demonstrated by FINCH and SCHNEIDER (1975): From proton NMR studies they calculated a "translational diffusion constant" of 10^{-9} cm^2/s for "bound water" as compared to the well-established self-diffusion coefficient of $\sim 10^{-5}$ cm^2/s for "free" water.

Role of lipids. Since membrane-spanning proteins interact with lipids in the hydrophobic membrane core, changes of fluidity in this domain might affect water transfer. SHA'AFI et al. (1968) could not demonstrate systematic changes of L_P in cholesterol-loaded and -depleted human erythrocytes. This result disagrees with cholesterol-dependent changes of other protein-related transfer processes, to be discussed in Sections IV C 6 and IV F. In contrast, alcohols and other amphipathic compounds enhance water transfer (SEEMAN et al., 1970). Since this would be expected for a transfer process involving the lipid phase (Sect. IV B 3), the effect probably represents an enhancement of that fraction of water transfer that occurs via the lipid domain.

3. Amide Transfer

The contribution of protein pathways to the transfer of small amides (formamide, acetamide) in human erythrocytes is indicated by the inhibitory action of PCMBS (NACCACHE and SHA'AFI, 1974). Kinetic details of the transfer are unknown. Low activation enthalpies (~ 12 kcal/mole), observed by GALEY et al. (1973), indicate that the transfer is impeded by lower energy barriers than that of the more lipophilic amides.

According to osmotic studies by KAPLAN et al. (1975) neither trypsin, which removes sialoglycopeptides from the membrane glycoproteins (CARRAWAY et al., 1971; CABANTCHIK and ROTHSTEIN, 1974), nor pronase at concentrations, which cleave a considerable amount of the 95,000-dalton (band 3) peptides (BENDER et al., 1971; TRIPLETT and CARRAWAY, 1972) affect acetamide permeability. The integrity of this major membrane-spanning protein fraction seems not to be required for the protein-related transfer of the amides.

4. Urea Transfer

Among the nonelectrolytes penetrating human and other mammalian erythrocytes much faster than predicted from their lipid solubility, urea and methylurea

are of particular significance. The transfer of these solutes is inhibited by PCMBS (MACEY and FARMER, 1970), which points to the contribution of proteins. In addition, competitive inhibition of urea transfer by thiourea, indicative of a common pathway of both solutes, has been demonstrated by WIETH et al. (1974). Since thiourea transfer, according to the same work, has a saturable component ($K_T = 15$–20 mM), in addition to diffusion via the lipid phase (indicated by alcohol-induced acceleration (HUNTER et al., 1965) the rapid transfer of urea and some of its derivatives in human erythrocytes seems to be restricted to a limited number of sites. The "facilitation", indicated by the high penetration rate is also reflected by a low temperature dependency (JACOBS et al., 1935).

As in the case of water and amides, a protein pathway of urea and thiourea transfer seems to be absent in chicken and other avian erythrocytes (WIETH et al., 1974; KAPLAN et al., 1974): Fluxes increase linearly with concentration up to 100 mM, are insensitive to phloretin (see below), and have an activation energy of 17 kcal/mole like other nonelectrolytes penetrating via the lipid pathway (Sect. IVB2). The absolute permeabilities in these species agree well with those of artificial phospholipid membranes as determined by GALUCCI et al. (1971).

Trypsin and pronase under the condition described above (Sect. IVC3) do not affect urea transfer (KAPLAN et al., 1975) which indicates that an intact 95,000-dalton protein is not essential for this transfer process.

5. Transfer of Polyhydric Alcohols

The transfer characteristics of polyhydric alcohols (polyols) migrating via protein pathways of the human erythrocyte membrane (ethylene glycol, glycerol and erythritol) are complex and seem to involve, in spite of the molecular homology, different routes, in addition to the lipid pathway. The contribution of the latter can be demonstrated in the presence of inhibitors which block the protein pathway (Sect. IVB). A number of the established characteristics of polyol transfer are compiled in Table 7.

a) Ethylene Glycol

The presence of an aqueous pathway for ethylene glycol in the human erythrocyte was suggested by the finding (GOLDSTEIN and SOLOMON, 1968) of a reflection coefficient lower than predicted from the diffusional permeability P_d. The dependency of transfer on pH and ionic strength (Table 7) also supports the concept of a protein pathway for ethylene glycol.

On the other hand, ethylene glycol transfer in human erythrocytes lacks certain characteristics expected for compounds penetrating via protein pathways: Alcohols and phloretin enhance transfer, instead of blocking it, and the covalent modifiers affecting the transfer of glycerol and erythritol (see below) are ineffective except for a minor inhibition by the water-soluble carbodiimide EDC. This behavior is reconcilable with the concept that ethylene glycol penetrates the human erythrocyte largely via the lipid phase, but has also access to a protein pathway. The lack of sensitivity to covalent inhibitors may indicate a very weak interaction between ethylene glycol and its protein pathway.

Table 7. Comparative characteristics of protein pathways for glycerol and erythritol and unspecified ethylene glycol pathway in human erythrocyte membrane

	Ethylene glycol	Glycerol	Erythritol
Concentration dependency	n.t.	Saturation at high concentrations (1) ($K_T \sim 1.5$ M)	Linear up to 0.40 M (3)
pH	Moderate enhancement between pH 5.9 and 6.4, higher pH values not studied (4)	Cooperative enhancement (Hill's $n \sim 3$) between pH 6 and 7, pH-independent above pH 7 (1, 2, 5)	No effect
Anion milieu (Cl^-, Br^-, SCN^-)	No effect	Shift of the range of pH-induced enhancement (1)	Slight inhibition by SCN^- (6)
Activation energy	?	Approximately 10–13 kcal/mole (2, 4)	22–24 kcal/mole (3, 4, 6)
Ethylene glycol	—	Inhibits noncompetitively (1)	Inhibits (900 mM 60%) (4)
Glycerol	Ineffective (900 mM) (4)	—	Inhibits (900 mM 40%) (4)
Ionic strength	Inhibits (4)	Shifts the range of pH-induced enhancement	n.t.
Aliphatic alcohols (butanol, hexanol)	Enhance (4)	Inhibit (1, 7)	Inhibit (3)
Phloretin	Enhances (4, 8)	Inhibits (4, 9)	Inhibits (3, 4, 6)
Amphipathic organic anions (e.g. salicylate, phenopyrazone)	n.t.	Shift the range of pH-induced enhancement (1)	n.t.
Cu^{++}	Ineffective (4)	Inhibits cooperatively (1, 5)	Ineffective (3, 6)
Hg^{++}	n.t.	Inhibits at low ionic strength (10), ineffective at normal ionic strength (4)	Inhibits at low concentration, reversal at higher conc. (>0.1 mM) (10)
Pronase	n.t.	Ineffective (1)	Ineffective (11)
FDNB	n.t.	Inhibits slightly (4, 12)	Inhibits (3)
PCMBS	Ineffective (9)	Inhibits (9)	n.t.
NEM (10 mM)	n.t.	Ineffective (4)	Inhibits
EDC (30 mM) (water-soluble carbodiimide)	Inhibits slightly (4) (20%)	Inhibits strongly (4) (100%)	Inhibits (4) (60%)

n.t. = not tested.

(1) DEUTICKE (1975c), (2) CARLSEN and WIETH (1976), (3) LA CELLE and PASSOW (1971), (4) DEUTICKE (unpublished results), (5) STEIN (1962b), (6) WIETH (1970), (7) HUNTER et al. (1965), (8) OWEN and SOLOMON (1972), (9) MACEY and FARMER (1970), (10) LEFEVRE (1948), (11) PASSOW (1971), (12) BOWYER and WIDDAS (1956).

b) Glycerol

Effects of H^+ and Cu^{++}. The transfer of glycerol has long been known to involve a specific pathway in erythrocytes of numerous mammalian species including man and rodents (JACOBS et al., 1935; DE GIER et al., 1966). In these species glycerol transfer is strongly pH-dependent, inhibited by low ($<10^{-5}$ M) concentrations of Cu^{++} and only slightly dependent on temperature ($E_a = 9$–13 kcal/mole). In other species (e.g., ox, pig, dog, cat) the temperature-dependency is more pronounced ($E_a = 20$ kcal/mole (Sect. IV B2)) but neither pH nor Cu^{++} have any effect. Results in favor of a simple lipid pathway of glycerol transfer in these species have been discussed in Section IV B2.

Between pH 6 and 7 the rates of glycerol transfer increase 100- to 1,000-fold (STEIN and DANIELLI, 1956; STEIN, 1958, 1962a, b; DEUTICKE, 1975c). The pH range and the midpoint of the activation curve indicate the titration of a histidine group. The slope of the curve is much steeper than expected for the titration of a single proton acceptor. Cooperative enhancement of permeability (P) describable by a Hill plot seems very likely. The Hill coefficient n can be obtained from the equation, $1-(P/P_{max}) = 1/1+(H^+/K)^{-n}$, where K is the dissociation constant of the proton-accepting group. n has been determined as 2 (STEIN, 1962b) or ~ 3 (DEUTICKE, 1975c; CARLSON and WIETH, 1976). Thus a minimum of two or three interacting charged residues seem to determine the rate of glycerol translocation.

This concept is further supported by the finding that Cu^{++} ions also inhibit cooperatively ($n \sim 2$–3, STEIN, 1962b; DEUTICKE, 1975c). The inhibition may result from the formation of Cu^{++}-histidine complexes (STEIN, 1962). In view of the strong inhibitory action of a carboxyl-blocking carbodiimide (see below) a Cu^{++}-histidine-carboxyl complex (GURD and WILCOX, 1956) should also be considered. The effect of Cu^{++} is not unique: other divalent cations (Zn^{++}, Ni^{++}, Co^{++}) inhibit glycerol transfer, but only at higher concentrations (WILBRANDT, 1941) and in a non-cooperative fashion indicative of a 1:1 interaction with membrane sites (DEUTICKE, 1975c).

Neither H^+ nor Cu^{++} inhibit glycerol transfer completely. The residual, inhibitor-insensitive flux most certainly occurs via the lipid pathway, since its characteristics agree with those of glycerol transfer in erythrocytes lacking the specific glycerol transfer system (Sect. IV B2).

The H^+ titration curve of the specific glycerol pathway is sensitive to numerous environmental parameters (DEUTICKE, 1975c). It is shifted to higher pH values at high ionic strength (Table 7), and by replacement of Cl^- in the medium by more strongly adsorbing anions (e.g., SCN^- or salicylate). These ion-induced displacements agree with the predicted changes for the pK′ values of a basic group.

Effects of small nonelectrolytes. A number of small hydrophilic nonelectrolytes (dimethylformamide, ethylene glycol, propandiols and even glycerol itself) displace the titration curve of glycerol transfer to the left at high concentrations (0.1–1 M) (Fig. 12). These nonelectrolyte effects are difficult to understand, since, on the other hand, the titration curve is insensitive to urea, which has a pronounced hydrogen-bonding capacity like the effective compounds, and

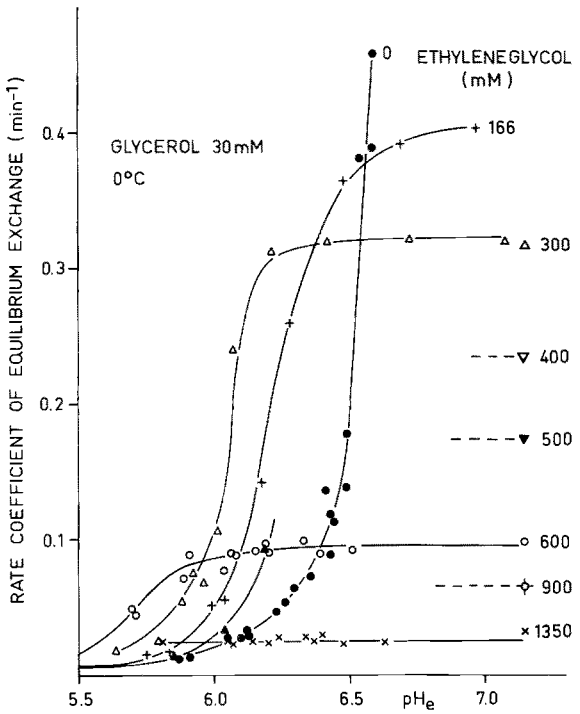

Fig. 12. Shift of the "titration curve" and inhibition of glycerol transfer by ethylene glycol. Tracer efflux measurements at concentration equilibrium (DEUTICKE, 1975c)

to hexanol, which is more lipophilic than the effective compounds. Thus, simple explanations related to hydrogen-bonding or hydrophobic interactions, as discussed for the effects of small nonelectrolytes on the structure of oligomeric proteins (ELBAUM and HERSKOVITS, 1974; SHIFRIN and PARROTT, 1975) have to be excluded. Changes of the dielectric constant of aqueous systems in the presence of the effective nonelectrolytes (WHITACKER et al., 1962) are quantitatively insufficient to account for the shift.

Besides displacing the titration curve, polyols and dimethylformamide reversibly inhibit glycerol transfer in the pH region of maximal activity (Fig. 12) (STEIN and DANIELLI, 1956; STEIN, 1962b; DEUTICKE, 1975c). This latter effect, however, is also observed with monohydric alcohols (JACOBS and PARPART, 1937; HUNTER et al., 1965)[8]. It is not yet clear whether the inhibition is competitive (STEIN, 1962b) or noncompetitive (DEUTICKE, 1975c). A competition for hydrogen-bonding groups in the transport pathway combined with noncompetitive perturbation of the transfer pathway or its lipid environment (Sect. IV B 3) would reconcile both observations. According to LACKO et al. (1974) and KRUPKA (1971), aliphatic alcohols inhibit facilitated hexose transfer also in a mixed fashion.

Competitive inhibition of glycerol transfer by other H-bonding compounds would indicate a limited number of transfer sites. The glycerol affinity of these

[8] The inhibition by alcohols disappears in the presence of Cu^{++}. The Cu^{++}-insensitive glycerol pathway is enhanced by alcohols, supporting its lipid nature.

sites, however, would have to be low in view of an apparent half-saturation constant of glycerol transfer > 1 M between 0 and 10° C (STEIN, 1962b; DEUTICKE, 1975c). Even this saturation, however, could be fortuitous since at high concentrations (> 1 M) glycerol, like other polyols, perturbs protein pathways in the erytrocyte membrane, e.g., those to arabinose, erythritol and anions (KIRK and GUNN, 1976; DEUTICKE, unpublished results). It may thus "shut off" its own, nonsaturable pathway, a view also supported by recent studies of CARLSEN and WIETH (1976).

Covalent modifiers. In view of its unique properties, the identification of the glycerol pathway is of particular interest. For this task, covalently bound inhibitors should be valuable. Glycerol transfer is inhibited by Hg^{++} and PCMB at low ionic strength (LE FEVRE, 1948). The more polar PCMBS, also inhibits at normal ionic strength (DEUTICKE, 1975c). Whether these inhibitions indicate the presence of SH-groups in the pathway is not clear. STEIN (1958) has reported the reversal of PCMB inhibition by histidine. Together with the dependency on ionic strength this would exclude SH groups as sites of inhibition. Inhibition by PCMBS, on the other hand, cannot be reverted by histidine, cysteine and other SH-reducing compounds (DEUTICKE, to be published). Thus SH groups in an environment accessible only to the polar sulfonate may be involved. A lack of inhibition by the more lipophilic SH reagent, NEM, supports this concept.

FDNB and TNBS, which block the pathway of anions (Sect. IVD4a) and monosaccharides (KRUPKA, 1971; JUNG, 1974; JUNG and RAMPAL, 1974) affect glycerol transfer only little, indicating a minor importance of superficial amino groups. Moreover, cleavage of proteins accessible on the outer surface of intact erythrocytes by high concentrations of pronase (Sect. IIID1, IVD4a) does not interfere with glycerol transfer (DEUTICKE, 1975c).

PCMBS, although a potent inhibitor, is not well suited for the identification of proteins due to the easy reversibility of its binding (SUTHERLAND et al., 1967). However, a water-soluble carbodiimide, EDC[9] has recently been shown to block glycerol transfer in human erythrocytes irreversibly to the same extent as Cu^{++} (DEUTICKE, to be published). The residual, EDC-resistant pathway has all the characteristics of the lipid pathway.

Carbodiimides are mainly known for their interaction with carboxyl groups (HOARE and KOSHLAND, 1967; MEANS and FEENEY, 1971; Table 8). In the presence of primary amines, carboxamides can be formed. It is not yet clear whether the inhibition of glycerol transfer involves such a phenomenon since exogenous amines are not required for the inhibitory action. Thus, either membrane amino groups are linked to membrane carboxyl groups by EDC, or the inhibition is due to the modification of one of the other possible binding sites of carbodiimides, e.g., tyrosyl, SH, or even amino groups (MEANS and FEENEY, 1971; HIRS and TIMASHEFF, 1972). In spite of its limited chemical specificity carbodiimide seems at present the most promising tool for the identification of the proteins involved in glycerol transfer.

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[9] 1-ethyl-3(3-dimethylaminopropyl) carbodiimide.

c) Erythritol

Investigations by BOWYER and WIDDAS (1956), LA CELLE and PASSOW (1971) and WIETH (1971) have shown that erythritol, although structurally very similar to glycerol, does not penetrate via the glycerol pathway but uses, in human erythrocytes, to some extent the system catalyzing the stereo-specific saturable transfer. The inhibition saturates at this level, indicating that the residual flux proceeds via another pathway, probably the lipid domain (Sect. IIIB), which appears to be the only pathway of erythritol transfer in other species, e.g., ox and pig (DEUTICKE, 1975a). The hexose concentrations required for half-maximal inhibition of erythritol transfer roughly agree with the half-saturation constants of their own transfer, as compiled by STEIN (1967). Erythritol transfer in the absence of hexoses, on the other hand, does not saturate between 0 and 400 mM, indicating a very low affinity to the hexose transfer system.

The hexose-sensitive fraction of erythritol transfer is blocked by fluodinitrobenzene, $HgCl_2$, phlorhidzin and phloretin (Table 7), well-known inhibitors of mediated hexose transfer. In addition, tetrathionate (50 mM) a specific oxidant of SH groups (MEANS and FEENEY, 1971) inhibits erythritol transfer (DEUTICKE, unpublished results). This renders support to the view that SH groups are involved in erythritol transfer. Whether or not the effect of fluodinitrobenzene also results from the blockade of SH groups as proposed by JUNG and RAMPAL (1974) or indicates the contribution of amino groups (BLOCH, 1974) is still an open question. Carbodiimides inhibit erythritol transfer to some extent (Table 7), in agreement with their inhibitory effect on glucose transfer (BLOCH, 1974).

Furthermore, the hexose-sensitive transfer of erythritol is inhibited by monovalent alcohols and polyols including ethylene glycol and glycerol (DEUTICKE, to be published) suggesting sensitivity of the pathway to perturbation of lipid-protein interactions. This interpretation is also supported by the effects of cholesterol demonstrated in Section IVC6. pH and ion milieu have little or no influence in contrast to their marked effects on glycerol transfer.

The further elucidation of the protein pathway of erythritol will be closely connected to the identification of the hexose pathway, which, although in progress (TAVERNA and LANGDON, 1973; JUNG and CARLSON, 1975; LIN and SPUDICH, 1974) is far from being accomplished.

6. Influence of Membrane Lipids

Transmembrane protein pathways are of necessity composed of intrinsic proteins. Consequently, the surrounding hydrophobic domain is likely to affect their properties. As outlined in Section IIIC2 and IVB4a, experimental variation of membrane cholesterol influences the fluidity of the lipid domain. In analogy to observations on various membrane-associated enzymes (PAPAHADJOPOULOS et al., 1973; COLEMAN, 1973; HALLINAN, 1974), a rise in membrane fluidity should stimulate protein-mediated transfer processes. Contrary to this expectation *cholesterol depletion* of human erythrocytes *inhibits* the protein-related transfer of glycerol and erythritol (DEUTICKE, to be

published), glucose (MASIAK and LE FEVRE, 1975), uridine (READ and MCELHANY, 1976). Inhibition of K^+ fluxes (POZNANSKY et al., 1973) and of anion transfer (Sect. IV F) have also been observed in cholesterol-depleted cells. Numerous protein pathways of the erythrocyte thus seem to require for their physiologic function a high ratio cholesterol/polar lipid. For the hexose-erythritol system this concept is also substantiated by the observation that the equilibrium fluxes of erythritol and arabinose are *enhanced* in *cholesterol-loaded* cells (DEUTICKE, in preparation).

This effect of cholesterol is somewhat unexpected in view of the rigidifying effect of high cholesterol levels in the erythrocyte membrane, postulated on the basis of spin resonance (KROES et al., 1972); rheologic (COOPER et al., 1975) and permeability (DEUTICKE and RUSKA, 1976) studies. It might be speculated that the presence of cholesterol relieves some sort of phospholipid-induced strain on protein structures involved in hexose transfer, and thus facilitates the translocation. Analogous phenomena have been described for membrane enzyme activities (HALLINAN, 1974).

7. The Dual Effect of Alcohols and Phloretin

In addition to its long-known inhibitory action on hexose transfer (WILBRANDT, 1950; LE FEVRE and MARSHALL, 1959), phloretin inhibits the transfer of small hydrophilic nonelectrolytes assumed to penetrate predominantly via protein structures, but accelerates the transfer of nonelectrolytes via the lipid pathway (OWEN and SOLOMON, 1972; KAPLAN et al., 1974). This dual effect of phloretin is also reflected in its binding to erythrocyte membrane proteins and lipids (JENNINGS and SOLOMON, 1975; EHRENSPECK, 1975). Phloretin thus mimics, although at much lower concentrations, the influence of simple alcohols, which are bound to membrane lipids and proteins (COLLEY et al., 1971) and have inhibitory effects on nonelectrolyte movements involving protein pathways. (HUNTER et al., 1965; KRUPKA, 1971; LACKO et al., 1973), while accelerating nonelectrolyte movements via the lipid pathway (Sect. IV B 3). Interestingly, the parallelism between phloretin and alcohols does not hold in all mammalian species: polyol transfer in bovine and porcine erythrocytes, e.g.,, is enhanced by alcohols but not affected by phloretin (DEUTICKE, unpublished). Thus, although the accelerating action of the two agents may result from a fluidization of the lipid domain, the effect of phloretin seems to be linked to structural requirements at the lipid-water interface not fulfilled in all species. Phloretin inhibition of protein pathways in the human erythrocyte, like alcohol inhibition, could result from its direct binding to the pathway, from the perturbation of its lipid environment, or both effects.

A dual effect of phloretin on lipid and protein pathways may also account for the observation that the phloretin-induced inhibition of nonelectrolyte transfer, which is most pronounced for the very hydrophilic compounds (urea, glycerol, formamide), becomes gradually less and turns into an enhancement of transfer with increasing hydrophobicity of the permeants (OWEN and SOLOMON, 1972). This pattern may simply reflect the transition from a high-

fractional contribution of protein pathways (inhibited by phloretin and alcohols) to a major contribution of the lipid pathway (enhanced by the two agents) in transfer processes involving both pathways.

D. Protein Pathways for Inorganic Anions

1. General Aspects

In view of the low anion permeability of artificial phospholipid bilayers, anions may be expected to permeate the lipid domain of the erythrocyte membrane only very slowly. For artificial membranes chloride tracer permeabilities between 5 and 50×10^{-12} cm/s have been reported (HAUSER et al., 1972); PAPAHADJOPOULOS et al., 1972; TOYOSHIMA and THOMPSON, 1975a, b). Even these low values are probably not due to the penetration of free anions, but involve electrically silent movements of anions bound to phospholipid head groups and translocated by a "flip-flop" of the complex (TOYOSHIMA and THOMPSON, 1975a, b), or moving as undissociated strong acid, unimpeded by energetic barriers, across the low dielectric membrane interior (BANGHAM, 1972). Although in particular the latter mechanism is relevant to the passive transfer of organic anions across the erythrocyte membrane, inorganic anions penetrate the membrane so fast that specialized protein pathways have to be claimed.

As a physiologically important characteristic these pathways convey to the membrane a conspicuous permselectivity of anions over cations. A chloride permeability of 10^{-3} cm/s at 37° C (calculated from isotope flux measurements of TOSTESON (1959) and DALMARK and WIETH (1972) contrasts with cation (Na^+, K^+) permeabilities of approximately 10^{-10} cm/s (TOSTESON and HOFFMAN, 1960). Very considerable permeability differences within the group of anions ($P_{Cl}:P_J:P_{PO_4} = 10^{-3}:4\times10^{-6}:3\times10^{-8}$ cm/s, calculated from DALMARK and WIETH (1972) and own data) constitute a further characteristic of the anion pathways.

These data refer to measurements of anion permeability by tracer techniques in the absence of net salt movements, i.e., under *equilibrium exchange* conditions. Due to the very low physiological cation permeability of the erythrocyte, these conditions reflect the in vivo situation, where Cl^- exchanges with HCO_3^- in the course of CO_2 transport in blood (HAMBURGER, 1891).

In contrast, anion permeabilities can also be computed from the rates of *net loss of anions* and cations from erythrocytes after disruption of the cation barrier by ionophorous antibiotics such as valinomycin or gramicidin (HARRIS and PRESSMAN, 1967; HUNTER, 1971; TOSTESON et al., 1973; WIETH et al., 1973), or by exposure to very low ionic strengths (WIETH et al., 1973). Although the calculation of permeabilities under these conditions requires certain assumptions such as the applicability of the GOLDMAN equation (HUNTER, 1971), there can be little doubt that the "net chloride permeability" thus obtained is lower by orders of magnitude than chloride exchange permeability and only about 100 times higher than K^+ permeability. The conclusion that anions pass the erythrocyte membrane at least by two pathways, an electrically silent "*exchange pathway*" and a current-carrying "*conductance pathway*"—responsible for the

net permeability—is also supported by conductance measurements (HOFFMAN and LASSEN, 1971; LASSEN et al. 1974): The membrane resistance of amphibian erythrocyte membranes was found to be at least 4, possibly 7 orders of magnitude higher than the resistance of 1 Ω cm^2 calculated from the tracer exchange fluxes of chloride. In case of the divalent anions, the difference between exchange and conductance pathway seems to be much lower (PASSOW et al., 1974).

The structural relationships between these two operationally defined anion pathways are far from clear, particularly since few data are available for the kinetics and properties of the conductance pathway. The further discussion will therefore focus on the structural aspects of the exchange pathway(s).

2. Influence of Environmental Parameters

pH. The transfer rates of divalent anions (sulfate, phosphate) have frequently served to characterize anion transfer, which is a passive process in the erythrocyte (PASSOW, 1964; DEUTICKE, 1967). Early observations of MOND (1927) indicated that a decrease of pH from 8.5 to 6.5 greatly enhances the net exchange of sulfate against chloride. This finding was substantiated by measurements of equilibrium tracer fluxes of divalent anions (RUMMEL and PFLEGER, 1958; PASSOW, 1961, 1969; DEUTICKE, 1970) and interpreted in terms of a "fixed charge hypothesis" of anion transfer.

In its most elaborate version (PASSOW, 1969; LEPKE and PASSOW, 1971) this concept postulated that the concentration of a penetrating anion at the surface of the membrane is determined by the density of positive, dissociable fixed membrane charges, probably amino groups, in front of the transfer rate limiting barrier. Anions were assumed to be distributed between the medium and the membrane domain bearing these charges according to Donnan equilibria. Changes of sulfate and phosphate fluxes with the concentration of H^+, sulfate (phosphate) and chloride predicted from the quantitative formulation of this hypothesis could be fitted to the experimental results. However, in addition to a proper choice of the concentration (2.5–3 M) and the dissociation constant ($K \sim 10^{-9}$) of the fixed charges, an exponential relationship between fluxes and intramembrane concentrations of the penetrating anions had to be assumed. This was taken to indicate an influence of anions on the properties of the barrier limiting the rate of anion transfer (DEUTICKE, 1970; LEPKE and PASSOW, 1971).

As a further complication, the fluxes as well as the permeability of divalent anions proved to reach a maximum at ~pH 6.5 and to decrease again at lower pH values (Fig. 13a). In view of this finding (DEUTICKE et al., 1968; SCHNELL, 1972), charged groups other than amino groups have to be postulated in the anion exchange pathway. This assumption is also supported by the pH-dependency of halide transfer. In contrast to the predictions of the fixed-charge concept, chloride exchange *permeability*[10]—measured at 0° C (DALMARK and

[10] Chloride *fluxes* behave differently: They increase between pH 5.5 and 7.0, but decrease again at high pH values if measured at physiological or lower chloride concentration (GUNN et al., 1973). This phenomenon is probably related to pH-dependent changes of the intracellular Cl^- concentration and of the Donnan distribution ratio (PASSOW and WOOD, 1974; DALMARK, 1975; FUNDER and WIETH, 1975).

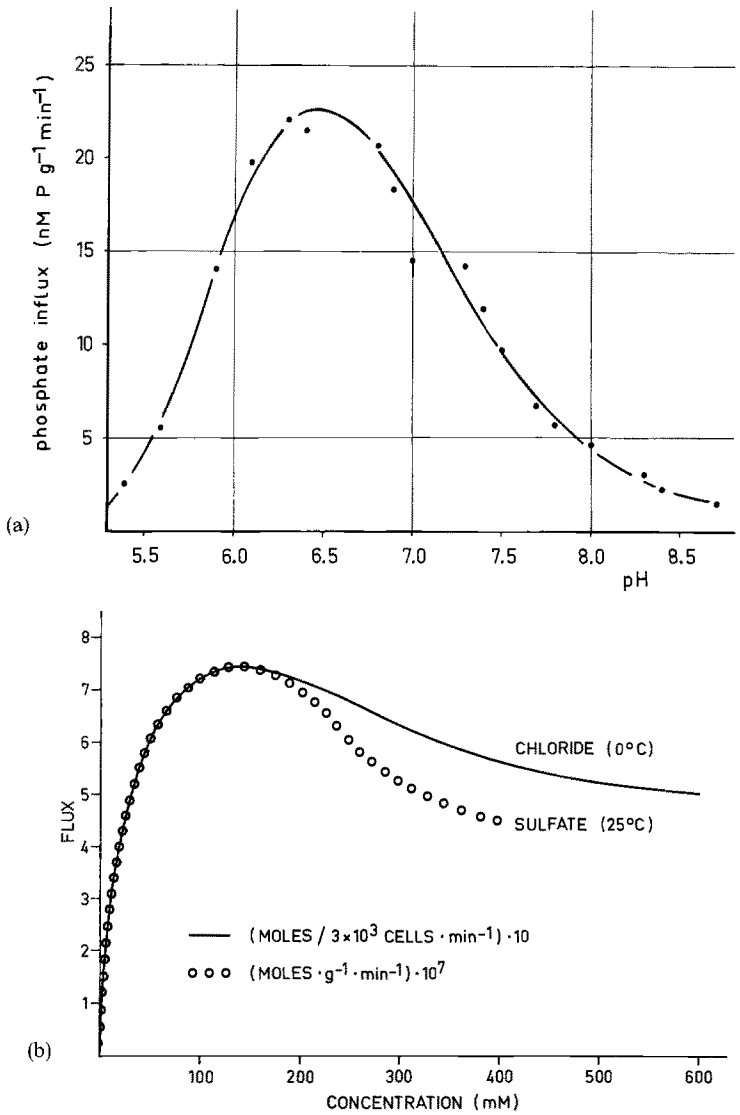

Fig. 13a and b. (a) pH-dependency of phosphate exchange in human erythrocytes. Tracer influx measured at 37° (DEUTICKE et al., 1968). (b) Concentration dependency of anion exchange fluxes in human erythrocytes at pH 7.2. Replotted from DALMARK (1975) for chloride and from SCHNELL et al. (1975) for sulfate. The cells were suspended in media containing increasing concentrations of the anion studied. No compensation for differences in osmolarity. Intracellular cation concentrations modified by polyene antibiotics

WIETH, 1972)—increases steadily with pH over the range from pH 5.5 to 7.0 and remains virtually constant between pH 7 and 9 (DALMARK, 1975; FUNDER and WIETH, 1975). Iodide permeability even increases continously between pH 6 and 9, although much less steeply than chloride permeability (PASSOW and WOOD, 1974). Mono- and divalent inorganic anions thus have in common a

remarkable inactivation of their transfer in the acid range. In the alkaline range, their transfer kinetics differ considerably. Taking for granted the presence of positive fixed charges in the anion pathway—which is strongly suggested by evidence dealt with below—this divergency seems difficult to explain as yet, in particular, since it is also observed in ghosts (SCHNELL, 1974; SCHNELL et al., 1975; FUNDER and WIETH, 1975) where charge asymmetries due to the Donnan equilibrium are absent.

Concentration dependency. The concept that positive fixed charges are involved in anion binding to the erythrocyte membrane prior to their translocation implies a limited number of such sites. This should lead to saturation kinetics. Such a phenomenon could not be demonstrated in experiments in which the concentration of the anion under study was elevated by equiosmolar substitution of Cl^- (DEUTICKE, 1967, 1970; LEPKE and PASSOW, 1971) since anion competition, discussed below, obscured the true concentration dependency. This difficulty was recently overcome by measuring fluxes under conditions of an isolated variation of the concentration of the anion under study (CASS and DALMARK, 1973; GUNN et al., 1973; PASSOW and WOOD, 1974; SCHNELL et al., 1975; DALMARK, 1975, 1976). Under these conditions, fluxes of sulfate, Cl^- and J^- reach a maximum (Fig. 13b). The concentration at half-maximal transfer (corresponding to an apparent Michaelis-Menten constant K_T) amounts to 30 mM for both Cl^- and sulfate (DALMARK, 1975; SCHNELL et al., 1975), although the corresponding fluxes differ by many orders of magnitude. K_T values for sulfate and chloride increase at alkaline pH, indicating a common lowering of the anion affinity at high pH in spite of the different pH dependencies of fluxes in this pH range.

Although the fluxes of sulfate and chloride reach a maximum, true saturation, as typical for simple, site-limited transfer processes, is not observed: At high concentrations (>200 mM) fluxes rediminish again (CASS and DALMARK, 1973; SCHNELL et al., 1975) and probably tend to zero at infinetely high anion concentrations (SCHNELL et al., 1975). This self-inhibition indicates a lowering of the maximal transport capacity by the transported anions and is probably mediated by the presence of "inhibitory binding sites" (SCHNELL et al., 1975; DALMARK, 1976).

Anion milieu. A limited number of anion-binding sites in the anion pathway is also indicated by pronounced effects of anions on anion transfer (DEUTICKE, 1967, 1970; WIETH, 1970; DALMARK and WIETH, 1972; PASSOW and WOOD, 1974; SCHNELL, 1974; SCHNELL et al., 1975; DALMARK, 1976). Equiosmolar replacement of chloride by other anions inhibits or enhances the equilibrium exchange of ions indicative of a pattern of increasing blockade of fluxes of mono- and divalent inorganic anions by other anions in the sequence malonate < lactate < acetate ≦ propionate < Cl ≦ Br < NO_3 < J < SCN, similar to the classic Hofmeister lyotropic series of increasing adsorbability (Fig. 16a). The sequence of transfer inhibition by inorganic anions differs from the sequence of anion permeabilities: As shown by DALMARK and WIETH (1972), self-exchange permeabilities decrease in the order $Cl > Br > SCN > J > SO_4$. Thus, anion-binding to the membrane as reflected by anion-inhibition is certainly not the only parameter determining the selectivity of the anion pathway.

As expected, the inhibitory effects of the inorganic anions are mainly competitive (GUNN et al., 1973; SCHNELL et al., 1975; DALMARK, 1976) indicating at least one common binding site for mono- and divalent anions in the pathway. In addition, however, noncompetitive components have also been demonstrated, leading to a decrease of the maximal transfer rates (DALMARK, 1976). Such noncompetitive effects, occurring at high concentrations of inorganic anions and probably responsible for the self-inhibition of anion transfer, are more pronounced and even characteristic for the inhibition produced by organic anions, dealt with in Section IVD4b).

Temperature dependency. The rate-limiting barrier for anion diffusion is probably different from the anion-binding region. In kinetic studies the significance of this barrier is illustrated by the very high temperature-dependency of anion translocation. Irrespective of the large differences in the absolute rates of transfer, temperature coefficients between 5 and 7 are obtained for mono- and divalent inorganic anions, equivalent to apparent activation energies of 30–35 kcal/mole (PASSOW, 1964; DEUTICKE, 1970; LEPKE and PASSOW, 1971; DALMARK and WIETH, 1972).

The reason for these high activation energies, which even exceed those for the transfer of hydrophilic nonelectrolytes (Sect. IVB2) and ions (PAPAHADJOPOULOS et al., 1971) across lipid bilayers, is obscure as yet. They are not affected by changes of pH (SCHNELL, 1972) and anion milieu (DALMARK and WIETH, 1972). Only extensive treatment with amino-reactive agents or pronase has been reported to lower the activation enthalpy (Sect. IVD4a).

Comparative aspects. Inorganic anion exchange in erythrocytes from other mammalian species, in spite of quantitative differences (GRUBER and DEUTICKE, 1973) seems to proceed by pathways similar to those in human erythrocytes. This can be concluded from a comparison between phosphate and Cl fluxes in 10 mammalian species (Fig. 14). With two exceptions the points fall on a straight line, which indicates that similar mechanisms of anion discrimination are operative. Moreover, phosphate transfer in erythrocytes from nine

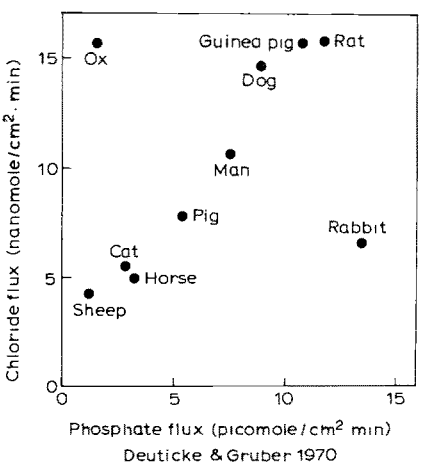

Fig. 14. Correlation between chloride and phosphate exchange fluxes in different mammalian species. Courtesy WIETH et al. (1974) with permission of the publisher

mammalian species has been shown to exhibit the same dependency on pH, temperature and anion milieu as in human erythrocytes (GRUBER and DEUTICKE, 1973).

3. Kinetic Models of the Pathway

pH-, concentration- and anion-dependencies of anion transfer obviously require the assumption of a limited number of transport sites. The kinetic details, however, can be fitted to a number of models of anion exchange which have extended or replaced the original fixed charge hypothesis. These models, which shall not be discussed in the present context, postulate alternatively:

1. Anions are translocated as complexes with positively charged, titratable "carriers"—defined in a very broad sense—(GUNN, 1972). The protonated groups were originally assumed to serve directly as binding sites, preferring mono- or divalent anions or being inactive in dependency on their own valency prior to the binding of the anions. A recent extension of this model claims that protonation determines the fraction of a nonspecified total pool of anion carrier available for anion transfer at a given pH (DALMARK, 1975). Complexes with monovalent anions are assumed to be neutral, complexes with divalent anions may also carry charge and thus move much slower across the hydrophobic barrier.

2. The transport of anions across the erythrocyte membrane is a diffusion process depending on the binding of anions to superficial adsorption sites. The anions penetrate the erythrocyte membrane via a proteinaceous region by a sort of "hopping mechanism". The unidirectional anion fluxes are determined by the fraction of sites occupied by anions at both membrane surfaces. The fraction of occupied sites, in turn, depends on the bulk anion concentration but is modified by surface potentials arising from cationic surface charges (SCHNELL, 1974a, b). The two oppositely directed unidirectional fluxes are coupled by the electrical potential differences across the main anion diffusion barrier of the cell membrane. As in case of Dalmark's model, the dissociable cationic groups do not act as the anion-binding sites.

In both models anion-binding to the surface or to carriers is regarded as a process preceeding the rate-limiting diffusion of the anion or translocation of the anion-carrier complex across a hydrophobic barrier in the membrane interior.

3. PASSOW and WOOD (1974) have proposed an alternative model in which the reaction between the penetrating anion and the binding sites in the membrane is the rate-limiting step. The rate coefficients for the binding of an anion to an empty site, to a site already complexed with an isotopic species of the same anion, or a site occupied by another anion, are assumed to differ from each other. This model accounts in particular for the anion competition and explains saturation as a replacement of OH^- by the penetrating anion.

All these models can incorporate many kinetic properties of anion transfer but none can explain all features at present. For this reason the kinetic data are not yet helpful for elucidating the structural basis of the anion pathway.

4. Structural Characteristics of the Pathway

a) Covalent Modifiers

Amino-reactive agents. The pH dependency of the transfer of divalent anions in the alkaline range (Sect. III C 2a) has been taken as evidence for the significance of amino groups, presumably in the ε-position of lysine, which could either act as direct binding sites for anions or indirectly determine the binding characteristics of the transfer pathway. Modification of membrane amino groups by amino-reactive agents (Table 8) has been used to test this assumption. FDNB, which increases erythrocyte cation permeability (BERG et al., 1965), was shown to inhibit irreversibly, although not completely, the transfer of divalent and monovalent inorganic anions (POENSGEN and PASSOW, 1971; WOOD and PASSOW, 1971). This reagent, however, is not amino-specific but also reacts with sulfhydryl, imidazole, and tyrosyl groups (MEANS and FEENEY, 1971). In the erythrocyte membrane, STEIN (1967) has demonstrated the dinitrophenylation of lysine as well as cysteine, histidine, and other unidentified groups. Moreover, FDNB combines with amino groups of phosphatidylethanolamine (POENSGEN and PASSOW, 1971; GORDESKY et al., 1973).

Nevertheless, its inhibitory action most likely results from the arylation of amino groups, since reagents of higher amino-specificity than FDNB also inhibit anion permeability. These include: (1) TNBS (ZAKI et al., 1971; KNAUF and ROTHSTEIN, 1971 b; DEUTICKE, 1975 b), which except for a very labile binding to SH groups appears to be exclusively bound to amino groups of proteins and phospholipids in the erythrocyte membrane (BONSALL and HUNT, 1971; (2) 2-methoxynitrotropine (PASSOW and SCHNELL, 1969), claimed to be amino-specific (TAMAOKI et al., 1967); and (3) maleic anhydride (MA) (OBAID et al., 1972) supposed to form—besides anionic amino-maleyl derivatives—only products labile at neutral and higher pH (MEANS and FEENEY, 1971).

In contrast, amidination of membrane amino groups by permeable and impermeable imidates, as described by WHITELEY and BERG (1974) (Table 8) has been reported not to affect anion permeability nor to protect against the inhibitory action of the potent "bimodal" inhibitors dealt with in Section IV D 4c (CABANTCHIK, 1976). In contrast to the other amino reagents,

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Footnotes to Table 8

* Proteins are symbolized by encircled Pr, showing major site of attack by modifiers. Other sites of attack in brackets.

[a] Discrimination between reaction of NH_2 or SH-groups is sometimes possible by "thiolysis" which cleaves S-dinitrophenyl but not N-dinitrophenyl derivatives (SHALTIEL, 1967; BLOCH, 1974).

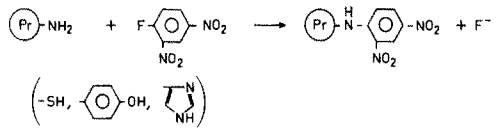
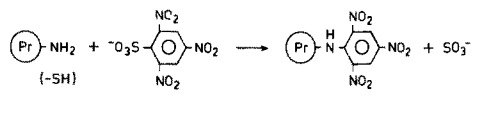
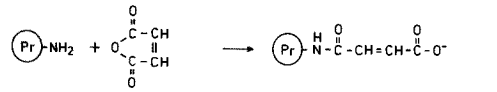
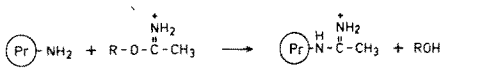
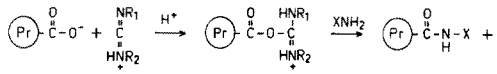
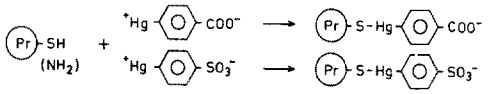
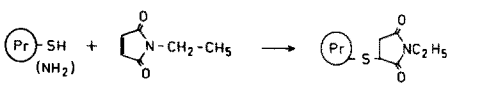
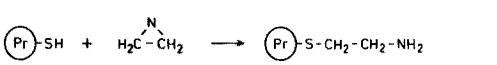
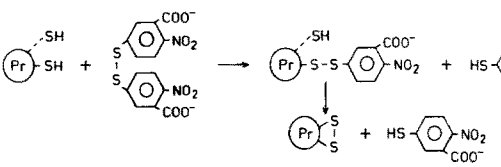
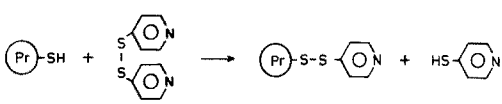
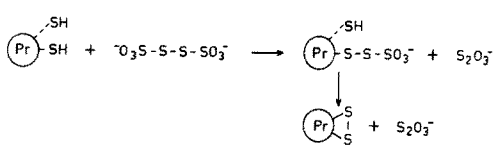
[b] WHITELEY and BERG (1974) used ethyl ($R = C_2H_3$) and isethionyl ($R = CH_2 \cdot CH_2 \cdot SO_3^-$) acetimidate.

[c] The reaction with SH-groups is highly favoured at low (< 7) pH values.

[d] Under suitable conditions the S-thionitrobenzene derivative may undergo disulfide interchange with additional SH-groups yielding intra- or intermolecular disulfide bonds.

[e] Besides *intra*molecular disulfide bonds, *inter*molecular disulfide bonds between membrane proteins arise in the presence of this agent (HAEST and DEUTICKE, 1976 b).

Table 8. Covalent modifiers of passive transfer pathways in erythrocyte membrane*

	1-Fluoro-2,4-dinitrobenzene[a]
	2,4,6-Trinitrobenzene sulfonate
	2-Methoxy-5-nitrotropone
	Maleic anhydride
	Acyl-acetimidates[b]
	Carbodiimides
	p-chloromercuribenzoate (pCMB) p-chloromercuriphenyl sulfonate (pCMBS)
	N-ethyl maleimide (NEM)[c]
	Ethylenimine
	5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB)[d]
	4,4′-Dithiodipyridine (DTDP)
	Tetrathionate[e]

imidates replace the modified membrane NH_2 group by an amidine, thus preserving the net number of positive charges. Whether or not this charge preservation is responsible for the lack of effects remains to be elucidated.

FDNB and MA rapidly enter the erythrocyte (POENSGEN and PASSOW, 1971; OBAID et al., 1972). FDNB binds to all major proteins of the membrane (ZAKI et al., 1975b). TNBS penetrates at best very slowly (GORDESKY et al., 1975; HAEST and DEUTICKE, 1975), but inhibits anion transfer almost as effectively as the other amino-reagents. This indicates a predominant arrangement of the amino groups of the anion exchange pathway at the outer membrane surface. STECK (1972) has demonstrated selective binding of TNBS to band 3 and glycophorin (Sect. III D 1) within the limits of resolution of SDS gelelectrophoresis. The conclusion that one of these proteins constitutes the anion pathway is also supported by the finding of ZAKI et al. (1975b) that stilbene sulfonates which specifically inhibit anion transfer, selectively prevent the binding of FDNB to band 3 and counteract its irreversible inhibitory action on anion exchange. Further support for the relevance of band 3 is provided by studies with "bimodal inhibitors", discussed in Section IV D 4c.

The mechanism of the inactivation of the anion pathway by amino group modification is not clear. Depending on the kinetic model (Sect. IV D 3) the binding of anions might be affected due to a loss of binding sites or a lowering of the surface potential. On the other hand, amino-reactive agents have been shown (RIGAUD et al., 1974) to affect the ESR spectra of spin-labelled proteins in the erythrocyte membrane. This finding suggests a possible indirect effect of amino-reactive agents via local conformation changes in the anion pathway subsequent to a modification of amino groups not directly involved in anion-binding. Such a mechanism of an "allosteric inhibition" has to be considered even more seriously in view of the inhibition of hexose transfer by these amino-reactive agents (BOWYER and WIDDAS, 1956; KRUPKA, 1971; JUNG, 1974; BLOCH, 1974) which is dependent on the conformational state of the transfer system (KRUPKA, 1972).

A further matter of debate concerns the nature of the residual transfer in presence of maximally inhibitory concentrations of amino-reagents. SCHWOCH et al. (1974) have shown that in FDNB-treated erythrocytes sulfate transfer is much less temperature-dependent than in unmodified cells. As a consequence, the transfer in modified cells is inhibited at high, but enhanced at low temperature. This may indicate, that, by inducing local conformation changes, the modifier not only perturbs the normal anion exchange pathway but also opens an additional pathway with a much lower resistance.

SH-reagents. Mercurials (PCMB, PCMBS) and other SH-reagents (NEM, DTNB, tetrathionate) enhance the cation permeability (ROTHSTEIN, 1970; KNAUF and ROTHSTEIN, 1971a, b) and block the hexose pathway (LE FEVRE, 1948; DAWSON and WIDDAS, 1963; VAN STEVENINCK et al., 1965; BLOCH, 1974; SMITH and ELMAN, 1974) of the erythrocyte membrane. In contrast, PCMBS does not interact with the transfer system for inorganic anions in a way interfering with its function (KNAUF and ROTHSTEIN, 1971a). The same is essentially true (DEUTICKE, unpublished results) for other SH reagents, such as DTNB and DTDP and ethyleneimine.

On the other hand, sulfate exchange is markedly inhibited by NEM (5–20 mM). In contrast to PCMBS and DTNB, NEM, but also DTDP rapidly penetrate the erythrocyte membrane (JACOB and JANDL, 1972; SMITH and ELMAN, 1974) and react with a considerable number of membrane SH groups in the intact erythrocyte (DEUTICKE, HAEST and KAMP, unpublished results). In view of the lack of inhibition by DTDP, the inhibitory action of NEM does not constitute unequivocal evidence that SH group modification inhibits anion transfer. There is even evidence for a covalent binding of this alkylating agent to amino groups (HOLBROOK and JECKEL, 1969).

Carbodiimides. EDC, a water-soluble carbodiimide (Table 8), irreversibly inhibits the transfer of sulfate at concentrations between 10 and 30 mM (DEUTICKE, to be published). As in case of glycerol transfer (Sect. IV C 5 b) the effect is not enhanced by the addition of exogenous amines and thus due either to internal crosslinking between adjacent protein carboxyl and amino groups, or to the modification of other side groups. Carbodiimides have been shown to inhibit Na^+-K^+- and Mg^{++}-dependent ATPases in isolated erythrocyte membranes (GODIN and SCHRIER, 1968). This effect, however, seems to result from the modification of chemically different groups since the presence of amines during the exposition to EDC almost completely prevents the inhibition. Moreover, an increase of osmotic fragility and an almost complete, rapid inactivation of acetylcholine esterase—at low concentrations of EDC—have been reported for intact erythrocytes by DUNCAN (1974). Since the inhibition of the anion pathway develops more slowly than that of choline esterase, the site modified in this system is probably located deeper in the membrane.

Proteases. Erythrocyte membrane proteins are in principle susceptible to proteolytic enzymes, as indicated by their extensive cleavage in ghosts (Table 4). Only a limited fraction is accessible in the intact erythrocyte, in particular to pronase, which degrades the major glycoproteins and cleaves the 95,000-dalton protein into split products of 65,000 and 35,000 daltons. The 65,000-dalton fraction seems to be confined to the membrane core, whereas the 35,000-dalton moiety disappears unless special precautions are taken (BENDER et al., 1971; CABANTCHIK and ROTHSTEIN, 1974 b).

In spite of these structural alterations of membrane-spanning peptides, protein pathways to nonelectrolytes are not perturbed functionally in intact erythrocytes by any of the proteases tested as yet (Sect. IV C). Moreover, neither active cation transport (WAGNER et al., 1974; KNAUF et al., 1974 a) nor the facilitated hexose transfer system (KAHLENBERG et al., 1972) are susceptible to externally applied pronase in intact erythrocytes, whereas the destruction of proteins accessible from the cytoplasmic membrane surface greatly interferes with these processes. The outward-facing domains of the constitutive proteins thus seem to be dispensable for these pathways.

The same is primarily true for anion transfer: Almost complete degradation of the glycoproteins and the 95,000-dalton fraction by pronase (<0.2 mg/ml) or chymotrypsin (CABANTCHIK and ROTHSTEIN, 1974 b; TRIPLETT and CARRAWAY, 1972) in intact human erythrocytes does not impair anion exchange. Very high concentrations, however, of pronase or papain induce a marked inhibition of sulfate transfer and enhance cation permeability (PASSOW, 1971). Less aggressive

proteases (trypsin, chymotrypsin) are ineffective from the outside. Trypsin has only moderate effects on anion transfer even if applied to the inner membrane surface in resealed ghosts (PASSOW et al., 1974), although the enzyme cleaves most proteins, including the 95,000-daltons fraction. The latter is split into a 58,000- and a 48,000-dalton fragment. In contrast to other protein fractions, both fragments are retained within the membrane, as would be expected for an intrinsic protein. Even the sensitivity toward inhibitors such as phloridzin and SITS (Sect. IVD4b, IVD4c) is preserved in these heavily modified membranes. Such findings suggest that considerable alterations of membrane protein do not necessarily affect the anion transfer system.

The inhibitory effect of pronase seems to involve the sites attacked by amino-reactive agents since there is no additivity of the effects of both modifiers (PASSOW, 1971). Pronase lowers the apparent activation energy of anion transfer like FDNB (SCHWOCH et al., 1974).

b) Reversible Inhibitors

In addition to its sensitivity to irreversible modifiers, the transfer of mono- and divalent inorganic anions is inhibited reversibly by a large variety of compounds (GERLACH et al., 1964; PASSOW and SCHNELL, 1969; DEUTICKE, 1970; SCHNELL, 1972; GUNN and COOPER, 1975). The spectrum of inhibitors (Table 9) comprises simple alcohols, fatty acids and alkylamines, phenol derivatives (salicylate, dinitrophenol), compounds known for their pharmacologic activities (local anesthetics, diuretics, dioxopyrazolidines, pyrimidopyrimidines) and inhibitors of protein-mediated transport phenomena, e.g., phlorizin. Some inhibitors are uncharged, others weak anions, others weak cations. Some are effective at very low concentrations and thus regarded as rather specific, others only act at much higher concentrations. So far the only recognized common property of these inhibitors is their amphiphilic structure, i.e., the presence of hydrophilic and hydrophobic domains in the molecule, which indicates a tendency to adsorb to interfaces.

The inhibition is predominantly noncompetitive for all compounds studied as yet (SCHNELL, 1972; GUNN and COOPER, 1975), including anionic inhibitors which might be assumed to compete with penetrating anions. More complex mechanisms must therefore be invoked to explain the inhibition. On the basis of kinetic studies, GERHARDT et al. (1973) have postulated a decrease of the fixed charge density in the presence of inhibitors. In more structural terms, inhibitors such as the alcohols, but also many others, might alter the anion pathway by perturbing lipid-protein interactions due to their incorporation into hydrophobic membrane domains. This interpretation is supported by the finding that alcohols accelerate transfer via the lipid pathway and inhibit numerous mediated nonelectrolyte transfer processes (Sect. IVB3).

Anionic inhibitors may contribute, in addition, an anion-repelling component to the membrane surface potential (FORTES and HOFFMAN, 1974). This effect would be analogous to the well-documented modification of ion conductances in artificial lipid bilayers by weak acids such as salicylate or 2,4-dinitrophenol (MCLAUGHLIN, 1972, 1973, 1975; SINGER, 1973). The direction of these changes

Table 9. Reversible inhibitors of anion exchange in human erythrocytes

	Concentration (mM) required for half-maximal inhibition of the transfer of		
	Phosphate (37° C) influx (1, 2)	Sulfate (37° C) efflux (3, 6)	Chloride (0° C) efflux
Dipyridamole	0.005	0.0007	0.005 (4)
Dioxopyrazolidines			
Phenylbutazone	0.2	0.1	
Phenopyrazone	0.13		
Furosemide	0.5		
Etacrynic acid	0.5		
Phloretin	0.25		0.0015 (8)
Phlorizin	0.55	0.6	
Phenol		12	
Benzoate		14	
Salicylate	2.5	1.0	
Salicylamide		3.0	
Dinitrophenol	1.0	1.0	
Trinitrocresolate		0.1 (5)	
Hexanol		3.2	
Decanoate	1.1		
Octanoate	7.0		
Octylsulfate	2.2		
Octylamine	1.0		
1,8-Anilino-naphtaline sulfonate (8-ANS)		0.15 (4)	0.025 (4)
Local anesthetics			
Cinchocaine	0.3		
Tetracaine	1.2	0.9	0.9 (7)
Hexobendine	0.68		
Papaverine	1.0		
Reserpine	0.2		

(1) DEUTICKE (1970), (2) DEUTICKE (1972), (3) SCHNELL (1972), (4) FORTES and HOFFMAN (1974), (5) GUNN and TOSTESON (1971), (6) DEUTICKE (unpublished results), (7) GUNN and COOPER (1976).

is consonant with alterations of the surface potential due to the adsorption of the charged species of weak acids. Even uncharged molecules, e.g., salicylamide which also inhibits anion transfer, may alter the surface potential due to changes of the dipole potential at the membrane-solution interface (MCLAUGHLIN, 1973). The contribution of such electrostatic mechanisms is also indicated by the observation (DEUTICKE, 1970) that the replacement of chloride by other anions affects the inhibitory potency of anionic inhibitors.

The inhibitory action of weak bases such as the local anesthetics cannot be explained by changes of the surface potential which should favour, but not inhibit, anion movements (MCLAUGHLIN and HARARY, 1976). Moreover, GUNN and COOPER (1975) have provided evidence that the protonated *and* the unprotonated species of a local anesthetic are inhibitory. The binding of such compounds to lipid membranes has been shown to involve electrostatic interactions with anionic groups, e.g., of PS (HAUSER et al., 1969; PAPAHADJO-

POULOS, 1972; PAPAHADJOPOULOS et al., 1975a) as well as hydrophobic interactions (CERBÒN, 1972; FERNANDEZ and CERBÒN, 1972) in particular with PS domains (PAPAHADJOPOULOS, 1975a). These are fluidized in the presence of local anesthetics. In the erythrocyte membrane, PS is thought to be localized at the inner membrane surface (Sect. III C1) and may interact preferentially with proteins (Sect. III C1). It is tempting to speculate that the effects of cationic inhibitors are in part due to their high affinity for PS, which causes a perturbation of the interaction of this phospholipid with proteins involved in anion transfer.

Evidence for different sites of action of anionic and cationic inhibitors is also provided by their antagonistic effects on erythrocyte shape: Anionic inhibitors produce crenated forms ("echinocytes"), cationic ones cup-shaped "stomatocytes" (DEUTICKE, 1968). In a concept recently put forward by SHEETZ and SINGER (1975), this difference has been ascribed to a preferential adsorption of anionic amphiphiles to the outer, and cationic ones to the inner membrane lipid layer due to electrostatic repulsion or attraction by the more negatively charged inner layer (Sect. III C1).

A possible asymmetry of inhibitor effects also follows from the observation that phloridzin as well as certain reversibly acting disulfonate inhibitors of anion transfer (see next Sect.) affect anion transfer only from the outer membrane surface (LEPKE and PASSOW, 1973; SCHNELL et al., 1973; ZAKI et al., 1975b; KAPLAN et al., 1976). This finding, which contrasts to the symmetric inhibition of hexose transfer by phloridzin (BENÈS et al., 1972), has been taken as evidence against an anion exchange mechanism involving a carrier which exposes identical sites alternatively to both faces of the membrane. On the other hand, however, it cannot be excluded that the asymmetric inhibitors are merely excluded from the transport system at the inner membrane surface due to the particular structural situation. This interpretation receives support from the observation that other disulfonate inhibitors act at both faces of the membrane (ZAKI et al., 1975b; KAPLAN et al., 1976).

c) Bimodal Inhibitors

Chemical modifiers and reversible inhibitors of anion permeability are unspecific in a dual sense. Firstly, their binding to the membrane is determined by parameters largely independent of the parameters governing the anion affinity of the anion exchange pathway. Secondly, many of these agents also affect the transfer of other solutes, in particular cation permeability, which is enhanced (PASSOW, 1969; DEUTICKE, 1970).

These disadvantages are encountered to a much lesser extent in a group of potent inhibitors, namely organic phosphates, sulfonates and particularly disulfonates (Table 10), which act as reversible or irreversible inhibitors depending on slight variations of their chemical structure or the experimental conditions. As was first worked out by CABANTCHIK and ROTHSTEIN (1972), a number of stilbene disulfonate derivatives which do not penetrate the erythrocyte membrane (MADDY, 1964; KNAUF and ROTHSTEIN, 1971a) are powerful, reversible and specific inhibitors of sulfate and chloride exchange. Further mono- and trisulfonate inhibitors have recently been studied by FORTES and HOFFMAN (1974), HO and GUIDOTTI (1974) and ZAKI et al. (1975b). Introduction of an

amino-reactive isothiocyano-group converts such compounds into irreversibly bound inhibitors which may be expected to label the anion pathway with some preference.

The same dualism of a reversible *and* irreversible, i.e., a "bimodal" inhibition, can be obtained with pyridoxal phosphate (CABANTCHIK et al., 1974b). This compound is known from enzymological studies (GLAZER et al., 1974) to form reversible Schiff bases with amino groups, particularly in the vicinity of phosphate-binding sites. Sodium borohydride reduces the Schiff base under formation of a stable covalent link to the amino group. Due to this mechanism of interaction, pyridoxalphosphate-labeling of membranes is highly amino-specific. Pyridoxal phosphate reversibly inhibits anion exchange ($K_i \sim 1$ mM), addition of borohydride makes the inhibition irreversible.

A third inhibitor of the bimodal type is the taurine derivative, NAP-taurine (=N(4-azido-2-nitrophenyl)-2-aminoethylsulfonate) originally introduced as a photodynamic membrane protein label by STAROS and RICHARDS (1974). In contrast to taurine (2-amino-ethylsulfonate), NAP-taurine reversibly inhibits anion exchange when added to erythrocytes in the dark (ROTHSTEIN et al., 1976). Exposure to light converts NAP-taurine into a nitrene compound (Table 10), which readily interacts with CH bonds in proteins, carbohydrates and lipids (STAROS and RICHARDS, 1974), i.e., with all neighbors in the region where it had been bound prior to photoactivation. After exposure to light, NAP-taurine inhibits anion transfer irreversibly.

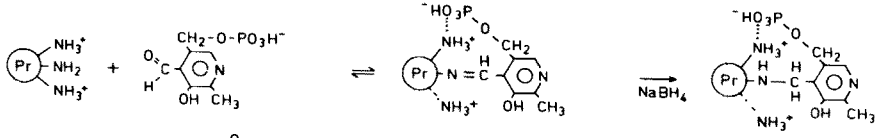
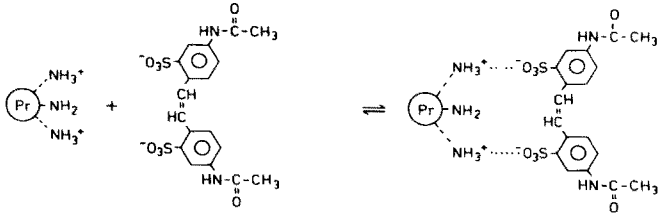
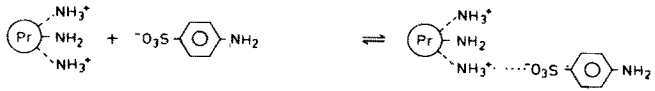
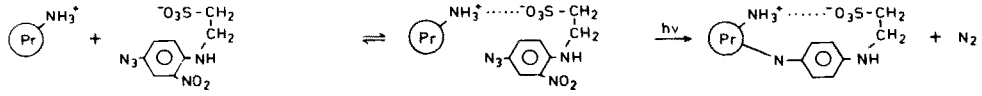
The extent of inhibition produced by the bimodal inhibitors is largely independent of the type of binding. The covalent modification of membrane amino groups seems thus not to be a constitutive element of the inhibition.

In contrast to the impermeable stilbene disulfonates, pyridoxal phosphate and NAP-taurine (STAROS et al., 1975) penetrate the erythrocyte membrane, presumably via the anion exchange pathway since their uptake into the cell is inhibited by DIDS (Table 10), the most potent disulfonate inhibitor of anion transfer (CABANTCHIK et al., 1975b), by dipyridamole, a potent reversible inhibitor of anion transfer, and by high concentrations of anions such as Cl^-, NO_3^- or $SO_4^=$ (ROTHSTEIN et al., 1976).

The analysis of membrane proteins after exposure of intact erythrocytes to radioactive-labeled bimodal inhibitors of the irreversible type, at concentrations sufficient to produce inhibition of anion exchange, has revealed a preferential, although not exclusive, binding to two groups of intrinsic membrane proteins, namely the 95,000-dalton fraction (band 3) and—to a minor extent—the glycoproteins, predominantly glycophorin. The binding to band 3 is suppressed by procedures which prevent the penetration of the reversible type of the inhibitor (ROTHSTEIN et al., 1976). Moreover, binding of one irreversible inhibitor prevents the binding of the other one. These and a number of other results which have been summarized by ROTHSTEIN et al. (1976) suggest that the bimodal inhibitors bind to a site, which is located on the 95,000-dalton protein and is a constituent, probably an anion-binding site, of the anion pathway.

Since this site is accessible to impermeable inhibitors such as DIDS it must be located in front of the main diffusion barrier to anions near to the outer membrane surface. On the other hand, after treatment of erythrocytes with

Table 10. Bimodal inhibitors of anion transfer in human erythrocytes

Reversible	Irreversible	
		Pyridoxal-5′-phosphate
		4,4′-Diacetamido stilbene -2,2′disulfonate (DAS)
		4,4′-Diisothiocyano stilbene -2,2′disulfonate (DIDS)
		1-Amino benzene-4-sulfonate
		1-Isothiocyano benzene-4-sulfonate
		N-(4-azido-2-nitrophenyl) -2-aminoethyl sulfonate (NAP-taurine)

low concentrations of pronase, which remove a superficial 35,000- dalton component from the 95,000-dalton fraction (see above), anion exchange is still inhibited by DIDS, and 85% of the DIDS bound are recovered in the 65,000-dalton fraction remaining in a hydrophobic domain of the membrane (CABANTCHIK and ROHTSTEIN, 1974b). Thus, the outermost segment of the 95,000-dalton fraction seems not to be required for the action of these inhibitors.

The bimodal inhibitors also interact to some extent with glycophorin and other membrane-spanning glycoproteins (CABANTCHIK and ROTHSTEIN, 1974a; CABANTCHICK et al., 1975a). This binding, however, is not affected by inhibitors of anion transfer. Moreover, the modifiers bound to this fraction can be removed by treatment with trypsin and pronase without any appreciable effect on anion exchange. In the light of these findings the major sialoglycoproteins are not very likely to be involved in anion transfer.

According to the data compiled in Section III D 1, the anion pathway, if localized in the 95,000-dalton fraction, might be a constituent of the intramembrane particles visualized by freeze-etch techniques. Results of protein crosslinking and ferritin-labeling studies in DIDS-treated ghost (ROTHSTEIN and CABANTCHIK, 1974) agree with this prediction. A further line of evidence has been based on the fact that band 3, as an intrinsic protein, can be extracted with some selectivity from ghost membranes by the detergent Triton-X 100 (YU et al., 1973). The protein extracted in this manner was incorporated into liposomes and found to bring about an enhancement of sulfate release which could be inhibited by pretreatment of the ghosts with DIDS and by addition of dipyridamole, a reversible inhibitor of anion transfer (BALSHIN et al., 1974; ROTHSTEIN et al., 1975).

The number of molecules of an irreversible modifier bound to the erythrocyte membrane in connection with the inhibition of anion transfer provides a rough measure of the number of anion transfer sites in a single cell. From linear relationships between binding and inhibition, 3×10^5 sites/cell have been derived as a probable upper limit (HO and GUIDOTTI, 1974; ROTHSTEIN et al., 1976)[11]. This value is lower than the number of "copies" of band 3 (9.4×10^5/cell). Thus the anion pathway is probably only one constituent of this major protein fraction which may also contain other pathways, e.g., that for water (Sect. IV C 2), hexoses (TAVERNA and LANGDON, 1973; LIN and SPUDICH, 1974), and active cation movements (KNAUF et al., 1974b). As compared to the number of sites for the latter function (250/cell) (HOFFMAN, 1969) the abundance of anion "channels" is very formidable.

5. Structural Models of the Pathway

A description of the anion exchange pathway which exceeds the assignment to a membrane protein fraction, has been put forward by CABANTCHIK and ROTHSTEIN (1972) and ROTHSTEIN et al. (1976). They propose that penetrating anions bind to a site bearing a number of closely apposed anion-binding groups, also able to accommodate the anionic groups of bimodal inhibitors. These sites

[11] Recent studies by ZAKI et al. (1975b) have provided evidence for a considerably higher number of binding sites on band 3 involved in the inhibition of anion transfer (1.7×10^6/cell).

Table 11. Reversible disulfonate inhibitors of sulfate exchange in human erythrocyte membrane

	$K_{0.5}$ mM[a]
Tetrathionate: $^{-}O_3S–S–S–SO_3^{-}$	1.2
Peroxodisulfate: $^{-}O_3S–O–O–SO_3^{-}$	40
Ethanedisulfonate: $^{-}O_3S–CH_2–CH_2–SO_3$	>100
Methanedisulfonate: $^{-}O_3S–CH_2–SO_3^{-}$	>100

[a] $K_{0.5}$ = concentration at half-maximal inhibition of sulfate tracer efflux. Experimental conditions: 37° C, pH 7.4, sulfate concentration 3.5 mM.

are assumed to be located in an (aqueous?) channel within the 65,000 segment of band 3 in front of a hydrophobic barrier, restricting the diffusion of anions. In view of its size and structural details (Sect. IIc, IIID1) the protein is unlikely to act as a mobile carrier. Preliminary evidence indicates (ROTHSTEIN et al., 1976) that it may undergo conformational changes in the course of anion translocation.

Further details concerning the geometrical arrangement of the amino groups of the anion-binding site might be obtainable by varying systematically the distance of the anionic groups of inhibitors. Such data are not yet available for the stilbene disulfonates. Anion transfer, however, can also be inhibited reversibly by higher concentrations of simple disulfonates (Table 11). The observation that tetrathionate with two sulfur atoms interposed between two sulfonate groups is more potent than its analogue containing two oxygen and one or two carbon atoms, may indicate a critical dependency of the binding of divalent anions on the distance of the negative charges. Assuming that the differences in the inhibitory potency of tetrathionate, peroxodisulfate, and ethandisulfonate are exclusively due to geometric parameters, the minimum distance between the anion-binding amino groups is probably similar or even larger than the distance of the two sulfonates in tetrathionate.

The effects of the noncompetitive, reversible inhibitors cannot yet be incorporated into this model unless one assumes that they "inactivate" binding sites by perturbing the spatial arrangement of the amino groups. The decrease of anion permeability at low pH (Sect. IVD2) may be due to a similar inactivation process.

E. Protein Pathways for Organic Anions

The transfer of organic anions may be expected to be more complex than that of inorganic anions, kinetically and with respect to the pathways used, for two reasons:

Firstly, most biologically occurring organic anions are weak electrolytes. Thus, nonionic diffusion of the undissociated acid across the lipid pathway has to be considered in addition to penetration of the free anion via protein pathways.

Secondly, the binding of organic anions will be governed not only be electrostatic forces but also by weaker interactions originating from the apolar part of the molecule.

1. Ionic Versus Nonionic Diffusion

The contribution of nonionic diffusion to the transfer of aliphatic anions has been studied qualitatively by DEUTICKE (1972, 1973). Phenomena coupled to the net uptake of undissociated weak acids into erythrocytes from *unbuffered* salt solutions were used as qualitative criteria (Fig. 15):

1. The net entry of undissociated acids goes along with a very rapid alkalinisation of the medium as a consequence of the disappearance of protons.

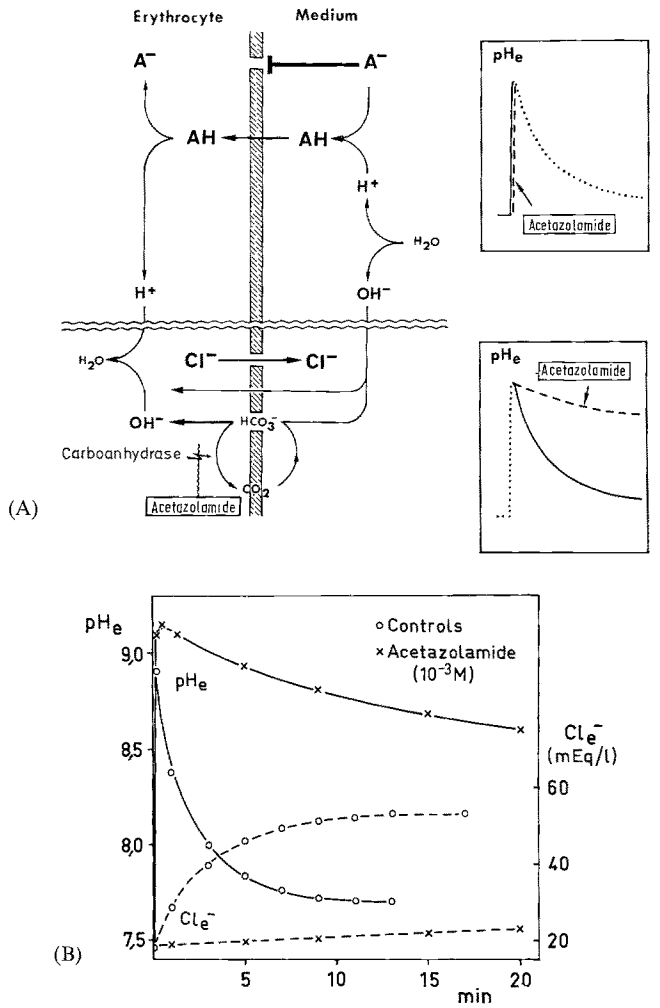


Fig. 15. (A) Processes accompanying the exchange of extracellular organic anions, penetrating by nonionic diffusion, against intracellular chloride. Experimental procedure: At 0 min 1 ml erythrocytes is rapidly mixed with 9 ml isotonic solution of organic sodium salt. The subsequent changes of extracellular pH (and chloride) concentration are measured in the absence or presence of acetazolamide. (B) Acetazolamide inhibition of the apparent "exchange" of an extracellular organic anion (caparonate) against intracellular chloride due to the mechanisms shown in Figure 15A. Experimental procedure as above. (From DEUTICKE, 1972, with permission of the publisher)

2. The subsequent exchange of the excess of extracellular OH^- ions left back by the protons, against intracellular Cl^- is sensitive to acetazolamide, an inhibitor of carbonic anhydrase, in contrast to the direct exchange of the anionic form of the organic acid, A^- with intracellular Cl^-. Carbonic anhydrase probably affects OH^-/Cl^- exchange, detectable by pH or Cl^- measurements (Fig. 15b), by catalyzing a reversible formation of HCO_3^-, which mediates OH^- movements. A direct exchange of A^-/Cl^- does not involve carbonic anhydrase and is therefore insensitive to acetazolamide.

On the basis of these criteria it could be demonstrated that nonionic diffusion contributes to the transfer of:

1. All simple aliphatic monocarboxylates (formate, acetate, etc.)
2. 2-Hydroxy-monocarboxylates $> C_4$, i.e., with the exception of glycolate and lactate
3. 2-Keto-monocarboxylates $> C_5$, i.e., with the exception of glyoxylate, pyruvate and α-ketobutyrate

In contrast, aliphatic dicarboxylates and monosulfonates, even those with long apolar chains, e.g., succinate or butane sulfonate, do not penetrate by nonionic diffusion. These results have been largely confirmed by AUBERT and MOTAIS (1975). They further demonstrated that aromatic monocarboxylates also penetrate in the undissociated form in contrast to the corresponding aromatic sulfonates.

In addition to nonionic diffusion, ionic diffusion of organic anions has also to be considered. If this process involves the exchange system for inorganic anions, the transfer should share kinetic properties and patterns of inhibitor sensitivity with that of inorganic anions. As yet this problem has been studied mainly for acetate, glycolate and lactate.

2. Acetate Transfer

Tracer flux measurements (DEUTICKE, 1973, 1975b) have confirmed that in human and many other erythrocytes acetate penetrates at least to a very high extent by nonionic diffusion. The equilibrium exchange is insensitive to the anion milieu, increases linearly with concentration and rises linearly (on a logarithmic scale) with decreasing pH as expected for nonionic diffusion. Transfer is enhanced by hexanol, indicating penetration via the lipid membrane phase. On the other hand, inhibitors of the inorganic anion exchange system inhibit the exchange of acetate to 20–40%. This may indicate a minor contribution of the specific anion exchange system. Pronase, however, at high concentration is ineffective. Thus, the bulk of the data indicate that in many mammalian erythrocytes unsubstituted aliphatic monocarboxylates do not have access to a pathway open to halides, divalent inorganic anions, and probably even 2-hydroxy- and 2-keto-derivatives of these simple fatty acids.

AUBERT and MOTAIS (1975) have proposed that in order to bind to the transfer system, an anion needs three adjacent functions capable of forming ionic or hydrogen bonds with a protein. This requirement would be fulfilled in anions containing a SO_3^-, a $CO\text{-}COO^-$, a $CHOH\text{-}COO^-$ or two closely apposed $COO^- \ldots COO^-$ groups, but not in anions containing only one COO^--

group. Although this particular "three-point attachment" hypothesis may not fully account for all experimental observations concerning the ionic permeability of organic anions, it clearly emphasizes the relevance of the configurational and structural properties of the polar domain of organic anions.

As an exception, the anion exchange system of rodent erythrocytes seems to transport acetate, as indicated by saturation kinetics, an anion dependency corresponding to that of inorganic anions, a pH-dependency irreconcilable with nonionic diffusion, and a pronounced sensitivity to reversible inhibitors of anion exchange (DEUTICKE, 1974). Thus, in spite of the many interspecific similarities of the anion transfer system there must be structural heterogeneities which render possible such differences.

3. Glycolate and Lactate Transfer [12]

a) Influence of Environmental Parameters

Lactate (CH_3–CHOH–COO^-) and glycolate (CH_2OH-COO^-) penetrate the human erythrocyte membrane about 50 and 100 times faster than sulfate, but 3 orders of magnitude slower than chloride.

The kinetics of lactate and glycolate transfer (DEUTICKE, 1976) resemble those of inorganic anions only up to a certain extent: Fluxes tend towards a maximum, although with half saturation constants higher than those for chloride and sulfate. Moreover, fluxes are also sensitive to the anion milieu. The anion pattern, however, differs from that observed in case of inorganic anion transfer (Fig. 16a).

Further peculiarities concern the pH dependency: Glycolate exchange has only a very flat maximum at ~pH 7 (Fig. 16b) and is thus much less pH dependent than chloride or sulfate exchange (cf. Sect. IVD2). The exchange of lactate increases continuously with lowering of pH from 8.2 to 5.8 without reaching a maximum. The slope of the flux versus pH relationship is smaller than would be expected in case of nonionic diffusion. Thus, the pH dependencies of the transfer of organic anions are different not only from those of inorganic anions but even from each other. The apparent activation energy for the transfer of glycolate (32 Kcal/mole) is almost as high as that for the transfer of inorganic anions, while the value for lactate (25.5 Kcal/mole) is somewhat lower. Taken together these results do not answer unequivocally the question whether the organic anions share the pathway of mono- or divalent inorganic anions.

b) Structural Characteristics of the Pathway

The situation becomes even more complex in the light of data obtained with reversible inhibitors and irreversible modifiers (DEUTICKE, 1976). Concerning their effects on sulfate, glycolate and lactate transfer, such agents fall into three classes (Table 12):

1. FDNB, EDC, pronase, reversible inhibitors of the "unspecific" type (e.g., phloretin, salicylate, tetracaine), but also the bimodal inhibitor pyridoxal phosphate inhibit the transfer of sulfate, glycolate and lactate with rather similar effectivity

[12] see Note added in proof, p. 97.

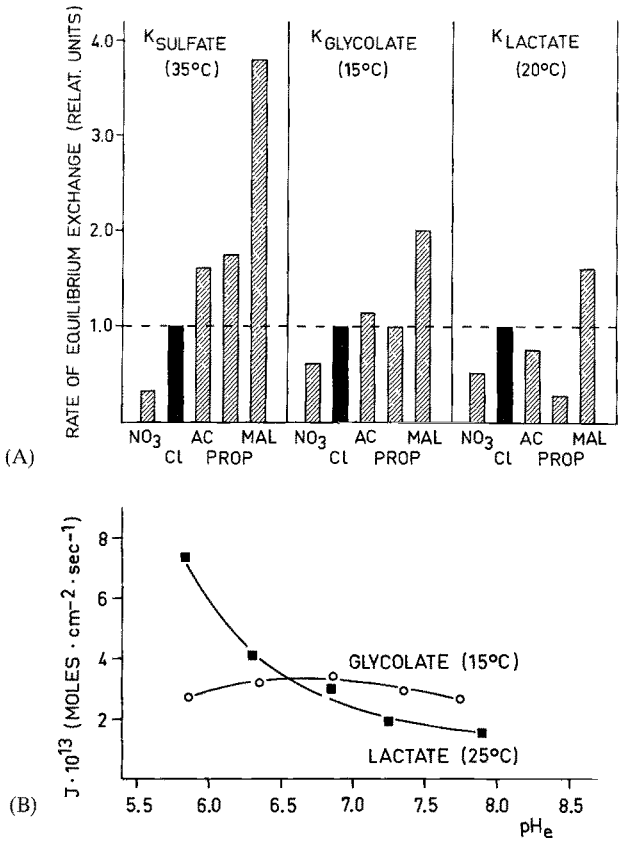


Fig. 16. (A) Influence of anion milieu on anion exchange fluxes in human erythrocytes. Anion variation by isoosmotic replacement of chloride by acetate (*ac*), propionate (*prop*), malonate (*mal*). Patterns for organic anion fluxes differ considerably from those for inorganic anions. (B) pH-dependency of the fluxes of organic anions measured at concentration equilibrium. Incubation media (mM): NaCl 40, KCl 100, sucrose 25, glucose 3, lactate (glycolate) 5.4, gramicidin D 5 µg/ml

2. Disulfonates such as SITS and tetrathionate inhibit sulfate transfer almost completely, glycolate transfer up to 70% extent, but have only a minor effect on lactate transfer (maximal inhibition ~25%)

3. Conversely, PCMBS and DTNB do not affect sulfate transfer (Sect. IV-D4a), inhibit glycolate transfer to a maximum of 30%, and lactate transfer to about 65%.

The complementary inhibitory patterns in 2. and 3., in combination with the kinetic peculiarities, may indicate that some organic anions penetrate the erythrocyte membrane via two, operationally defined, pathways: The "normal" exchange pathway and an additional, specialized system.

The sensitivity to SH-reagents clearly distinguishes these two pathways. In view of the binding characteristics for the two almost impermeable inhibitors (VAN STEVENINCK et al. 1965; CHAN and ROSENBAUM, 1969) only a small population of superficial membrane SH-groups ($\leqq 10^6$/cell) seems to be relevant for

Table 12. Characterisation of different protein pathways of transfer for inorganic and organic anions by their different sensitivities to reversible and irreversible inhibitors

Inhibitor (mM)	% Inhibition of equilibrium exchange of		
	Sulfate	Glycolate	Lactate
FDNB (3.5)[b], pH 7.4, 60 min, 37°	92	83	83
Pronase 2.5 mg/ml[b], pH 7.4, 60 min, 37°	86	67	68
EDC (25)[b], pH 6.0, 60 min, 37°	52	52	35
Phloretin (0.6)[a]	90	91	94
Salicylate (2)[a]	68	68	68
Tetracaine (1)[a]	56	37	37
Pyridoxalphosphate (5)[a]	86	40	65
SITS (0.5)[b], pH 7.4, 30 min, 4°	78	66[c]	25[c]
Tetrathionate (10)[a]	>95	65[c]	23[c]
Hg^{++} (0.05)[a], pH 7.4, 37°	10[b]	25[c]	55[c]
pCMBS (0.1), pH 7.4, 10 min, 37°	10[b]	25[c]	65[c]
DTNB (5), pH 8.0, 90 min, 37°	5[b]	30[c]	60[c]

[a] Inhibitor added at the beginning of the efflux period.
[b] Cells pretreated with the inhibitor for the time periods indicated.
[c] Maximal inhibition obtainable.

the transfer of organic anions. These SH-groups are probably located on the 95,000-dalton protein fractions, as indicated by DTNB binding studies of BROWN et al. (1975). The organic anion pathway therefore may be located very close to the inorganic anion pathway. It seems even possible that, structurally, both pathways are nearly identical, if one assumes that all anions share a common pathway, in which the transfer of organic anions requires the conformational intactness of certain SH-group containing domains, which is not required for inorganic anion transfer.

Due to the partly apolar nature of the organic anions, the interaction with the proteins relevant for their transfer may involve to a higher extent nonelectrostatic forces and more hydrophobic protein domains. This assumption is supported not only by model studies on artificial ion exchange membranes (SHOHAMI and ILANI, 1973) but also by data concerning the influence of hexanol on the transfer of glycolate and lactate (DEUTICKE, 1975b). In contrast to the fluxes of sulfate, which are markedly inhibited by aliphatic alcohols up to prelytic concentrations, the transfer of glycolate and lactate is inhibited at low but enhanced reversibly at higher concentrations. With lowering of pH the accelerating effect of alcohols becomes more pronounced and finally dominates by far. Since alcohols enhance nonelectrolyte transfer via the lipid phase (Sect. IV B 3) this acceleration of anion transfer may indicate the relevance of hydrophobic protein structures or lipid-protein interactions, since nonionic diffusion via the lipid phase must be discarded from the list of possible explanations (Sect. IV E 1).

F. Relevance of Membrane Lipids for the Anion Pathways

Obviously, the 95,000-dalton peptide fraction is presently the best candidate for the exchange pathways of inorganic anions and probably also of organic anions. This fraction strongly interacts with the lipid domain of the membrane (YU and STECK, 1975a, b). Consequently, the nature of the surrounding lipids may affect the anion transfer systems due to lipid-protein interactions. Two observations bear on this point:

1. Species differences in the rates of phosphate transfer (Sect. IVD2) are closely correlated with differences in membrane lipid composition (GRUBER and DEUTICKE, 1973). The permeability increases linearly with the double bond index of the phospholipids in the same fashion observed for nonelectrolyte transfer. This might indicate that the transfer is facilitated by increasing membrane fluidity. On the other hand, experimental lowering of the double bond index in rat erythrocytes by dietetic means (Sect. IVB4b) does not affect anion permeability (DEUTICKE and HAEST, unpublished results). A positive correlation of phosphate permeability with the membrane contents in phosphatidylcholine and a negative one with sphingomyelin levels, which also emerge from the comparative studies, may indicate a causal significance of the phospholipid head group regions for the function of the anion exchange system.

2. Membrane cholesterol affects the anion pathway. The pattern of the alterations induced by elevation or reduction of cholesterol levels again reflect the differences between inorganic anions and lactate.

Sulfate and glycolate transfer are largely independent of membrane cholesterol levels. In contrast, lactate transfer is inhibited in cholesterol-depleted and enhanced in cholesterol-loaded erythrocytes. These changes qualitatively agree with those of the hexose transfer system, as discussed in Section IVC6.

In addition to affecting the properties of a protein pathway, membrane phospholipids may, to minor extent, also be more directly involved in anion exchange. The demonstration of an electrically silent Cl^- exchange in artificial bilayers even of a very pure lecithin (TOYOSHIMA and THOMPSON, 1975a, b) seems to indicate that chloride bound to the positively charged choline head group of lecithins may, in the course of the very slow flip-flop of phospholipids (KORNBERG and MCCONNELL, 1971; ROTHMAN and DAVIDOWICZ, 1975), be translocated across the hydrophobic core of the membrane. Although the contribution of this process to the anion exchange in the erythrocyte is qualitatively only of minor importance, it has to be regarded conceptually as an additional, independent pathway of anion exchange.

V. Concluding Remarks

In the preceding sections it was attempted to characterize transport pathways across the rythrocyte membrane and their experimental alterations in terms of known features of membrane composition and organization. In the author's

opinion the varying success of such an attempt reflects not so much the different kinetic complexities of the transfer processes discussed, but is a measure of the "resolution" to which the structural analysis of the assumed pathway has advanced. Many features of transfer via the lipid domain could be interpreted in terms of the material properties of that pathway, in particular, its hydrophobic sections. Nevertheless, it must be kept in mind that the picture of the lipid pathway presented above, in putting special emphasis on the role of the hydrophobic interior of the bilayer, may to some extent reflect the present lack of information about the rôle of the bilayer/water interface. A better understanding of the arrangement of the phospho- and glycolipid head groups and their interaction with cholesterol and water may probably lead to considerable revisions of concepts concerning the localization of the barriers in the lipid pathway.

It has also become evident that the characterization of protein pathways is in a less advanced state. Apparently, only a small number of protein fractions, as defined by gel electrophoresis, have the disposition required for a transfer pathway. This may explain why attempts to relate transfer processes to defined classes of proteins have so far provided disappointingly uniform results. The available data suggest that the 95,000-dalton fraction, which may or may not be homogeneous, serves as the pathway for processes as different as water diffusion, mediated hexose transfer, or anion exchange. If this turns out to be true one has to invoke the same or at least very similar proteins in transfer processes responding in a completely different way to milieu parameters as well as covalent and noncovalent inhibitors. Assuming that the conformation of a protein pathway is altered by inhibitors, a different inhibitor- and milieu-sensitivity of transfer processes involving the same protein may indicate that within this macromolecule, different routes are available for various permeants. Alternatively, it might be hypothesized that conformational changes in a single route are not "seen" to the same extent by various permeants.

Evidence that the 95,000-dalton fraction may be closely connected with other proteins and constitute supramolecular complexes visisble as membrane-intercalated particles (Sect. III D 1) has been rationalized in terms of "permeaphores" (PINTO DA SILVA and NICOLSON, 1974) facilitating in specialized membrane regions the translocation of hydrophilic and charged solutes which have only very limited access to the lipid pathway. Generalizing, such permeaphores would be the structural equivalent of the "carriers" and "pores" conceived on the basis of kinetic and thermodynamic data.

The available data on the structural identity of protein pathways do neither prove nor disprove the permeaphore concept. To test its validity and to answer the more basic question, whether the 95,000-dalton fraction may be regarded as a protein or class of proteins specialized on the mediation of passive transfer processes, will require the further systematic assignment of kinetically defined transfer processes to membrane proteins serving as pathways. Glycerol transfer with its singular properties deserves particular interest in this context.

An ultimate understanding of the function and organization of protein pathways, however, will not be possible without taking into account the surrounding lipid phase. The effects of membrane cholesterol content and of the phospholipid composition on protein-mediated transfer processes (Sect. IV C 6,

IVF) make it very likely that the fluidity of the hydrophobic environment in the membrane interior as well as the lipid configuration and charge at the membrane/water interface adjacent to a protein pathway are important determinants of its function. The detailed evaluation of these effects and their assignment to structural properties of the erythrocyte membrane lipid domain is likely to become possible within the near future. The study of the erythrocyte as a model may thus further contribute significantly to the solution of problems difficult to investigate in more complex membrane systems.

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List of Abbreviations

PC	Phosphatidylcholine (lecithin)
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PI	Phosphatidylinositol
PA	Phosphatidic acid
Sph, S	Sphingomyelin
PAS	Periodic acid—Schiff
ESR	Electron spin resonance
NMR	Nuclear magnetic resonance
ORD	Optical rotatory dispersion
SDS	Sodium dodecyl sulfate
TNBS	2,4,6-trinitrobenzene sulfonate
FDNB	1-fluoro-2,4-dinitrobenzene
PCMB	Para-chloromercuribenzoate
PCMBS	Para-chloromercuribenzene sulfonate
NEM	N-ethylmaleimide
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
DTDP	4,4′dithiodipyridine
EDC	1-ethyl-3(3-dimethylaminopropyl) carbodiimide
SITS	4-acetamido, 4′-isothiocyano-2,2′ stilbene disulfonate
DIDS	4,4′-diisothiocyano-2,2′ stilbene disulfonate
DAS	4,4′-diacetamido-2,2′ stilbene disulfonate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
L_p	Hydraulic conductivity
P_d, P	Permeability coefficient

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Note Added in Proof:

Recent studies in the authors laboratory have demonstrated that lactate transfer in human and many other mammalian erythrocytes is stereospecific, the L-isomer being transported about 2–20 times faster than the D-isomer, depending on the species. The specificity appears to result from different affinities for the same transfer system. L-lactate transfer is inhibited more strongly by sulfhydryl reagents (maximal inhibition by PCMBS 90–95%) than D-lactate (maximal inhibition 65% [Table 12]).

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The Gradient Hypothesis and Other Models of Carrier-Mediated Active Transport*

R.K. CRANE

Contents

* Dedicated to Professor CARL F. CORI on the occasion of his 80th birthday.

I. Introduction

Coupling of organic solute transport to ion active transport as explicitly formulated by CRANE (1960, 1962) and CRANE et al. (1961) for Na^+ and extended by MITCHELL (1963) to H^+ appears now to be widely regarded as a fundamental biological principle. Mechanisms based on this principle have been described in diverse cells from microorganisms to mammals and appear to be perhaps the chief means by which living forms transduce the energy yield of metabolism into support of the cellular accumulation of organic foodstuffs. Coupling in these mechanisms is generally made possible by the formation of a ternary complex of ion, solute, and membrane transport carrier which can respond to the transmembrane electrochemical potential gradient established by the ion pump. Thus, one of the terms used to identify the concept is "The Gradient Hypothesis".

At intervals during the growth of experimental support for the Gradient Hypothesis there have been excellent and authoritative reviews written from several viewpoints (STEIN, 1967; SCHULTZ and CURRAN, 1970; HAROLD, 1972; MITCHELL, 1973). These need not be replicated. However, there are several reasons why a fresh perspective of the origins, development, and current standing of the Gradient Hypothesis would be timely. Firstly, research in this field is rapidly approaching the end of one trail and the beginning of another. Past research has been largely descriptive and phenomenological with insight into mechanism depending mostly upon analysis of transport kinetics as observed with intact cells or natural membranes. Present research foretells the future in the various demonstrations that mediators of ion transport, both active (RACKER and FISHER, 1975) and passive (ROTHSTEIN et al., 1976; HO and GUIDOTTI, 1975) and of passive sugar transport (KASAHARA and HINKEL, 1976), can carry out their work when removed from their native setting and reconstituted into artificial membranes. This has also now been accomplished for gradient-coupled transport (CRANE et al., 1976a) and the turning point in research is clearly at hand. It is still for the future to yield an understanding of transport at the level of the interaction of identified functional groups of purified molecules. However, the era of phenomenology is coming to a close, and one purpose of this review is to update the experimental support for the Gradient Hypothesis as a background for studies at the molecular level. Secondly, the Gradient Hypothesis is a biophysical concept distinct from the biochemical traditions of the time, as were particularly exemplified by MITCHELL (MITCHELL and MOYLE, 1958) who supported an "essentially orthodox biochemical approach" and took the view (MITCHELL, 1957, 1959) "that metabolic energy is generally converted to osmotic work by the formation and opening of covalent links ... exactly as in enzyme-catalyzed group transfer reactions". The Gradient Hypothesis proposed a transfer of energy without the intervention of covalent intermediates. This dichotomy of viewpoint led to early and continuing perceptual difficulties. One purpose of this review is to trace the origins and development of the concept of noncovalent energy transduction. Thirdly, the Gradient Hypothesis arose directly out of precedent studies on carrier transport and was an extension of them. It was addressed to the properties of translocating binding sites and not to vectorial enzymes or group translocating

catalysts such as proposed by MITCHELL (1959, 1961a, 1961b) in the chemiosmotic hypothesis. The difference is fundamental. The Gradient Hypothesis and the chemiosmotic hypothesis are individual concepts as MITCHELL several times (e.g., MITCHELL, 1963, 1970) and others more recently (SIMONI and POSTMA, 1975) have tried to make clear. One purpose of this review is to further clarify the distinction by recalling the mobile carrier background and basis of the Gradient Hypothesis.

Inasmuch as the time-frame of focus for various aspects taken up in this review is as much as 75 years, it would obviously be hopeless to try to justify every point by full reference or to do justice to each and every investigator whose experimental or conceptual efforts have influenced the development of the Gradient Hypothesis. Moreover, the review will be limited to a discussion of sugars and amino acids as substrates for the process since the critical studies have been largely done with these chemical species.

This review is written as much for the general reader as for the specialist in membrane transport.

II. Background

A. The Carrier Concept

1. The Exchange-Diffusion Model

LUNDEGÅRDH'S original proposal of carrier function (LUNDEGÅRDH, 1940) is vividly recalled by the recent report of TYSON et al. (1976). It was LUNDEGÅRDH'S idea that charged components of the plasma membrane bind ions of opposite charge and occasionally turn around in the membrane with the result that the bound ion is delivered to the aqueous environment at the alternate membrane surface. TYSON et al. have now proposed that some phospholipids of the plasma membrane carry out precisely this operation.

Following LUNDEGÅRDH, DAVSON and REINER (1942) proposed that the membrane component involved in Na^+ uptake was "enzyme-like" whereas USSING (1947) conceived of the component as a Na^+-binding particle suspended in the membrane matrix and moving about in a Brownian fashion reminiscent of the ideas of OSTERHOUT (1935). No matter which conception one takes, however, the fundamental property of the ion-translocating component can be formalized in the same way, as in Figure 1.

From the representation in Figure 1 it is intended to be clear that the carrier, C, cannot move except with the ion attached. It acts only to exchange ions across the membrane in a compulsory fashion and net flux never occurs when the exchange is between the same ion species. USSING observed this process with radiosodium and gave it the name *exchange diffusion* (USSING, 1948). The concept of carrier, as it developed from studies of non-electrolytes, is importantly different.

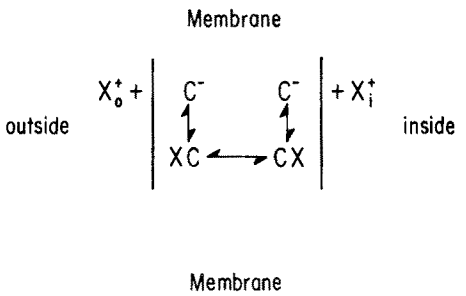


Fig. 1. The exchange-diffusion model

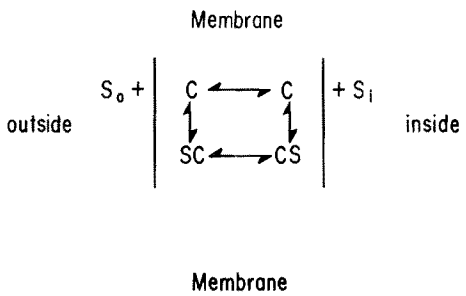


Fig. 2. The Widdas mobile carrier model

2. The Widdas Mobile Carrier Model

Many studies of sugar permeability in red cells by others as well as themselves were interpreted by LE FEVRE and DAVIES (1951) in terms of simple equilibria between the sugars and a membrane component with which the sugars must interact during transport. WIDDAS (1952) added critically to this line of thought with a kinetic analysis of sugar permeability in sheep placenta, which fitted the earlier work with other cells as well as his own data and can be expressed as in Figure 2.

In the Widdas model, the carrier, C, in contrast to the exchange-diffusion carrier, can translocate whether it is free or combined with substrate. This model is still in use as a fundamental expression of uncomplicated mobile carrier transport or facilitated diffusion as defined by DANIELLI (1954).

3. Predictions from the Widdas Model

Predictions from the Widdas model include net flow and an exchange diffusion which is competitive rather than compulsory. More importantly, theoretical lines introduced into the field by ROSENBERG (1948) and developed by WIDDAS (1952) and others (STEIN, 1967) predicted the coupling of diffusional flows based upon the properties implicit in the model. One such prediction is the uphill transport

of one substrate for the carrier induced by the counterflow, downhill of a second substrate (WIDDAS, 1952; ROSENBERG and WILBRANDT, 1957). This prediction was confirmed for sugar pairs with fluoride-treated rabbit red cells by PARK et al. (1956) and human red cells by ROSENBERG and WILBRANDT (1957); it was confirmed for arsenate vs. inorganic phosphate in bacteria by MITCHELL (1954). The other prediction is accelerative exchange diffusion, which was first observed by HEINZ (1954) in his studies of the uptake of labelled glycine by Ehrlich ascites tumor cells preloaded with non-radioactive glycine (see also HEINZ and WALSH, 1958). As explained by STEIN (1967) accelerative exchange diffusion depends upon a ratio of translocation rates $CS/C > 1$. STEIN (1967) has also made it clear that the case USSING (1949) analyzed is actually the limiting situation of accelerative exchange diffusion wherein the free carrier cannot move. In the Widdas model this is equivalent to a ratio of translocation rates, $CS/C = \infty$.

B. The Meaning of "Mobile Carrier"

1. Carrier vs. Fixed Site

The success of the predictions mentioned above led to the wide acceptance of the "carrier" rather than the "fixed-site" model, although attempts to produce a satisfactory "fixed-site" model continued. For example, a "fixed-site" model in which entry and exit flows occur by separate and independent pathways (Fig. 3) was shown to be able to account for some of the "carrier" phenomena; e.g. counterflow, but it could not account for the effect of non-penetrating inhibitors which when used to block entry also block exit without themselves entering the cell (STEIN, 1967). A mathematically more successful non-carrier model which treats the red cell membrane as a lattice with a complex array of fixed binding sites situated within water-filled channels which span the membrane has been produced (NAFTALIN, 1970) in order to explain certain anomalies in the kinetics of sugar transport by red cells. A related model with fewer necessary postulates has also been produced (LE FEVRE, 1973). However, two routes for sugar entry and exit in red cells have been identified, and simpler models involving two carriers seem as successful (EILAM, 1975; GINSBURG and STEIN, 1975; GINSBURG and RAM, 1975), though a final form of hypothesis has not yet been achieved. KASAHARA and HINKLE (1976) have reported the reconstitution of glucose transport in sonicated liposomes by the incorporation of a protein fraction from human red cells. Studies with this system should help resolve the conflict which has developed because of the "distinctly dissonant behavior" (LE FEVRE, 1975a) of red cell sugar transport.

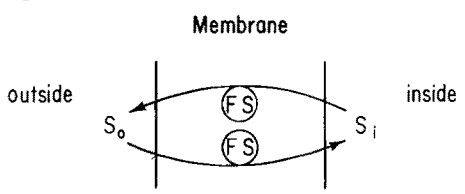


Fig. 3. An early fixed-site model

2. "Carrier" as a Kinetic Concept

Although more complex models have been developed (LE FEVRE, 1975a, 1975b), the Widdas model of "carrier" transport continues to be useful for many cells, especially in those situations where there appears to be a single route for entry and exit of substrate. Kinetic analyses of the Widdas model have generally assumed Michaelis-Menten kinetics as they might be applied to an uncomplicated enzyme-catalyzed equilibrium with a $K_{eq}=1$ (STEIN, 1967). Using simple notation as in (1) it is evident that carrier-mediated translocation

$$\begin{array}{c} \text{FAST} \quad \text{SLOW} \quad \text{FAST} \\ S + E \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P \\ S_o + C \rightleftharpoons S_oC \rightleftharpoons CS_i \rightleftharpoons C + S_i \end{array} \tag{1}$$

across the membrane is formally equivalent to enzyme-catalyzed transformation of substrate; they are the slow or rate-limiting reactions in the sequence.

In the enzyme reaction $ES \rightleftharpoons EP$, all physical transformations are ignored by the kinetic equations. In the same way, any physical transformations in translocation, $S_oC \rightleftharpoons CS_i$, are ignored. The assumed slowness of translocation may be a function of distance; it may equally be a function of the mechanics of binding site reversal. The point to be made is that no particular degree of translocational movement through the membrane is assumed in a kinetic analysis of carrier transport although illustrative models have sometimes given the impression that there is. "Mobile Carrier" is a distinction in kinetics, not of molecular organization.

3. "Functional" vs. "Anatomic" Thickness of a Membrane

The point made above has been and still is frequently misunderstood insasmuch as in model building attention has been drawn to the physical or anatomic thickness of the membrane bilayer (e.g. PATLAK, 1957; SINGER, 1974) rather than to what might be called its *functional thickness*. DANIELLI (1954) very early appreciated that the same apparent kinetics are to be observed whether the carrier is thought to shuttle, rotate, or circulate and a simple extension of his ideas shows that the apparent physical separation of the transmembrane aqueous compartments has not been a kinetic issue for carrier transport. The functional thickness of a membrane depends solely upon the particular molecular mechanism by which a carrier operates, imbedded, however, it may be in the membrane. The carrier molecule may span the full thickness of the membrane (BRETSCHER, 1971) but its operating center may be as discrete in space as that of a Patlak "gate"; kinetics will not make the distinction (PATLAK, 1957). Thus in the sense used here, any model in which the substrate binding site appears to alternate in membrane sidedness is a carrier model. The same sense, though in far more rigorous language, may be gained from a definition given by WYSSBROD et al. (1971).

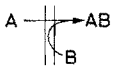
C. Active Transport and Energized Transport

1. Transmembrane Asymmetrics

Concepts of membrane transport developed more or less parallel with the growing understanding of cellular biochemical transformations (e.g. GUZMAN-BARRON, 1943; KREBS, 1943), and the introduction of powerful concepts of the energetics of group transfer reactions (e.g. KALCKAR, 1941; LIPMANN, 1941) and concepts of membrane transport did not avoid being strongly influenced. Active transport has been traditionally defined (ROSENBERG, 1948; ROSENBERG, 1954) as the net transmembrane movement of a chemical species against its own prevailing electrochemical potential gradient as in (2) and it is a phenomenon which clearly requires being supported by an energy source in addition to the heat of the environment. However, the mechanism by which metabolic energy is transduced in support of active transport is not specified by the existence of the phenomenon. Hence, those mechanisms first imagined were generally the mechanisms conveniently at hand from studies of cellular metabolism. That is why it is more revealing at this point to discuss Energized Transport rather than active transport.[1]

(2)

What the term Energized Transport is intended to mean is any membrane translocation process which requires the transduction of metabolic energy. Thus, Energized Transport includes both active transport, as defined above, and vectorial membrane functions in which the chemical species is changed during translocation by the formation of a chemical bond,



(3)

the breaking of a chemical bond,

(4)

or the translocation of only a portion of the chemical species with again the breaking of a chemical bond.

(5)

Energized Transport thus includes those mechanisms which clearly depend on carrier function and those mechanisms which may not; its essential feature is the maintenance or production of a transmembrane asymmetry whether of electrochemical potential, as in active transport, or of chemical species.

[1] HARRIS (1972) in encountering the same difficulty chose the term, energy-dependent transport.

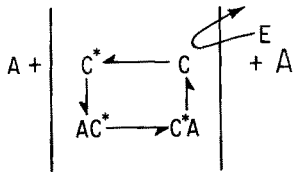


Fig. 4. Representation of energy transduction to the carrier

2. The Question of Energy Transduction

Since to produce or maintain a transmembrane asymmetry requires work, metabolic energy must be transduced. The questions are, in what form and in what way.

The question of in what form will be left till later. The question of in what way is important at this point and the question may be rephrased to be more specific; e.g. which is the receptor in energy transduction, the carrier or the substrate? In those cases where the chemical species is changed, the simplifying judgement may be made that the substrate is the receptor and the transduction of metabolic energy should be accountable in the net sum of the chemical reactions involved, whatever they may be, as enthalpy, entropy, or both. In those cases where the chemical species is not changed, the simplifying judgement may be made that the receptor is a component of the membrane; i.e. the carrier with which the translocated species interacts during translocation. In the carrier situation, energy transduction is ordinarily understood as the production of a transmembrane asymmetry of the carrier energetically inverse to the asymmetry of substrate which it supports. Without specification of mechanism or of energy form, a notation which may be used is shown in Figure 4 where C* is a higher energy state of the carrier than C, produced by interaction of C with some energy donating product of metabolism, E.

As will be seen, distinction between the substrate as receptor and the carrier as receptor was a fairly late advance in the field.

D. Hypotheses of Energy Transduction to the Substrate

1. Sugars

For nearly 60 years, studies of sugar transport were carried out almost exclusively with animal cells and a conceptual problem was thereby introduced which was only very slowly unraveled. Today it is widely appreciated that among commonly studied mammalian tissues only the epithelial cells of the small intestine and the kidney proximal tubules are capable of the transport of sugars against their chemical potential gradient [the less commonly studied choroid plexus also does this but that discovery was not made until 1964 (Csaky and Rigor, 1964)], and that all other mammalian cells contain in their membranes sugar transport systems that operate, at least predominantly, by facilitated diffusion. In the early

days, however, it was not so easy to see that mechanisms proposed for the energy transduction needed in the intestine and the kidney might not be appropriate when applied to, say, the red cell where energy transduction was not an issue.

In 1899, HÖBER recognized the need for the cells lining the small intestine to do work in support of the absorption of glucose and galactose. He found a rate of sugar absorption from the intestine of the dog higher than would correspond to the relative rate of diffusion predicted by the application of Fick's Law. Consequently, he proposed that sugar entering the cells was removed with the effect of steepening the downhill concentration gradient between the lumen of the intestine and the blood vessels. In Figure 5 this is called the Diffusional Sink.

It is worth recalling that, in HÖBER's time, no special qualities of the plasma membrane were invoked. The membrane was generally viewed as an inert matrix the permeability of which depended upon being maintained in a "normal" state

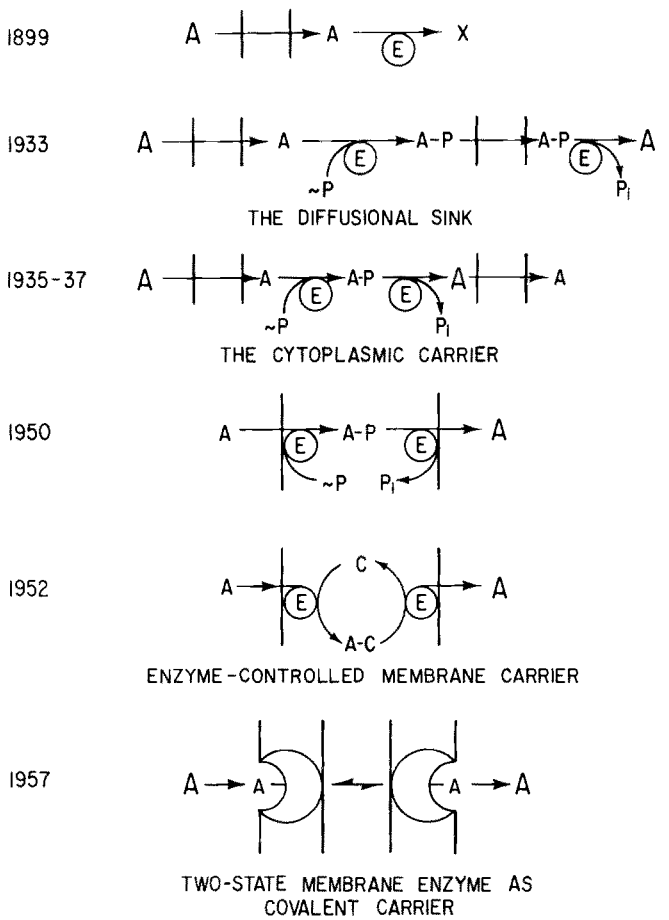


Fig. 5. Hypotheses of energy transduction to the substrate

but which otherwise was explainable from physicochemical laws and principles and not by "vital cell activity" (GOLDSCHMIDT, 1921). Comparisons between sugars with respect to their absorption changed this simplistic view. Extensive studies showing that stereoisomeric sugars are absorbed at different rates (NAGANO, 1902; CORI, 1925) served to underline the emerging concept of "the selective permeability of living membranes" (CORI, 1925). CORI noted especially that the absorptive rates for glucose and galactose were maximal at low concentrations whereas the rates for other sugars tested increased as the concentration was increased.

Mindful of these absorptive differences and of the fact that some sugars, like glucose, but not other sugars, like xylose, were metabolized by the epithelial cells, VERZAR (1931) revived HÖBER's proposal and WILBRANDT and LASZT (1933) proposed that the required metabolic reaction was phosphorylation. The glucose ester was assumed to be reconverted to free glucose after passing through the basal membrane of the intestinal cells (see Fig. 5). The Diffusional Sink hypothesis was left behind as it became clear that the problem was actually not to explain rapid down-the-gradient diffusion but rather to account for up-the-gradient accumulation.

Until WEARN and RICHARDS (1924) confirmed LUDWIG's early insight, it was widely believed that the glomerulus of the kidney acted as a selective filter and that glucose was absent from the urine because it did not leave the blood. WEARN and RICHARDS showed that the glomerular filtrate contained glucose at the same concentration as the blood; hence, the absence of glucose from the urine must reflect work done by the tubules (WHITE and SCHMITT, 1926; WALKER and HUDSON, 1937) in moving sugar up the concentration gradient into the bloodstream. Also the similarity in phlorizin inhibition of sugar absorption by the intestine (NAKAZAWA, 1922) to its inhibition of sugar reabsorption by the kidney (VON MERING, 1888, 1889; WALKER and HUDSON, 1937) and similarities in the respective structural specificities for kidney reabsorption (HAMBURGER, 1922; HÖBER, 1933) as compared to intestinal absorption led to the proposition that the processes in the two organs were similar, though active transport by the intestine was actually not demonstrated until 1939 (BARANY and SPERBER, 1939).

LUNDSGAARD (1935) and KALCKAR (1937) proposed, for both organs, phosphorylation at one end of the cell and dephosphorylation at the other. DRABKIN (1948) further elaborated this proposal with the assumption that phosphorylation was due to hexokinase and dephosphorylation to phosphatase. WILBRANDT (1954) has discussed this as the cytoplasmic-carrier mechanism (Fig. 5). KALCKAR (1941) had recognized that the species crossing cell membranes was glucose and not its ester, but what was not recognized until MCDOUGAL et al. (1960) was that the sugar gradient across epithelial tissues was established at the first; i.e. the brush border membrane and not the second; i.e. the serosal membrane. It was on this demonstration that the cytoplasmic carrier hypothesis in any form finally foundered (however, see NORDLIE and SOODSMA, 1966).

Prior to this time, however, WILBRANDT (1954) had entered strong arguments against the cytoplasmic carrier hypothesis on the basis of the assumptions about permeability constants and back-diffusion that would be needed. He favored the idea of the formation of a substrate-carrier complex before the first membrane

of an epithelial tissue was passed. In large measure his preference was based on the fact that, with the exception of uphill transport, all the features of active glucose transport by the intestine and kidney could be demonstrated in the case of glucose transport through the red cell membrane. WILBRANDT's 1954 concept of the enzyme-controlled membrane carrier mechanism described in ROSENBERG and WILBRANDT (1952) is illustrated in Figure 5. At first, it was proposed by ROSENBERG (WILBRANDT, 1950) that a membrane soluble derivative of glucose was formed by the first enzyme and decomposed by the second. Later (WILBRANDT, 1954), it was proposed that a carrier-substrate complex was the intermediary.

Throughout these studies of sugar transport in animal cells, the development of a unifying concept was hindered, as already mentioned, by the fact that only the epithelial tissues of the gut and kidney were known to carry out active sugar transport. Single cell populations such as the red cell (WILBRANDT, 1954) or the Ehrlich ascites tumor cell (CRANE et al., 1957) carried out only facilitated diffusion. Active transport of sugars in single cell populations was first seen in bacteria (RICKENBERG et al., 1956).

The development of a unifying concept was also hindered by the continued belief in and search for enzyme catalysis of the formation and breaking of covalent substrate bonds which extended throughout studies on transport from animal cells as discussed above to bacteria (MITCHELL, 1957, 1959) (Fig. 5). The search for a non-covalent mechanism began when CRANE and KRANE (1956) overturned the prevailing phosphorylation-dephosphorylation hypothesis by finding that sugar analogs incapable of undergoing phosphorylation were nearly as well transported by in vitro hamster intestine as glucose. Some other reactions possible to imagine were also excluded in a later study (CRANE and KRANE, 1959). My summation in 1958 (CRANE and KRANE, 1959) was that "most of the properties of the intestinal active transport system could be as well accounted for by hypothesis, which relate transport processes to membrane phenomena ... as by hypotheses, which include chemical reactions" and in 1959 (CRANE, 1960), "a purely chemical mechanism, ... in which the energy requirement for the movement of sugar against a concentration difference is satisfied by chemical conversion and reconversion, seems unattractive."

A chemical hypothesis for animal cells was tried once more, this time involving the membrane enzyme, trehalase (SACKTOR, 1968). However, the membrane location of trehalase (MALATHI and CRANE, 1968) was not appropriate to the proposed mechanism nor was trehalase to be found in the kidneys of all of even related species (VAN HANDEL, 1969). The hypothesis appears now to have been abandoned (BECK and SACKTOR, 1975). In bacteria, a chemical hypothesis for energy transduction to substrate accumulation was developed by KABACK and BARNES (1971) according to the two-state enzyme concept of MITCHELL (1957) (Fig. 5); carrier function was postulated as being carried out by electron transfer intermediates. This hypothesis also has been abandoned (SCHULDINER and KABACK, 1975; RAMOS et al., 1976).

2. Amino Acids

The situation with respect to amino acids was different from the very beginning. In 1913, VAN SLYKE and MEYER (1913) discovered an "uphill" movement of chemically unchanged amino acids from the blood into the several tissues examined. In context with the times, they attributed this energy requiring phenomenon to adsorption of the amino acids onto some intracellular polymeric structure. However, for later researches the need for energy transduction at the cell membrane was clarified. GALE (1947) and his co-workers discovered amino acid active transport in bacteria. CHRISTENSEN and RIGGS (1952) found the Ehrlich ascites tumor cell to be particularly well-suited to the study of the animal cell phenomenon discovered by VAN SLYKE.

Distinct chemical mechanisms for the support of amino acid transport are not to be found in the early literature presumably because concepts of amino acid activation developed late although HÖBER and HÖBER (1937) noted a demand for a specific metabolic reaction to initiate the specific transport. The first clear chemical hypothesis was introduced in 1970 (ORLOWSKI and MEISTER, 1970). This hypothesis proposed a γ-glutamyl cycle involving the ubiquitous membrane enzyme, γ-glutamyl transferase as the carrier. However, it now seems probable that this proposal is not correct since, in an individual with γ-glutamyl transferase deficiency and showing glutathionuria and glutathionemia, there was no apparent deficit to amino acid transport (SCHULMAN et al., 1975). To be true, the enzyme deficit was shown only in skin fibroblasts and to be more certain it would better to see whether γ-glutamyl transferase is also missing from the brush border membrane of an intestinal biopsy (CRANE et al., 1976d). However, a direct test of the hypothesis based upon its prediction that 1 mol of glutathione is degraded per mol of amino acid transported has yielded negative results (YOUNG et al., 1975).

Assuming on this basis that the γ-glutamyl cycle mechanism is moot, as seems most likely, it then appears at this writing that there are no chemical hypotheses for active transport of *free* sugars or *free* amino acids which are outstanding and still to be considered. The energy for active transport in these cases is not transduced to the substrate.

E. Hypotheses of Energy Transduction to the Carrier

1. Covalent Hypothesis

The existence of the membrane Na^+ pump was an early and strong, but generally unappreciated hint that a carrier could be the receptor for energy transduction in the support of membrane active transport; Na^+ does not form covalent bonds. In whatever way the Na^+ pump might have been formulated as carrier-mediated, the interaction of Na^+ was required to be non-covalent with an energized form of the carrier as in Figure 4 (e.g. SHAW, 1954) and similar non-covalent interactions of sugars or amino acids could be readily formulated from existing knowledge of enzyme substrate interactions of these species (e.g. SOLS and CRANE, 1954).

Consequently, the question of energy transduction to the carrier was not whether, but how.

Energy transduction to a non-electrolyte carrier was first clearly proposed by COHEN and MONOD (1957) after it had been demonstrated that thiogalactosides accumulated by *Escherichia coli* were free in the intracellular fluid and not bound. The action of "permease" (RICKENBERG et al., 1956) was formulated as a mobile carrier system (COHEN and MONOD, 1957) the exit phase of which was inhibited when "coupled to an energy donor". However, SKOU's (1957) success in the search for the energy donor of the Na^+ pump apparently strongly influenced the thinking about the energy donor for permease active transport. In 1960 (KEPES, 1960) and as late as 1970, (KEPES, 1970) the formulation by the Paris school for permease was equivalent to the many formulations of the Na^+-pump in which ATP is used to produce an energized carrier as in Figure 4 (e.g. POST et al., 1965).

2. Non-covalent Hypothesis

Once again, covalent coupling is seen as a preferred mode of energy transfer. Indeed, the covalent concept for epithelial cells which originated with CSAKY (CSAKY et al., 1961) continues to be revived (KIMMICH, 1970, 1973). Yet, ROSENBERG (1948, 1954) had made it clear that the additional forces needed to support active transport may be any forces that can be coupled to the transport; "The additional forces may be represented by gradients of potential or analogous thermodynamical entities. Gradients, as, for instance, the gravitational gradient, that of pressure, of temperature or of other chemical potentials, act as additional forces when the corresponding quantities mass, volume, entropy, or other chemical components move coupled with the substance in question" (ROSENBERG, 1954).

Thinking along these same lines; "the energy requirement for absorption against a concentration difference is independent of mechanism" (CRANE, 1960), I reviewed the known requirements of sugar active transport in the intestine; namely 1. the process exhibits MICHAELIS-MENTEN kinetics 2. it requires energy, 3. it probably does not include a chemical reaction, 4. free sugar accumulates within the cells, and 5. the presence of Na^+ is required.

This led to the proposal that "sugar absorption is coupled to a second and different energy-dependent transport process such as Na^+ transport, and does not itself require a direct energy input" (CRANE, 1960).

F. Early Observations of the Effects of Na^+ on Active Transport Systems

1. Sugars

In hindsight, a great deal of wisdom is often to be found. Although the statement referred to just above in postulating the coupling of the membrane transport of sugars to the membrane active transport of ions appears to be the earliest statement of the principle in the literature, REID (1900, 1902) noted effects of

NaCl on the uptake of sugar by the small intestine which he referred to as a "chemical excitation" nearly 60 years earlier. REID was quoted by GOLDSCHMIDT (1921) but was then long forgotten by the time RIKLIS and QUASTEL (1958) observed that in the complete absence of Na^+, active sugar absorption by the guinea pig intestine, perfused in vitro, ceased. CSAKY and THALE (1960) confirmed this observation as did BIHLER and CRANE (1962). However, CSAKY related the phenomenon to a direct interaction with the Na^+ pump or with ATP (CSAKY et al., 1961) whereas on the basis of our early experiments showing an effect of Na^+ on the anaerobic entry of sugars (in KLEINZELLER and KOTYK, 1961a, p. 464) I proposed indirect coupling as already cited.

2. Amino Acids

In hindsight, CHRISTENSEN (1970) has found a number of the early experiments of his laboratory which a dozen or so years later he felt could be interpreted as an effect of Na^+ on amino acid uptake by duck red blood cells and Ehrlich ascites tumor cells. However, it was not Na^+ but the reciprocal effects of K^+ which received attention and suggested to CHRISTENSEN and his colleagues (e.g. CHRISTENSEN and RIGGS, 1952) the possibilities either that K^+ and amino acids share a common step in the transport process or, following FLECKENSTEIN'S (1948) ideas on the energy available in ion gradients, that "the energy for amino acid concentration is drawn from the store of potential energy inherent in the asymmetric distribution of potassium." Though not so specified at the time this would, in mobile carrier terms, have been interpreted, as RIGGS et al. (1958) later did, as a concentration of amino acid by *exchange diffusion* with potassium.

A particularly serious disadvantage to interpretation of any of the early experiments on the ion events associated with amino acid uptake was the use generally of 1 to 3 h incubation periods for the analysis of events that were, so far as amino acid was concerned, half over in 3 min and substantially completed within 15 min (RIGGS et al., 1958). In the single short-interval study reported by this group (RIGGS et al., 1958), the relative extent of glycine concentration attained was directly related to the cellular $[K^+]$ and inversely related to the extracellular $[K^+]$; Na^+ was not measured. Also, it is essential to the formulation of a membrane hypothesis to study entry dissociated from accumulation. This was not done.

Nonetheless, in the discussion of their paper, there is to be found, among four possibilities suggested by RIGGS et al. (1958) as an explanation of the reciprocal ion effects, what is now believed to be the correct explanation; "the excess of Na influx could occur in the form of a complex between the carrier, glycine and sodium ion". This phrase is frequently quoted and labeled an hypothesis. However, reading further in the discussion it is found that this possibility was rejected because it was not supported by data obtained with pyridoxal, indoleacetate and the like in CHRISTENSEN'S laboratory. The K^+ effect was favored because an Na^+ effect was viewed as incorrect. For example, when this work was next reviewed (CHRISTENSEN, 1960), a variety of possible energy sources including the gradient of potassium ion were suggested as the support for active transport. However, Na^+ movements and coupling with the Na^+ pump were not among

them except by a footnote added in proof after our formulation of the Gradient Hypothesis had been made (CRANE et al., 1961 and discussions on p. 463 and 465).

Although CHRISTENSEN (1970) has labelled his preference for K^+ as opposed to Na^+, "odd", the preference appears to have been based on his experimental data and would thus seem natural. He has also suggested (CHRISTENSEN, 1970) that the ideas of others on Na^+ coupling were dependent on his prior views. However, from the foregoing, it ought to be clear that others in the field worked entirely independently. If there were effects of Na^+, their potential meaning was certainly obscure to CHRISTENSEN and his colleagues; any effects themselves were unknown elsewhere. The first experiments which can be said to demonstrate an Na^+-effect on amino acid entry into Ehrlich ascites tumor cells were published by HEINZ and colleagues in 1963 (KROMPHARDT et al., 1963).

G. Formulations of a Non-Covalent Mechanism

1. Convective Coupling with the Na^+ Circuit

The first attempt to formulate non-covalent coupling of organic solute transport to an ion circuit was based upon a view that a different spatial arrangement of properties common to all body cells may produce a new kind of activity; "All animal cells that have been examined seem to possess in their membranes structure-specific sites through which sugars may enter and other sites out of which Na^+ is 'pumped'. In cells such as the erythrocyte which have a relatively smooth limiting membrane, these sites, whatever their location on the surface, are probably about equidistant from the main intracellular compartment. These cells do not actively transport sugar. In the intestinal and tubular epithelial cells, on the other hand, there is a typically rough or brush-like border on the side facing their respective lumina which is composed of finger-like, presumably cytoplasm-filled, projections called microvilli. These cells carry out active transport of sugar. Could this be the result, one may ask, of the distribution along the brush border in a specific way of the same kind of structure-specific sugar entry sites and sites of Na^+ pumping present in other cells?" (CRANE, 1960).

The mechanism then proposed is diagrammed in Figure 6 in which Na^+, Cl^-, H_2O, and substrate enter at the tip of the microvillus and Na^+, Cl^-, and

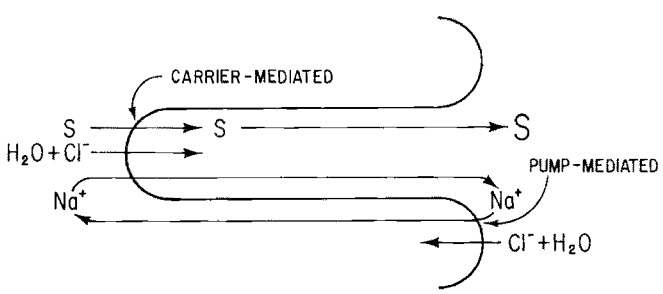


Fig. 6. A convective model for non-covalent coupling with the Na^+ circuit. Drawn from a description given by CRANE (1960)

H_2O exit at the base due to the pumping of Na^+. Substrate accumulates because of the resulting convective current. Because of the effects of Na^+ on anaerobic sugar entry, we abandoned convective coupling as a membrane mechanism although its basic elements have survived in a physiological concept of an External Fluid Circuit operating in the villus as a whole (CRANE, 1965, 1976). Convective-coupling itself seems to have been revived in a recently proposed convective-diffusive model (NAFTALIN and HOLMAN, 1976).

2. Diffusional Coupling with the Na^+ Circuit: The Gradient-Coupled Model

Although the convective-coupling model was unsatisfactory, reviewing it serves to illustrate the wide range of possibilities that were under consideration as the concept of non-covalent coupling was being worked out in 1959. A few months later (CRANE et al., 1961) non-covalent coupling with the ion circuit was specifically formulated as occurring between an outwardly directed Na^+ pump and a Widdas model carrier with two specific binding sites rather than one. The concept is illustrated in Figure 7 by the simplified version published in 1962 (CRANE, 1962). In this version, the brush border membrane enzyme concept also introduced in 1960 (CRANE et al., 1961) was left out and a comparison with the red cell was added in an effort to improve understanding. Later (CRANE, 1965) in an attempt to achieve greater clarity, the carrier was depicted as in the lower part of Figure 7 and explained as a kinetic, not a physical model.

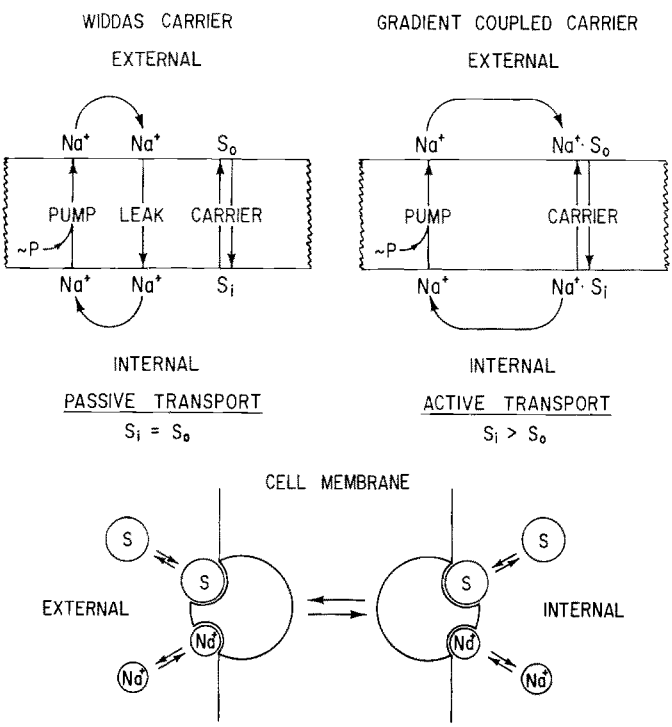


Fig. 7. The gradient-coupled model, Upper, after CRANE (1962). Lower, after CRANE (1965)

The model in Figure 7 when coupled to the Na^+ pump fits the carrier asymmetry notation used in Figure 4 in the following way:

$$\begin{aligned} C^* &\rightleftharpoons C \\ NaC &\rightleftharpoons C + Na \rightarrow \end{aligned} \tag{6}$$

The question has been raised many times of why the Na^+ pump was placed in the brush border membrane and not the serosal membrane as is now done for the epithelial cells we studied. There are two reasons. Firstly, the issue of pump location was not resolved clearly in 1960 (CURRAN, 1960). Being aware of the frog skin work of KOEFOED-JOHNSON and USSING (1958), the analogy with a serosal membrane pump such as had been suggested for the kidney by CORT and KLEINZELLER (1956) was noted (CRANE, 1960). However, sugar active transport occurred at the brush border membrane. Secondly, work with impermeant anions persuaded us that Na^+ moved without a counterion and that continued operation of the carrier could be expected to lead to an accumulation of Na^+ at the inner surface of the brush border membrane unless it were rapidly removed. It was felt that such an unlikely event; i.e. Na^+ accumulation without a counterion, would be relieved by placing the Na^+ pump close by in the same membrane (CRANE, 1962).

3. Diffusional Coupling with the H^+ Circuit

HORECKER et al. (1961) studied the effect of 2,4-dinitrophenol on galactose accumulation by *E. coli* and came to the conclusion that this compound blocks primarily the accumulation of substrate rather than its entry as had been previously thought. MITCHELL (1961c) found that 2,4-dinitrophenol (and other uncouplers of oxidative phosphorylation) acted as a proton ionophore to catalyze the equilibration of protons through the membranes of mitochondria and bacteria. Putting these observations together, MITCHELL (1963) formulated the bacterial permease as a "hydrogen ion-coupled sugar-translocation system" in a manner precisely conforming to my formulation of the Na^+ system except that MITCHELL assumed the proton gradient to be established by electron transport rather than by an ATP-using proton pump similar to the Na^+-pump.

III. The Characteristics of the Basic Model

In a later section, the model will be refined so that variations in kinetics, the question of coupling ratios, and other matters may be considered. However, my interest at this point is to focus on the general predictions implicit in the model with respect to the ion pump and to the *cis*- and *trans*membrane relationships of ion $[X^+]$ and substrate [S] concentration. For this purpose, the refined model to be discussed later (see Fig. 18) may be reduced to a basic model as in Figure 8 which matches the diagrams in Figure 7.

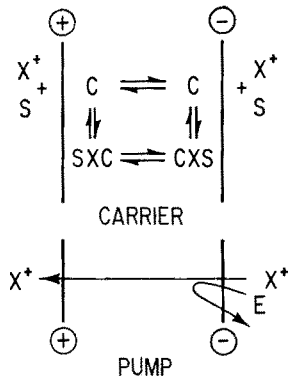


Fig. 8. The basic model of the gradient hypothesis

A. The Role of the Pump: Equilibrium vs. Steady State

It is clear from a consideration of this model that the final state of the system will depend on whether the pump is on or off and what limits of adjustment of $[X^+]_i$ are available to the cellular system under study. When the pump is off, the final state should be an equilibrium with Keq in the vicinity of 1 provided that the normal cellular ion gradients cannot be maintained for very long with the pump off. When the pump is on, a transmembrane electrochemical potential gradient for X^+ can be maintained and the final state will be a quasisteady state which reflects this gradient. The level of the steady state will be a function of $[X^+]_o$, the rate at which entering X^+ is ejected by the pump and the stability of the size of the intracellular pool of X^+, that is $[X^+]_i$. If an increased rate of pumping is more or less precisely adjusted by the rate of X^+ entry, $[X^+]_i$ will be fairly stable and low. Under these circumstances, a series of steady-states can be achieved with differing ratios of $[X^+]_o/[X^+]_i$ by adjustments of $[X^+]_o$.

When the pump is off, transient intermediate states can be achieved by experimental manipulation which are in the normal or the reversed direction of either X^+ or S or both.

B. The Role of the Gradient

The transmembrane electrochemical potential gradient has two components; the chemical potential which depends upon the concentration or activity ratios $[X^+]_o/[X^+]_i$ and $[S]_i/[S]_o$ and the electrical membrane potential which can influence the transmembrane activity ratios of the ion, the mobility (and perhaps the local intramembrane concentration) of the ion if it travels through some portion of the membrane as an ion-stream free of the carrier, and the mobility of the carrier if the carrier is charged in one of its forms. Since membrane potentials are characteristically + outside and − inside, it follows that a loaded carrier may be positively charged ($C\overset{+}{S}X$) and a free carrier may be negatively charged, ($C^- + S + X^+ \rightarrow CS$) in order to provide additional forces supporting substrate influx. Both may occur in the same system; e.g., $C^- + S + 2X^+ \rightarrow C\overset{+}{S}X$, in which

case energy obtained from the membrane potential will be doubled (COCKBURN et al., 1975). A negatively charged loaded carrier ($C\bar{S}X$) would respond in support of efflux.[2]

In formal terms, the electrochemical potential gradient, $\Delta\bar{\mu}X^+$, may be described as

$$\Delta\bar{\mu}X^+ = \Delta\Psi + \Delta\mu X^+ \tag{7}$$

where $\Delta\Psi$, the electrical component, equals E_mF; that is, the membrane potential, E_m, times Faraday's constant. $\Delta\mu X^+$, the chemical component, is a function of the transmembrane activity ratios of X^+ expressed as $RT \ln [X_i^+]/[X_o^+]$. Correspondingly, the gradient of the neutral substrate, $\Delta\mu S, = RT \ln [S_o]/[S_i]$. The distribution of the potential gradient between electrical and chemical forces in different systems will be discussed later. However, it should be noted that when purely chemical forces are at work in the basic model, the system will be stable when $RT \ln [S_i]/[S_o] = RT \ln [X_o^+/[X^+]_i$ or, more trivially, when $[S_i]/[S_o] = [X_o^+]/[X_i^+]$.

C. Predictions from the Basic Model

From the above considerations, the basic model predicts the following characteristics for a gradient-coupled system:

1. The unidirectional flux rates of both S and X^+ should be functions of the cis-values of [S] and $[X^+]$, whether the pump is on or off.

2. X^+ and S should translocate in some stoichiometric ratio whether the pump is on or off.

3. With the pump on, the final state should be a steady-state in which $[S_i]/[S_o]$ depends on the transmembrane chemical potential gradient of X^+; i.e., $[X_o^+]/[X^+]_i$ or the electrical potential gradient, E_mF, or both.

4. With the pump off, the final state should be an equilibrium in which if $[X^+]_o \simeq [X^+]_i$, then $[S_i] \simeq [S_o]$.

5. In any final state, the opposing unidirectional flux rates should be equal; that is, $J_{S_{in}} = J_{S_{out}}$ and $J_{X_{in}^+} = J_{X_{out}^+}$.

6. With the pump off, substrate and ion transmembrane movements and ratios in a transient state should reflect the electrochemical forces imposed by the manipulation.

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[2] In order to round out this discussion it may be pointed out that charged substrates may also contribute to energy transduction from the electrochemical potential gradient. Widdas model binary complexes, $C\bar{S}$ and $C\dot{S}$, will respond to the membrane potential; e.g. as in lysine transport by *Staphylococcus aureus* (NIVEN et al., 1973). Also, the effect can be greatly increased by chemical modifications which alter the pK of the α-amino group so that it is reversibly protonated by translocation between the differing pH's of the external and internal environments (CHRISTENSEN et al., 1973, 1974; CHRISTENSEN and HANDLOGTEN, 1975). Protonation of a negatively charged substrate, $C^- + S^- + X^+ + H^+ \rightarrow CSXH$, would provide an additional response to the transmembrane chemical gradients which would be important provided the intermediate complex, CSX, cannot return S^- to the external medium in response to the membrane potential. Other possibilities may be imagined with predictable results.

IV. Experimental Support for the Basic Model

A. Cellular Na^+-Systems: Sugars

Except for flux rates of Na^+ and stoichiometry, the above characteristics of the model were very early verified by experiment and formed the basis for the reiteration of the Gradient Hypothesis in 1962 (CRANE, 1962).

1. Active Transport but not Equilibration Depended upon the Normal Operation of the Na^+ Pump

CSAKY and THALE (1960) and CSAKY et al. (1961) found that specific inhibition of the intestinal Na^+ pump with cardiac glycosides inhibited active transepithelial transport of sugars. CRANE et al. (1961) extended this to show that similar specific inhibition of the Na^+ pump had no effect on transmembrane equilibration. This finding dissociated the pump from the membrane transport carrier.

2. The Initial Rate of Active Substrate Transport Was Dependent upon the External Ion Concentration

Both RIKLIS and QUASTEL (1958) and CSAKY and THALE (1960) found that total replacement of Na^+ with K^+ or other alkali cations abolished transepithelial active transport. However, RIKLIS and QUASTEL found no rate dependence on $[Na^+]$ above the lowest concentration tested; 30 mM, and CSAKY and THALE made no test of concentration. BIHLER and CRANE (1962) studied the entire range of $[Na^+]$ from 0–217 mEq and found a graded active transport response at early time periods. Also, BIHLER and CRANE studied entry through the brush border membrane and not transepithelial movements.

3. Varying Steady-State Levels of Active Substrate Transport Were Achieved by Varying $[Na^+]_o$

BIHLER and CRANE (1962) found steady-state levels of internal substrate to be achieved and to depend on the level of $[Na^+]_o$ as shown in Figure 9. The level of $[S]_i$ increased with $[Na^+]_o$ and as would be required, a steady-state already achieved at a lower level of $[Na^+]_o$ was moved to a higher steady-state by an increase in $[Na^+]_o$.

The fact that the steady-state achieved actually reflected bidirectional movement across the membrane was established by measurements of the substrate exchange rate in the steady state (Table 1). The exchange rate increased with $[Na^+]_o$ and the fraction exchanged per unit time was the same over the entire range of $[Na^+]_o$ used. It was noted that exchange flux measured in the steady state was about the same as initial rates measured in other experiments.

4. The Levels of the Steady-State Depended on the Transmembrane Electrochemical Potential Gradient

The data of BIHLER and CRANE (1962) also reasonably fitted the expectations of a dependence of the level of the steady-state on the transmembrane $\Delta[Na^+]$ as may be rudely shown by calculation and comparison of the ratios $[S]_i/[S]_o$ and $[Na^+]_o/[Na^+]_i$ (Table 2).

The correspondence of the ratios in Table 2 would suggest that the dominant force in brush border membrane sugar translocation by hamster small intestine is chemical rather than electrical.

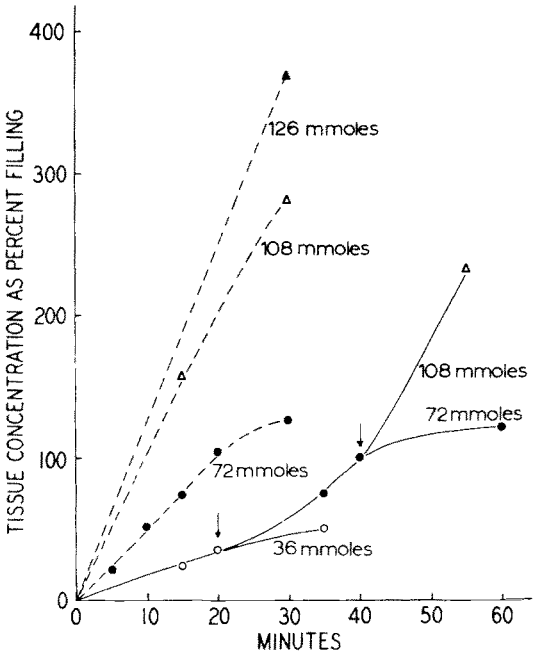

Fig. 9. Demonstration of steady states and their dependence of $[Na^+]_o$. Strips of hamster small intestine were incubated with 1,5-anhydro-D-glucitol, 5 mM, at the Na^+ concentrations and for the time periods indicated. (From BIHLER and CRANE, 1962)

Table 1. The influence of Na^+ concentrations on the steady-state tissue concentration of 1,5-anhydro-D-glucitol and on the exchange rate of 1,5-anhydro-D-glucitol in steady-state

Medium Na^+ (mM)	Tissue 1,5-AG (mM)	Exchange rate (mmol/l tissue water/min)	Fraction exchange (%/min)
145	18.0	0.69	3.8
108	13.8	0.51	3.7
72	9.6	0.37	3.8
36	4.0	0.14	3.6
0	2.1	0.07	3.2

External 1,5-anhydro-D-glucitol was 5 mM. The incubation to achieve the steady-state was for 40 min. From BIHLER and CRANE (1972).

Table 2.

$[Na^+]_o$	$[S]_i/[S]_o$	$[Na]_o/[Na^+]_i$
145	3.6	3.6
108	2.8	2.7
72	1.9	1.8
36	0.8	0.9

Data from Table 1.

In these calculations, $[Na^+]_i$ is assumed to be 40 mEq as found at $[S]_o=[S]_i$ by interpolation of the data. $[Na^+]_i$ measured with no sugar being transported varied from 32 mEq at $[Na^+]_o=$ 36 mEq to 50 mEq at $[Na^+]_o>96$ mEq, (BOSACKOVA, 1963; BOSACKOVA and CRANE, 1965). Actual $[Na^+]_i$ at values of $[Na^+]_o>36$ mEq may be somewhat higher than 40 mEq which would tend to reduce the ratio $[Na^+]_o/[Na^+]_i$. However, $[Na^+]_i$ is not corrected for the intracellular activity coefficient which might tend to decrease the ratio (LEE and ARMSTRONG, 1972). Also KOOPMAN and SCHULTZ (1969) have shown for rabbit ileum that $[Na^+]_i$ is not different whether or not sugar transport is taking place unless the sugar is galactose.

5. With the Na^+ Pump Off, Transmembrane Equilibration Occurred with Characteristics Appropriate to a Widdas Model Carrier Interacting with Both Substrate and Na^+

KRANE and CRANE (1959) had shown that galactose transport by slices of rabbit kidney was inhibited by phlorizin whether transport was energized or not. The same was found for sugar transport in the intestine (CRANE et al., 1961). It was also well known that phlorizin at the concentrations used interacted at the membrane and not intracellularly (CRANE, 1960) and strictly competitively with actively transported sugar substrates (ALVARADO and CRANE, 1962). These observations were taken as evidence of carrier function at the membrane.

BIHLER et al. (1962) demonstrated that anaerobic transmembrane equilibration of sugars was Na^+-dependent and that substrate exchange occurred at equilibrium. They also showed that this Na^+-dependent equilibration had the same substrate specificity as active transport; the entrance of actively transported sugars was enhanced by the replacement of K^+ with Na^+. The entrance of sugars not actively transported was not.

CRANE (1964) demonstrated that a reversal of the Na^+ gradient caused the uphill efflux of previously equilibrated substrate (Fig. 10) as is predicted from the countertransport characteristics of the Widdas model.

6. The Entry of Na^+ is Substrate Dependent and Occurs in Some Stoichiometric Ratio with Substrate

BIHLER and I recognized the electrogenic implications of the fact that Na^+-dependent transport occurred in the presence of impermeant anions (BIHLER and CRANE, 1962) and enlisted the help of D. Tosteson in an attempt to find

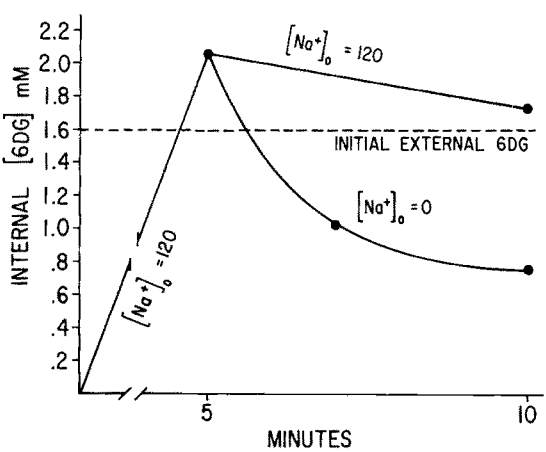

Fig. 10. Na^+-dependent substrate counterflow. Drawn from data of CRANE (1964). Isolated villi from hamster small intestine were preincubated with 6-deoxy-D-glucose for 5 min with $[Na]_o = 120$ mEq then transferred as indicated. Media contained 0.05 mM 2,4-dinitro-0-cresol and were anaerobic; i.e. 95% N_2, 5% CO_2 to suppress energy-yielding metabolism. Internal concentration of 6-deoxyglucose at 5 min is slightly higher than external concentration. This may be attributed to incomplete equilibration between $[Na^+]_i$ and $[Na^+]_o$ and a small effect of the residual gradient

some expression of this in the transmural PD. We were not successful. Others, however, were.

Substrate-dependent entry of Na^+ was very early seen (BARRY, MATTHEWS, SMYTH, and WRIGHT, 1961; CLARKSON, CROSS, and TOOLE, 1961) by means of measurements of the effects not only of glucose but also of actively transported non-metabolizable sugars and phlorizin on the transmural PD. However, this interpretation of the observations was not fully clarified (BARRY et al., 1964) until SCHULTZ and ZALUSKY (1964a) showed 1. that ΔPD equaled the short circuit current of Na^+ and 2. demonstrated that the Na^+ pump was on the serosal side, not the mucosal side of the preparation.

The now classic experiment of SCHULTZ and ZALUSKY (1964b) in demonstrating the substrate effect on Na^+ influx is shown in Figure 11. The non-metabolizable sugar, 3-methyl glucose, increased Na^+ influx and this increase was inhibited by phlorizin. Sugar influx itself was, however, not measured, hence stoichiometry was not tested at the time. However, these findings led SCHULTZ and ZALUSKY (1964b) to make an appropriate, for the intestinal epithelial cell, rearrangement of the carrier and pump in the Gradient Hypothesis so that the pump was given a serosal location.

Using a technique introduced by SCHULTZ et al. (1967) whereby the mucosal surface of rabbit ileum could be uniquely exposed to the bathing medium and unidirectional fluxes across the brush border membrane could be rapidly and reproducibly measured, GOLDNER et al. (1969) showed that influx of Na^+ took place with 3-MG in a ratio of 1 over a five-fold range of $[Na^+]_o$.

ROSE and SCHULTZ (1971) confirmed that Na^+ contributes to the conductance across the brush border membrane and that the increased Na^+ influx which takes place when actively transported sugars or amino acids are present brings

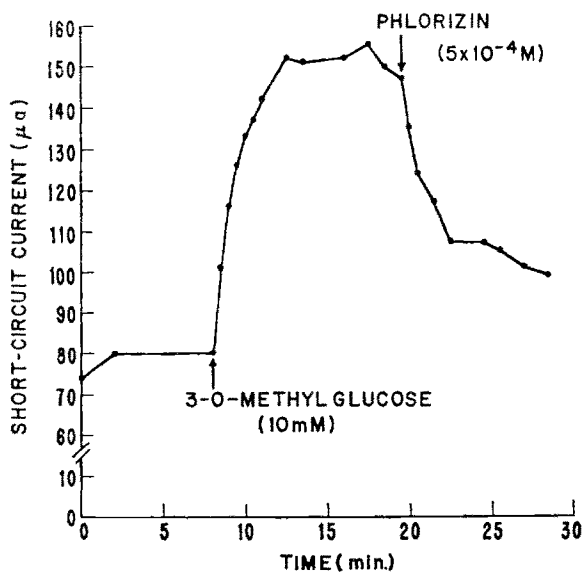

Fig. 11. Time course of the Short Circuit Current in an experiment with isolated rabbit ileum (From SCHULTZ and ZALUSKY, 1964b)

about a marked depolarization of the brush border membrane potential. The Na^+ influx is electrogenic.

B. Cellular Na^+-Systems: Amino Acids

The probability that amino acids responded to the Na^+ gradient similarly to sugars was very early indicated. NATHANS et al. (1960) and CSAKY (1961) found that Na^+ was required for amino acid uptake by the intestine. KROMPHARDT et al. (1963) demonstrated that external Na^+ controlled glycine entry in Ehrlich ascites tumor cells. ROSENBERG et al. (1965), using the non-metabolized analog, α-aminoiso-butyric acid (AIB) showed the effects of Na^+ both on accumulation and on carrier-mediated entry in rabbit jejunum and supported our conclusions described above for sugar.

However, the basic model received really substantial support from the elegant experiments of VIDAVER using pigeon red cells and "ghosts" prepared from them (VIDAVER, 1964a, b, c, d). VIDAVER's experiments were the first anywhere to demonstrate stoichiometry between S and X^+ and the first to demonstrate that a gradient-coupled carrier would respond to the membrane potential.

1. The Stoichiometry of Na^+ and Glycine Effect Fitted the Kinetics of the System

Glycine entry into pigeon red cells was found to have two components; a sodium-independent component linear with glycine concentration and a sodium-dependent component with Michaelis-Menten kinetics and an effect of external $[Na^+]$ corresponding to the predicted entry of 2 Na^+ with each glycine.

VIDAVER measured increments of Na^+ entry over a nearly three-fold range of Na^+-dependent glycine entry and found, within very modest experimental deviation, the predicted value of 2 Na^+/glycine (VIDAVER, 1964d).

Several years later, CURRAN et al. (1967) found the stoichiometric ratio of 1 for alanine and Na^+ entry across the brush border membrane of the rabbit ileum when saturating levels of Na^+ were used. SCHAFER and JACQUEZ (1967) found a ratio of 1 for AIB and Na^+ entry into Ehrlich ascites tumor cells.

2. Transmembrane Movements of Glycine Responded to the Transmembrane Electrochemical Potential Gradient

Using lysed and restored cells, VIDAVER (1964b) showed that glycine entry was enhanced when the initial conditions were $[Na^+]_o > [Na^+]_i$, that glycine exit was enhanced and took place against a concentration gradient when the initial conditions were $[Na^+]_o < [Na^+]_i$, and that glycine entry and exit were equal when $[Na^+]_o = [Na^+]_i$. The measured distribution ratios approximately satisfied the conditions $([Na^+]_o/[Na^+]_i)^2 = [S]_i/[S]_o$ (STEIN, 1967).

VIDAVER (1964d) also showed that Na^+-dependent glycine transfer could respond to the membrane potential. He used toluene disulfonate to produce a Cl^- Donnan potential with equal concentrations of Na^+ across the membrane

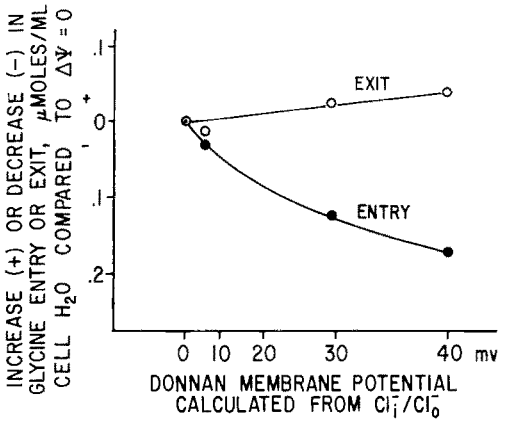

Fig. 12. Total glycine entry and exit with lysed and restored pigeon red cells plotted against Donnan Membrane Potential calculated from $[Cl^-]_i/[Cl^-]_o$. Redrawn from VIDAVER (1964b)

of lysed and restored cells. The potential established was opposite in sign to the normal K^+ gradient potential of these cells. Hence, it would be predicted that entry would be slowed and exit increased as the potential increased. This was the result obtained, as shown in Figure 12.

C. Cellular H^+-Systems

Although MITCHELL immediately appreciated the applicability of the concept of non-covalent coupling with an ion circuit and began very soon to revise (MITCHELL, 1961d, 1963) his earlier views on coupling as strictly a covalent chemical process (MITCHELL, 1959, 1961b), experimental verification of the basic model in H^+-coupled bacterial systems lagged greatly behind the Na^+-coupled mammalian systems.

1. Demonstration of the Carrier Basis of Active Transport

A carrier basis for bacterial active transport of galactosides was predicted by COHEN and MONOD (1957). KOCH (1964) modeled sugar transport in *E. coli* as carrier-mediated based upon much of his own work as well as others. However, the breakthrough for bacterial systems was provided by WINKLER and WILSON (1966). These workers showed that in cells poisoned with azide or dinitrophenol, active transport stopped but translocation of lactose and nitrophenyl galactoside continued to occur, mediated by what appeared to be membrane carriers of the Widdas type. As shown in Figure 13, the effect of the inhibitors was to reduce the K_t of exit. Other experiments showed that the K_t of entrance was not affected. In discussing the energy source for active transport, WINKLER and WILSON considered the possibility of ion gradients and specifically eliminated an Na^+-gradient from consideration. However, they do not seem to have clearly appreciated the probability that azide and nitrophenol in their experiments worked

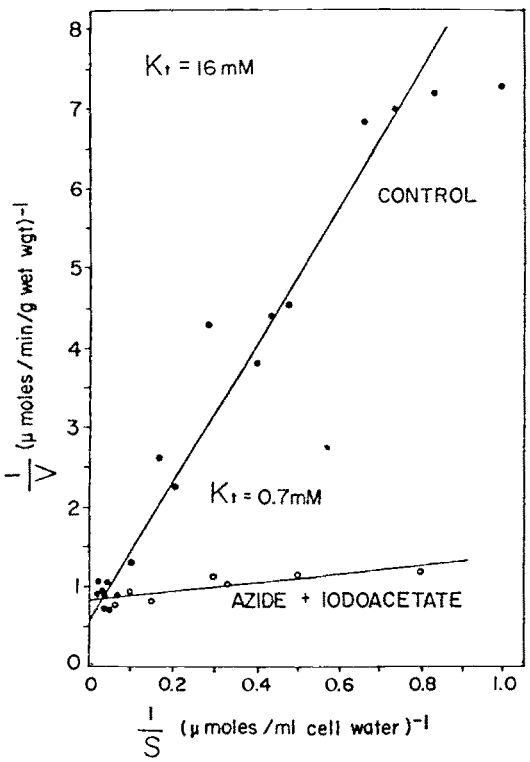

Fig. 13. Effect of azide and iodoacetate on the exit K_t for lactose in *E. coli.* Cells were preloaded with ^{14}C-lactose and exit rate was measured. (From WINKLER and WILSON, 1966)

because they collapsed the pH gradient (MITCHELL, 1961c). PAVLOVSOVA and HAROLD (1969) directly demonstrated that proton uncouplers abolish galactoside accumulation and, equally important, do not decrease cellular ATP levels.

2. Demonstration of Stoichiometry between the Entry of H^+ and Galactoside

WEST (1970) demonstrated an inward flow of H^+ together with the inward transport of lactose in *E. coli.* WEST and MITCHELL (1973) quantitated this effect. Shown in Figure 14 is the time course of net inward H^+ and lactose translocation under anaerobic conditions into *E. coli* depleted of carbohydrate reserves and non-metabolizing. At the earliest time period measured, net H^+ lagged behind net lactose. However, extrapolation to zero time indicated an initial inflow of both species at a ratio of 1.

3. Demonstration of the Responsiveness of Galactoside Transport to the Transmembrane Electrochemical Potential Gradient of H^+

KASHKET and WILSON (1973) showed that a loss of protons from the suspending fluid occurred simultaneously with uptake of thiomethylgalactoside by *Strepto-*

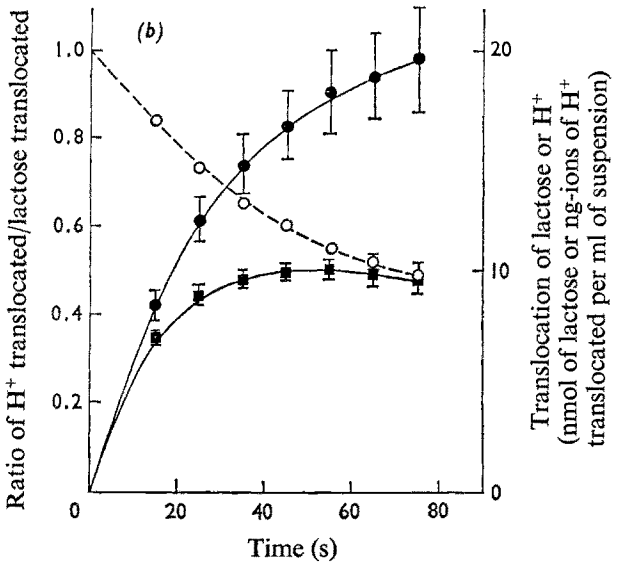

Fig. 14. Correlation between ^{14}C-lactose inflow and net inflow of H^+ into anaerobic *E. coli.* ●, lactose translocation; ■, effective H^+ translocation; ○, stoichiometric ratio, H^+/lactose

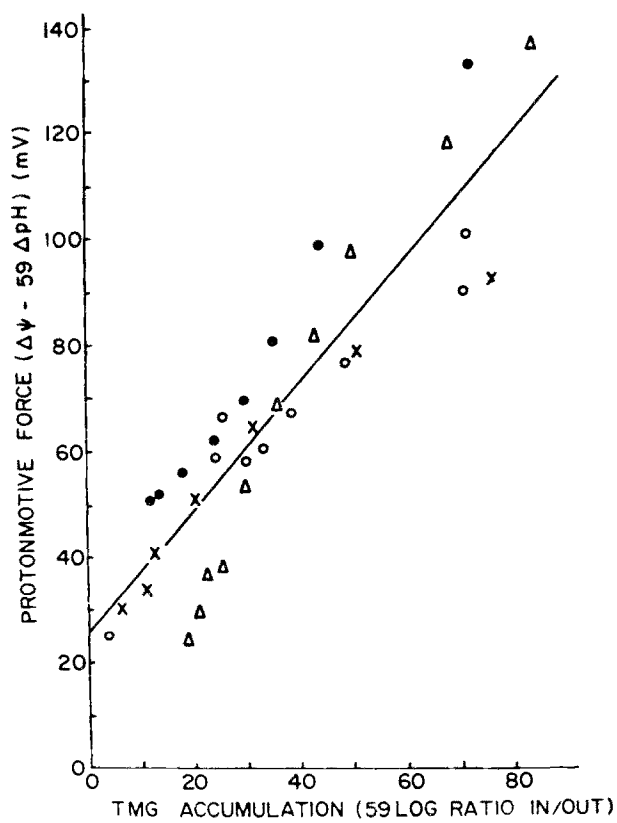

Fig. 15. Correlation between thiomethyl galactoside accumulation ratio and the transmembrane elec trochemical potential gradient of H^+, $\Delta\bar{\mu}H^+$. (From KASHKET and WILSON, 1973)

coccus lactis and that the extent or galactoside accumulation was influenced by the transmembrane pH gradient. They also showed with K^+ rich cells in which addition of the K^+ specific ionophore, valinomycin, induced a membrane potential, inside negative, that there was direct relationship between thiomethylgalactoside accumulation and the transmembrane electrochemical potential gradient of H^+ (Fig. 15). Theoretically, the line drawn should intercept zero. However, there were indeterminate factors in the activity of intracellular K^+ and the $[K^+]$ at the membrane surface both of which would tend to overestimate the membrane potential, ψ, and cause an intercept on the ordinate as was found.

A more recent study of galactoside accumulation in *E. coli* (FLAGG and WILSON, 1976) adds support to these conclusions.

D. Membrane Vesicle Systems

The use of vesiculated plasma membranes for the study of membrane transport phenomena was introduced by KABACK (KABACK, 1960; KABACK and STADTMAN, 1966). Studies with these bacterial and related mammalian preparations have proved them to be generally a powerful experimental tool. In the present case, the characteristics of gradient-coupled transport observed with intact cells seem to be preserved.

1. Intestinal Brush Border Membrane Vesicles

HOPFER et al. (1973) prepared a vesiculated preparation from isolated brush border membranes of rat small intestine. This preparation contained an intact glucose transport system by means of which the uptake of D-glucose as compared to L-glucose into an intravesicular space was specifically stimulated by Na^+, inhibited by phlorizin and D-galactose, and showed the counterflow property of a Widdas model carrier. The Na^+-dependent system is specifically located in brush border as compared to baso-lateral membranes (MURER et al., 1974) and a closely similar system for L-alanine has also been demonstrated (SIGRIST-NELSON et al., 1975). The vesicles also contain a Na^+/H^+ exchange system (MURER et al., 1976).

The properties of these membrane vesicles with respect to induced ion fluxes also closely approximate predictions from prior experiments with intact tissue; the Na^+-dependent transport systems for glucose (MURER and HOPFER, 1974) and for L-alanine (SIGRIST-NELSON et al., 1975) are electrogenic; which is to say that Na^+ only moves without a negative counterion on the carrier. In the intact cell, active substrate transport is supported by the removal of entering Na^+ by the Na^+-pump. In vesicles, which lack a pump, only an equilibrium and not active transport can ordinarily be achieved as the Na^+-gradient slowly dissipates mediated in part by the carrier and in part by various unspecified leaks. Being electrogenic, Na^+ flux with the carrier in vesicles is limited by the rate at which a negative counterion can move across the membrane in the same direction or a positive counterion can move in the opposite direction. However, by providing a means for a rapid carrier-mediated dissipation of the

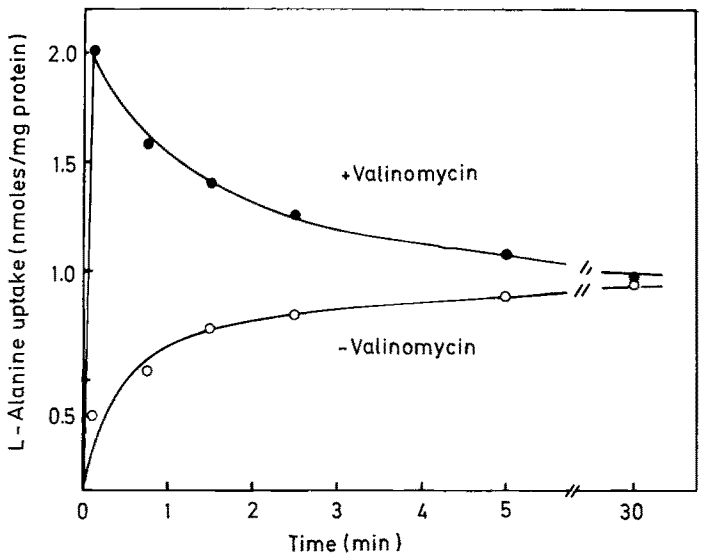

Fig. 16. Effect of valinomycin on L-alanine transport in K^+-preloaded isolated brush border membrane vesicles from rabbit kidney. L-alanine uptake was initiated by adding K^+-preloaded vesicles to an Na_2SO_4 solution containing L-[3-^{3}H2 alanine (1 mM). (From SIGRIST-NELSON et al., 1975)

Na^+ gradient, a transient active substrate transport can be demonstrated with vesicles.

In different experiments, concentration gradients of the lipophilic anion, SCN^-, of K^+, or of H^+ were established across the vesicular membrane between the inner space and the suspending medium. In the presence of external Na^+ and an inward downhill gradient of SCN^- a transient overshoot of substrate entry above the equilibrium level was seen. A similar overshoot was produced by increasing the membrane conductance for K^+ by use of the K^+-specific ionophore, valinomycin, or for H^+ by use of the proton ionophore, carbonyl cyanide p-trifluoromethoxy phenyl hydrazone (CF-CCP). In each case, the inward driving force of the Na^+-gradient was released in support of an active transport of substrate which was transient and subsided as the driving force dissipated as would be predicted from the basic model. A supporting membrane potential is also created using ionophore by induced K^+ or H^+ efflux. A typical experiment is shown in Figure 16.

2. Kidney Tubular Brush Border Membrane Vesicles

A role for Na^+ in kidney sugar reabsorption was indicated by the findings of KLEINZELLER and KOTYK (1961 b) in extension of the work of KRANE and CRANE (1959) on galactose active transport by rabbit kidney cortex slices. A close similarity of the kidney to the intestine was further indicated by ouabain inhibition of glucose reabsorption in a heartless dog preparation (CSAKY et al., 1965). Kidney slice preparations also have been shown to transport amino acids (ROSENBERG et al., 1961) by Na^+-dependent processes (FOX et al., 1964; THIER

et al., 1967). However, work with slices has been difficult to interpret (KLEINZELLER, 1970; SEGAL and ROSENHAGEN, 1974) owing to the impossibility of knowing which tissue membrane is responsible for the properties observed. The problems of the slice technique were overcome, as so well reviewed by ULLRICH (1976), by the introduction and use of microperfusion and electrophysiologic methods which permitted the characteristics of the brush border tubular membrane to be specifically studied and Na^+-dependent substrate reabsorption to be demonstrated. However, with respect to the presence and operation of gradient-coupled carriers in the brush border membrane, work with brush border membrane vesicle preparations seems to provide the clearest evidence.

The first report of such studies (BUSSE et al., 1974) showed Na^+-dependent glucose uptake but suffered the problem that glucose was metabolized by the preparation. In more recent studies, glucose (KINNE et al., 1975; ARONSON and SACKTOR, 1975; BECK and SACKTOR, 1975) and L-phenylalanine (EVERS et al., 1976) were translocated into kidney brush border membrane vesicles by Na^+-dependent transport systems with characteristics closely similar to those described above for intestinal brush border membrane vesicles.

In these experiments addition of valinomycin or CF-CCP had the same effect of supporting a transient active transport of substrate due to their mediation of an electrogenic ion efflux for K^+ and H^+, respectively. In addition, BECK and SACKTOR showed that when channels for Na^+ alternative to the carrier were provided in the vesicular membrane with either electrogenic properties, gramicidin, or electroneutral properties, nigericin, Na^+-dependent glucose uptake was greatly diminished. As expected a large proportion of the driving force of the Na^+-gradient was dissipated unproductively through these channels.

3. Pigeon Red Cell Membrane Vesicles

Membrane vesicles were prepared by VIDAVER and his colleagues (LEE et al., 1973) from pigeon red cells which, as discussed above, were used so successfully by VIDAVER in early experiments on Na^+-dependent glycine uptake in both intact and "ghost" preparations. Glycine uptake by these vesicles resembled glycine entry into intact cells; that is, Na^+ was required in the medium and glycine uptake depended upon glycine concentration and $([Na^+]_o)^2$. However, a substantial proportion of the vesicles appeared to be inactive in transport and more definitive tests of the basic model could not be made.

4. Ehrlich Ascites Tumor Cell Membrane Vesicles

COLOMBINI and JOHNSTONE (1974a, 1974b) have prepared plasma membrane vesicles from Ehrlich ascites tumor cells. Using this preparation they have found an exchange-diffusion system for amino acids and an Na^+-dependent AIB uptake system with some of the properties of the basic model. Influx of AIB was specifically stimulated by an inward Na^+ chemical gradient. When the gradient was dissipated by use of gramicidin, AIB uptake was inhibited. When the gradient was added to by a valinomycin induced K^+ efflux, AIB uptake was enhanced.

However, a twofold AIB accumulation ratio of AIB was found in the absence of a transmembrane electrochemical potential difference of ions. It was also found that ouabain appeared to act directly on amino acid transport and to reduce the Na^+-gradient induced AIB uptake. The effect of ouabain was interpreted as being directly on Na^+-dependent uptake. This finding creates some difficulties. If it may be assumed that K^+ interaction with the Na^+-binding site of the carrier (EDDY et al., 1967) has structural meaning, it is possible to imagine that K^+ and ouabain interact in this case as in Na^+-K^+, ATPase (HANSEN, 1974), on a common site. However, AIB accumulation in the absence of a gradient taken together with the ouabain effect suggests the possibility that the Ehrlich ascites cell membrane possesses a second AIB uptake system which satisfies its energy requirement by a different mechanism.

5. Bacterial Membrane Vesicles

Studies with the membrane vesicle preparations from bacteria also provide support for the Gradient Hypothesis.

As is well known, KABACK's early interpretation of the experimental results with *E. coli* membrane vesicles was in terms of two-state enzyme model (KABACK and BARNES, 1971). As research has progressed, however, this proposal has been left behind (RAMOS et al., 1976) in favor of the model provided by the Gradient Hypothesis. Consequently, the following description of results will be referred to the basic model insofar as they can be so interpreted. The number of questions addressed and the speed with which experimental answers have been obtained with microbial membrane vesicles is simply remarkable.

BARNES and KABACK (1970) showed that ATP was not the energy donor for β-galactoside transport by *E. coli* vesicles but that uptake activity was coupled to membrane-bound D-lactate dehydrogenase (LDH). REEVES et al. (1973) confirmed this by reconstitution. When LDH extracted with guanidine-HCl from wild type vesicles was added to a vesicle preparation from an LDH negative mutant strain both D-lactate oxidation and D-lactate dependent active transport were reconstituted. The question of energy source seemed to be resolved. The question remained, how were LDH and active transport coupled.

SHORT et al. (1974) showed that reconstitution could be carried out with a homogeneous preparation of LDH and that the maximal transport activity conferred on the mutant vesicles was of similar magnitude to transport activity in wild type vesicles. More significantly, however, studies with dansylgalactoside indicate that there were more transport sites than LDH sites, by seven- or eightfold in reconstituted vesicles and 15-fold in wild type. Consideration of other D-lactate activated carriers would, of course, increase this excess. It is also significant that the position of LDH in the membrane did not determine its function. In native vesicles, LDH is inside, it is not accessible to antibody. In reconstituted vesicles, it is outside (SHORT et al., 1975). In view of these findings, it seemed unlikely that LDH is a direct participant in the transport process.

A series of experiments led to the conclusion that LDH activates transport by generating a membrane potential through proton transport. REEVES (1971) had demonstrated that D-lactate oxidation by vesicles resulted in transient pH

changes. LOMBARDI et al. (1973) confirmed the production of these changes and showed that they could be abolished by the proton ionophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP). KABACK et al. (1974) showed that CCCP inhibited lactose and amino acid transport. PATEL et al. (1975) found that when the proton permeability of vesicles was increased by extraction with chaotropic agents, there was no membrane potential and no transport. Both were restored when the permeability changes were reversed by treatment with carbodiimide.

Other experiments have shown that substrate transport responds to the membrane potential in at least two ways. 1. In a series of experiments with dansylgalactoside (SCHULDINER et al., 1975a, b; 1976) and azidophenylgalactoside (RUDNICK et al., 1975) both of which bind to the transport carrier but are not themselves transported, it could be demonstrated that binding occurred only when the vesicle membrane was "energized" by substrate oxidation. This has been interpreted as indicating either a negative charge on the carrier or perhaps less likely, that $\Delta\Psi$ directly affects carrier-substrate affinity. 2. HIRATA et al. (1974a) found that proline accumulation by K^+-loaded wild type *E. coli* vesicles could be driven by a valinomycin induced membrane potential. In mutants defective in proline transport, proline accumulation was not found whereas glycine and thiomethylgalactoside accumulation proceeded as expected. SCHULDINER and KABACK (1975) have measured the membrane potential generated by oxidation of lactate or reduced phenazine methosulfate by means of the distribution of the lipophilic probe, triphenyl-methylphosphonium ($TPMP^+$). Consistent with the basic model, steady state levels achieved for lactose, proline, glutamic acid, and glycine were directly related to $\Delta\Psi$ as measured by $TPMP^+$. However, the measured $\Delta\Psi$ fell short of providing enough energy to sustain the substrate accumulation gradients found. Tests of ΔpH as an additional energy source were negative. Thus to rationalize these conflicting data, SCHULDINER and KABACK have proposed that $2H^+$ are taken up for each cycle of the carrier thus doubling the response to the membrane potential as has been found for the proton-driven yeast glycine system by SEASTON et al. (1976).

Further studies on membrane vesicles by KABACK and his colleagues which give additional support to the Gradient Hypothesis are presented in a later section.

6. Liposome Vesicles

Gradient-coupled carriers of the hamster intestinal brush border membrane (CRANE et al., 1976b) and of the rabbit kidney brush border membrane (CRANE et al., 1976c) have been reconstituted. The property of Na^+ enhanced, phlorizin and galactose inhibited, D-glucose (but not L-glucose) uptake has been conferred upon liposome vesicles by combining them with triton X-100 extracted proteins of hamster brush border membranes or rabbit kidney cortex brush border membranes. Figure 17 shows a reconstitution experiment with hamster intestine membrane proteins.

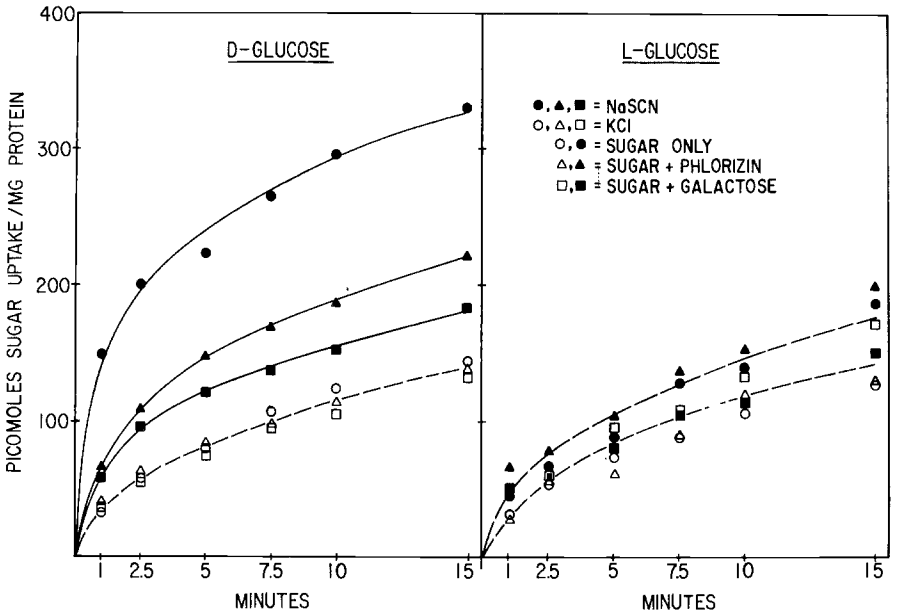

Fig. 17. Reconstitution of specific Na^+-dependent D-glucose transport in liposome vesicles. Liposomes contained KCl. D- or L-glucose were 0.1 mM. Phlorizin when added was 5 mM, galactose when added was 10 mM

E. Conclusions About the Basic Model

In one way or another, in one cell or another, and in natural membrane and liposome vesicles, all of the predictions of the basic model have been satisfied by experiment. It is reasonable to conclude that the Gradient Hypothesis has been, in principle, proved. However, there are variations in the performance of gradient-coupled systems from cell to cell and with different substrates, perhaps particularly with Na^+-coupled systems, which are puzzling and have given rise to questions relating to whether the electrochemical potential gradient is the sole source of energy for substrate accumulation in these systems. As will be seen in the next section, many of these questions seem to be related to underlying complexities of gradient-coupled transport and of other membrane activities rather than to whether the hypothesis is right or wrong.

V. Kinetics and Coupling: The Refined Model

A. General Considerations:

1. Formulation of the Refined Model

In order to understand the Gradient Hypothesis in more depth and as it may vary from one cell to another and with substrate, the basic model must be

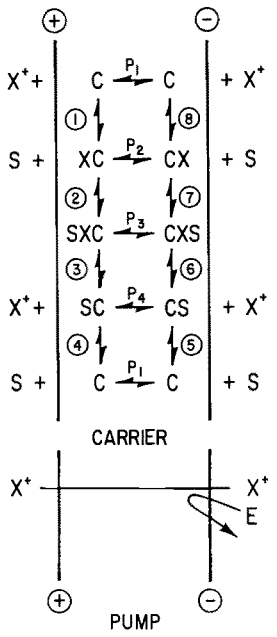

Fig. 18. The refined model of the gradient hypothesis

expanded to provide for 1. the effects of the movement of carrier in any of its forms, free or combined and 2. the effects of any required order of addition of ion and substrate. For this purpose, a refined model using a formulation closely similar to that of SCHULTZ and CURRAN (1970) is given in Figure 18. In this figure, the refined model is described by a totally unrestricted carrier which may translocate empty, P_1, with ion or substrate alone, P_2 and P_4 or with both together, P_3, as in the basic model. The refined model shows the fully-loaded carrier, CXS, as formed with either ion or substrate added first. Also, as a first approximation, the refined model assumes no difference in the translocation rate for any of the forms; that is, $P_1 = P_2 = P_3 = P_4$ although the probability that some but not all carrier forms are charged and responsive to the membrane potential suggests immediately that this simple state of total equality of translocation rates may never actually occur.

The refined model has been well and thoroughly analyzed by STEIN (1967), SCHULTZ and CURRAN (1970), HEINZ et al. (1972) and JACQUEZ (1972) and the reader interested in having the kinetic equations is referred to these fine papers. The purpose here is not to repeat these analyses but to focus attention on the varied characteristics of different systems which these analyses have led us to expect or which they can explain.

2. Alternative Membrane Pathways:

Also, it follows that in order to understand the model in a practical as well as a theoretical way, it is necessary to take into account the other properties

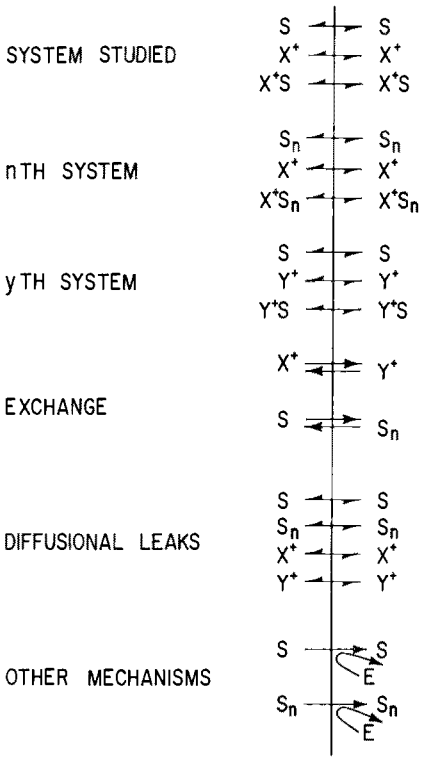

Fig. 19. Some of the alternate pathways that may exist in cell membranes

of the particular membrane in which the model is thought to operate. The presence of more than one gradient-coupled system, as for example, for both sugar and amino acid, for more than one variety of each of these, or for unrelated compounds may provide additional portals for entry and exit of substrate and ion that may have the same or different kinetics as the system under study. A different ion may be involved. Also, there may be additional alternative pathways for substrate and ion movement which can act as leaks regardless of their actual nature. Exchange diffusion pathways such as for Na^+/H^+ (MURER et al., 1976) come readily to mind. Indeed, there may be active transport processes with energy transduction mechanisms other than gradient coupling. These considerations are illustrated in Figure 19.

B. The Kinetics of Gradient-Coupled Carriers

As explained by HEINZ et al. (1972)[3] the general kinetic model of a gradient-coupled carrier is highly complex but may realistically be reduced to three types;

[3] After completion of this manuscript GECK and HEINZ (1976) have published an important extension of the kinetics of gradient-coupled carriers to include the effects of electrical potentials.

namely, 1. Affinity-type (or K_m) models in which the affinity of the carrier for S and X^+ is modified by its interactions but its mobility is not changed, 2. Velocity-type (or V_{max}) models in which mobility is changed without modification of affinity and 3. Mixed-type models in which both affinity and mobility changes occur.

HEINZ et al. (1972) have also considered direct and indirect (quasi-allosteric) variations for each of these types. The direct variations are those in which only a single form of the carrier exists which is directly modified by the interaction. The indirect variations are those in which two or more conformational states of the carrier coexist in equilibrium and have different affinity interactions with S and X^+ and any modifiers which may be present. They may also have different mobilities. HEINZ et al. (1972) showed that the indirect variation of the velocity-type model cannot lead to substrate accumulation, though the indirect variations explain the findings for the other types as well as do the direct variations.

The approach by SCHULTZ and CURRAN (1970) emphasizes the effects on kinetics of the order of addition of S and X^+ and the influence on experimentally measured kinetic parameters of differences in the mobilities of the different carrier forms. JACQUEZ (1972) considers the effect of the membrane potential on the mobility of charged forms of the carrier.

C. Coupling and Energy Transduction

Effective coupling of the flows of S and X^+ is achieved only through the mediation of the carrier form, CSX, as in the basic model. Assuming that S and X^+ each have only one kind of individual interaction with the carrier, the coupling coefficient of the basic model is 1; the coupling efficiency is 100 percent. The refined model (Fig. 18), however, suggests that measured coupling efficiencies may be less than 100% owing to the possibility of uncoupled flows of S and X^+ mediated by CS and CX^+, respectively. These uncoupled flows are called *inner leaks* by HEINZ (1974) and correlate with the operation of an ungeared activated carrier in other systems. The alternate pathways in Figure 19 also represent flows that are not coupled to the system under study. HEINZ (1974) calls these *outer leaks*.

As is obvious, the final state achieved by a gradient-coupled system will depend on 1. the particular cell studied, 2. the particular substrate, 3. the proportion of carrier in the various forms at the concentration of S and X^+ used, 4. the mobility of the various carrier forms, 5. the presence and interactions of other substrates and ions with the carrier binding sites for S and X^+ and 6. the operation of all other portals through which S and X^+ may translocate. Depending on their identity and characteristics under the experimental conditions imposed, these other portals may add to or subtract from the transmembrane gradients of S and X^+ and the membrane potential.

It is clear that if the proportion of the total flows of S and X^+ which occur by means of CSX is not known, the thermodynamic efficiency of the system cannot be evaluated.

VI. The Kinetics of Real Systems

A. The Kinetic Types Found

Real gradient-coupled systems vary so widely in their characteristics that they will be discussed illustratively rather than exhaustively. Furthermore, no real system has yet been adequately described kinetically and such an accomplishment is in any case improbable for some time to come. Fundamentally, the reason for this is the relative inaccessability of the inner surface of the membrane to the same kinds of experimental manipulation as may be applied to studies of interaction at the outer surface. Consequently, studies of the kinetics of efflux are rare and it is usually assumed that interactions of the carrier are symmetrical across the membrane with respect to measured kinetic parameters. Where this assumption has been put to test (e.g. VIDAVER and SHEPHERD, 1968), it has been found wrong.

Thus any statement on the kinetics of real systems must currently be viewed as qualitative. With this understanding it may then be noted that all three of the models of HEINZ et al. (1972) have been observed. Among affinity-types for sugar transport are hamster (CRANE, 1965) and rat (LYON and CRANE, 1966), jejunum and rabbit kidney vesicles (ARONSON and SACKTOR, 1975). Affinity-types for amino acid transport include alanine uptake by rabbit ileum (CURRAN et al., 1967) glycine transport by pigeon red cells (VIDAVER and SHEPHERD, 1968) and phenyl-alanine transport in rabbit kidney vesicles (EVERS et al., 1976). Examples of the velocity-type model are 3-O-methyl glucose uptake by rabbit ileum (GOLDNER et al., 1969) and Na^+-dependent proline transport by membrane preparations of *Mycobacterium Phlei* (HIRATA et al., 1974b). The mixed-type is well exemplified by amino acid systems other than for glycine in red cells (WHEELER et al., 1965; WHEELER and CHRISTENSEN, 1967) and amino acid systems generally in the Ehrlich ascites tumor cells (INUI and CHRISTENSEN, 1966; EDDY et al., 1967).

At the present time, a discussion of kinetics along the lines developed above must be, for the most part, limited to Na^+-coupled systems; experimental studies on H^+-coupled systems with respect to the discrimination of $[H^+]$ effects on affinity or velocity have been too few to permit a generalization, although MITCHELL (1963, 1967) has vigorously predicted the occurrence only of affinity type systems for both H^+ and Na^+.

It would seem that studies with H^+-coupled systems may be difficult to do in ways that can be analyzed clearly. $[H^+]$ may, like $[Na^+]$ in some systems, produce substrate affinity effects by interaction with its specific carrier binding site but in addition $[H^+]$ may also produce such effects via dissociable groups within the substrate binding site as well as on the concentration of the binding form of dissociable substrates. $[H^+]$ may, like $[Na^+]$ in some systems, also produce velocity effects but these may be due to a change in dissociation of groups located non-specifically elsewhere on the carrier as well as of interaction of H^+ at a specific carrier binding site.

In spite of these inherent difficulties, KOMOR and TANNER (1974b) have studied pH effects on the proton-coupled hexose transport system in chlorella (KOMOR and TANNER, 1974a). Their analysis indicates that it is an affinity-type system.

SEASTON et al. (1976) have assessed proton-coupled glycine transport in yeast as primarily a velocity-type system.

B. Inner Leaks:

Since affinity-type systems have been identified by the ability of substrate at high concentrations to translocate without Na^+, they all have an inner leak of substrate mediated by CS, the dimensions of which depend upon $[Na^+]_o$. The occurrence of inner leaks of Na^+ mediated by CNa^+ is not so clearly demonstrated. In the glycine system studied by VIDAVER and SHEPHERD (1968) it was demonstrated that CNa^2 did not translocate. In the chlorella hexose system (KOMOR and TANNER, 1974b) CH did not appear to translocate. There is thus no inner leak for X^+ in these systems. However, in the rat intestinal glucose system studied by LYON and CRANE (1966), effects of $[S]_o$ on the K_m for Na^+ were found as measured by transmural potential changes; the apparent K_m for Na^+ was in the range 0–3 mEq for the reaction $CS+Na \rightarrow CSNa$ and in the range 190–232 for the reaction $C+Na \rightarrow CNa$. These findings suggest that in the rat there is no required order of addition of S or Na^+, that CNa can translocate and that there is an inner leak of Na^+. Assuming reliability for these data obtained by potential measurements, it may be concluded that the presence or absence of an inner leak for Na^+ in affinity-type systems is not predictable and must be established by experiment. The same is probably also true for other type systems.

C. Coupling Coefficients

As discussed above an experimentally determined coupling ratio will reflect the kinetics of the system and the experimental conditions. Figure 20 clearly illustrates

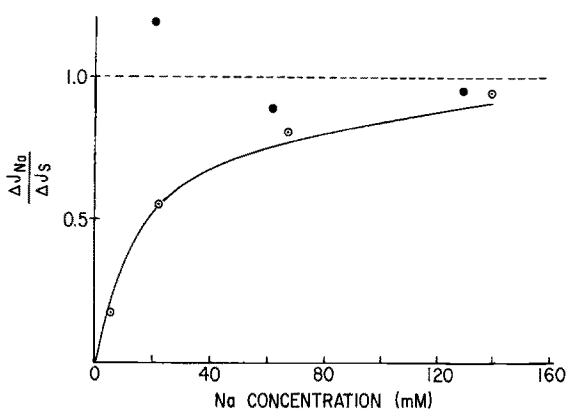

Fig. 20. Ratio of substrate-dependent Na^+ influx to substrate influx as a function of $[Na]_o$. Open circles, L-alanine; closed circles, 3-0-methyl-D-glucose. Points are experimental values. Solid curve was constructed by calculation from the equation $[Na^+]_o/K_2+[Na^+]_o$ using $K_2=17$ mM. Alanine experiments from CURRAN et al. (1967). 3-0-methyl-D-glucose experiments from GOLDNER et al. (1969)

this point. With the same tissue; rabbit ileum, CURRAN's group studied a velocity-type system; 3-O-methyl-D-glucose uptake, and an affinity-type system; alanine uptake. In this particular velocity system, CS and CNa do not translocate and there is no preferred order of addition of S or Na^+; the system has no inner leaks. In this particular affinity system, on the other hand, S adds first and the CS complex can translocate, creating an inner leak which is reduced as $[Na^+]_o$ is increased. As shown in Figure 20, the coupling coefficient of the velocity system is always 1; the coupling coefficient of the affinity system varies between zero when $[Na^+]_o=0$ and 1 when $[Na^+]_o=\infty$. These two situations are the extremes between which all systems would be expected to fall, corrected only for whether more than one ion is bound to the carrier as in the pigeon red cell or yeast glycine systems.

D. Outer Leaks

The data from which Figure 20 was constructed were corrected for outer leaks of Na^+ as measured by substrate-independent Na^+ flux. In the alanine system, the outer leak of Na^+ was 7 times the flux of Na^+ on the alanine carrier at 37° and 38 times at 23°. It is a large correction. A similarly large correction for the outer leaks of Na^+ in studies of AIB uptake by Ehrlich ascites tumor cells has been found to be necessary (HEINZ and GECK, 1974).

Outer leaks caused by Na^+-dependent substrate entry by means of more than one transport system have been shown for glycine in rabbit reticulocytes (WHEELER and CHRISTENSEN, 1967) and in pigeon red cells (EAVENSON and CHRISTENSEN, 1967). In fact, such Na^+-dependent outer leaks for amino acids appear to occur with great frequency (CHRISTENSEN, 1969).

Na^+-independent outer leaks for amino acids have been identified as occurring by exchange diffusion (JACQUEZ, 1973). For some amino acids, e.g. phenylalanine, cycloleucine, and methionine, the exchange diffusion flux appears to be additive to the Na^+-dependent flux and this would be important to correct for. An exchange-diffusion flux also occurs for AIB, but the K_m is high and this flux is not additive with the gradient-coupled flux.

The problem of outer sugar leaks for gradient-coupled sugar transport would not appear to be severe in intestinal and kidney brush border membrane (CRANE, 1960; HONEGGER and SEMENZA, 1973; ULLRICH, 1976) though in bacteria it may well be (HAROLD, 1972).

E. Trans Effects

Trans effects are a characteristic of carrier–mediated transport which complicate transport kinetics in ways that are not obvious from the simple analogy with enzyme kinetics that is usually assumed. They are caused by the presence of S, X^+ or a modifier on the *trans* side of the membrane during a kinetic measure-

ment. SCHULTZ and CURRAN (1970) point out that the only condition that insures the absence of *trans* effects is the very special case where all species of the carrier translocate and all coefficients of translocation are equal. In any other case which is probably all real cases, exact kinetic measurements can only be made in a completely defined system under true initial rate conditions; that is, at zero translocation. What this means in a practical way is 1. that corrections for the progress of the reaction which may be applicable to enzyme kinetics (JOHNSTON and DIVEN, 1969; LEE and WILSON, 1971) are not applicable to transport and 2. that the transport worker must be satisfied for the time being with inexact kinetics.

To illustrate, some *trans* effects are easily understood by reference to Figure 19. In a case where CS and CX do not translocate, the presence of X^+ or S in the transcompartment will sequester the carrier on the *trans* side of the membrane in an inactive form at least until the *trans* concentrations of entering S and X^+ rise to saturating levels for the formation of CSX. In a case where only one of these, CX or CS, does not translocate, a preferential efflux-driven cycling of the carrier by X^+ or S may be predicted in addition to a sequestration effect. Where both translocate but with different mobilities, cycling of the carrier and thus entry rate will be different depending upon the *trans* species and their concentrations.

Such *trans* effects are predicted by the kinetic analyses provided in the reviews referenced earlier. A recent, excellent paper demonstrating *trans* effects on one-way fluxes of AIB in Ehrlich ascites tumor cells (JACQUEZ, 1975) may be consulted for review of the problem and further references.

F. The Role of Potassium

K^+ plays diverse roles in gradient-coupled transport. As is well known, the high intracellular concentration of K^+ supports the membrane potential and part of the driving force for gradient-coupled transport. However, K^+ has other, more direct interactions in some systems and these are clearly not identical (SCHAFER, 1971). K^+ interacts directly with the amino acid carrier in Ehrlich ascites tumor cells (EDDY and HOGG, 1969) and the sugar carrier in hamster small intestine (CRANE, 1965). There are no important effects of K^+ for amino acid transport in rabbit ileum, pigeon red cells, or rabbit reticulocytes (SCHAFER, 1971).

In the Ehrlich ascites tumor cell, external K^+ competitively inhibits the action of Na^+ (EDDY and HOGG, 1969) and undoubtedly forms a CK complex. The efflux of intracellular K^+ seen during glycine uptake (EDDY et al., 1967) is thus assumed to be mediated by the gradient-coupled glycine carrier. However, the affinity of the CK complex for glycine is very low and the rate of translocation of CKGly is negligible (EDDY et al., 1967). Thus, in the Ehrlich ascites tumor cell, a circuit may be set up in which both Na^+ influx and K^+ efflux may contribute to the diffusion forces supporting substrate transport (EDDY, 1968a; EDDY and HOGG, 1969). The situation has been said to be similar in yeast cells operating with a proton-driven carrier (EDDY et al., 1970a, 1970b).

In hamster small intestine external K^+ also competitively inhibits the action of Na^+ (CRANE et al., 1965).

However, there is no evidence for an efflux of K^+ during sugar uptake. Consequently, it was assumed that "intracellular K^+ would interfere with Na^+ interaction for outward movement of the carrier", though not itself efflux, and contribute to the total gradient to which the carrier could respond (CRANE, 1965). SCHAFER (1971) and MORVILLE et al. (1973) have disputed the possibility of K^+ involvement in the energy gradient without translocation. However, it seems probable that this negative view is based upon the particular assumptions contained in the model systems considered. What I had in mind was based upon observations in a real system; the affinity-type sugar transport system of hamster small intestine.

In order to sustain a simple perception of what may be expected to happen in an affinity-type system operating across a membrane without regard to any other influences, it is best to illustrate by combining two figures used earlier (CRANE, 1965) as is done in Figure 21. In Figure 21 the anticipated effects of [S] and [Na] are plotted in the reciprocal Lineweaver-Burk form. Any horizontal line represents a state of equal velocities. The condition for the final state of a gradient-coupled system is that influx and efflux are equal and if, for simplicity, influx and efflux are considered to be mediated only by CSX, the equality holds for both substrate and ion. Hence, the point where the line, $[Na]_o$, intersects a given velocity (the horizontal line) represents the inward velocity with a given external substrate concentration, $[S]_o$. Where the line $[Na]_i$ intersects the same velocity, the internal substrate concentrations, $[S]_i$, for the final state is established. The line, $[Na]_i + K^+_i$ represents the effect of $[K^+]$ on the relationship 1/v vs. 1/S at a given $[Na^+]$ as was demonstrated in a real system (CRANE et al., 1965; CRANE, 1965). Where this line crosses the line of equal velocity, a demand is

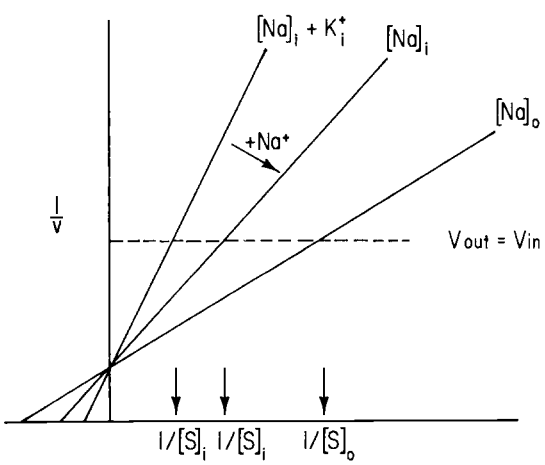

Fig. 21. Predicted effect of $[K^+]_i$ on substrate accumulation in hamster small intestine plotted in the Lineweaver-Burke transformation of the MICHAELIS-MENTEN expression for enzyme kinetics. For description see text

seen for a higher $[S]_i$ in the final state. Alternatively, the lower $[S]_i$ could be achieved by raising $[Na]_i$ which would shift the line $[Na]_i + K^+$ toward and finally to the line $[Na]_i$.

From this diagram, it is evident that high $[K]_i$ can have two effects in an affinity-type system; namely (1) the final state will be achieved at a higher $[S]_i$ and/or (2) the final state will be achieved at a higher $[Na^+]_i$ than if K^+ were not present internally. In either case comparison of the energy available from the Na^+-gradient with the energy needed by the substrate gradient will show a requirement for a greater energy transfer from the Na^+ gradient than is actually the case. This extra energy is provided by the fact that $[K^+]$ is asymmetric across the membrane; that is by the $[K^+]$ gradient.

In a velocity-type system, $[K]_i$ cannot have the same effect. However, it need not be assumed to be without effect. As the Widdas model is formulated, sequestration of carrier at the inner surface membrane in an inactive form would reduce the total available carrier for mediation of the coupled fluxes of S and X^+ but it would not change the energetics of the system. However, if one assumes a transport model in which K^+ sequestration at the inner surface leads to a decrease in maximal efflux velocity but not of influx velocity, effects of K^+_i are readily predictable. In such a case, assuming $[K]_i$ to be maintained at a high value and $[X]_i$ to be stabilized at a low value by the continued operation of the X^+-pump, the disproportionation of flux would be continuously maintained and would support an accumulation of substrate in addition to that provided by the X^+ gradient and the membrane potential. Assuming rough equalities of ion affinity for the carrier, the permissible limits of $[H^+]$, about 10^{-4}–10^{-8} M, as compared to those of $[K^+]_i$, about 10^{-1} M, makes it seem possible that such an effect might be particularly pronounced in systems utilizing proton gradients. In the proton-driven yeast glycine system (SEASTON et al., 1976) glycine efflux is strikingly low as compared to influx and when intracellular conditions are approximated in the suspending medium; i.e. high [K], influx is inhibited.

In the velocity-type situation as in the affinity-type situation, energy is required to maintain a dislocation of the transport system from its predicted equilibrium position with $[X]_i$. The dislocation is asymmetric because of the asymmetry of $[K^+]$. Thus, it seems appropriate to speak of and consider the response as being to the K^+ gradient even though K^+ does not propel the carrier.

In a recent paper, ALVARADO (1976) has concluded that in the guinea pig, affinity effects such as described above are not sufficient to account for the effects of intracellular K^+ on sugar transport and had proposed an additional effect of K^+ which he has called the "capacity" effect. It is not clear that ALVARADO'S capacity effect is different from the second of the above two proposed effects of the affinity of K^+ for the carrier. However, if it is different the fact that K^+ effects are not ubiquitous (see above) would suggest that any additional K^+ effect is a species-related complexity of gradient-coupled transport in the guinea pig which would not require a serious revision of the general gradient hypothesis as ALVARADO has proposed.

VII. The Energy Available vs. the Energy Needed

Studies discussed above under the basic Model (e.g. Table 2 and Figure 15) indicated that substantial proportions of the energy needed to support substrate accumulation are obtained by coupling with the transmembrane electrochemical potential gradient. However, along the way, other studies (e.g. JACQUEZ and SCHAFER, 1969) suggested the possibility that under some circumstances not all of the energy was obtained from the gradient and that the non-gradient energy might be substantial. This question has been actively and directly pursued in a few laboratories. As successive experimental revisions have been made, correspondence between the gradient energy available and the energy needed has been more closely approximated.

A. The Intestinal Sugar System

ARMSTRONG et al. (1973) have compared the transmembrane electrochemical potential gradient with galactose accumulation across the brush border membrane of bullfrog small intestine. In the steady-state after 2 h of incubation, external ion activities were measured as was the membrane potential. Internal ion activities were assumed to be the same as in a previous study (LEE and ARMSTRONG, 1972). Galactose concentrations were measured and galactose was assumed not to be appreciably metabolized. Based on these measurements, the work performed, $\Delta\mu$gal, was estimated to be 3400 joules/mole galactose. The energy available from the Na^+-gradient and the membrane potential together, $\Delta\bar{\mu}$Na, was estimated to be 5800 joules/equiv. Na^+. An additional, approximately equal amount was found in the K^+ gradient after correction for the membrane potential.

There seems to be more than enough energy available and this correspondence is, on the face of it, a satisfactory result in terms of the gradient hypothesis. However, that is true only if it is assumed that galactose is indeed poorly metabolized by bullfrog intestine and that the conditions employed measure the contribution only of the carrier form, CSX, to the transmembrane distributions of Na^+ and galactose. Unfortunately, neither the fate of galactose nor the kinetics of the gradient-coupled sugar system in this tissue have been examined.

B. The Ascites Tumor Cell AIB System

HEINZ et al. (1975) have recently reviewed the extensive and painstaking work that has gone into the determination of the electrochemical potential driving forces available in Ehrlich ascites tumor cells and have concluded that "a complete solution of the problem of energy supply for amino acid transport in these cells in terms of the gradient hypothesis seems to be in reach".

HEINZ et al. (1975) reviewed the major arguments against the gradient hypothesis which were raised by early work:

1. The electrochemical potential gradient appeared to be grossly inadequate to account for the amino acid accumulation observed (JACQUEZ and SCHAFER, 1969).

2. Metabolizing cells accumulate glycine about 3 times more effectively than inhibited cells at the same ion distribution (EDDY, 1968) and this effect of metabolism might indicate the direct participation of ATP (see also JOHNSTONE, 1974).

3. When the transmembrane ion gradients are experimentally abolished or inverted within certain limits, active uptake of amino acids nonetheless takes place apparently against an opposing driving force (SCHAFER and HEINZ, 1971).

HEINZ et al. then go on to describe how more recent work and insight has refuted or weakened the assumed meaning of these findings:

1. PIETRZYK and HEINZ (1974) found that $[Na^+]_i$ had to be greatly corrected for sequestration in the nucleus which in Ehrlich cells seems to be in direct communication with the outside. When so corrected the revised data led to calculations for the driving force in the Na^+ gradient "approximately adequate to drive the observed accumulation of 2-aminoisobutyrate".

2. HEINZ and GECK (1974) found that previous calculations of coupling efficiency between transport of AIB and Na^+ entry when corrected for the substantial outer leaks of Na^+ gave values suggesting an adequate transfer of energy between the ion and substrate gradients.

3. GECK et al. (1974) have shown that the effects of metabolism are not mediated by direct interaction of ATP with AIB transport in Ehrlich cells.

4. Studies with tetraphenylphosphonium, which distributes across the membrane in response to the membrane potential, strongly suggest that the Na^+ pump in the ascites cell is K^+-activated and electrogenic. Reasoning from this, HEINZ et al. suggest that transport observed earlier to take place against the prevailing ion gradients was driven by the membrane potential. In the earlier work, $\Delta\Psi$ was assumed to be reduced by the conditions imposed. In the revised work, $\Delta\Psi$ would now be expected to have been increased by those same conditions. The effects of metabolism which formerly suggested the participation of ATP may now be explained by effects of the membrane potential.

C. Bacterial Membrane Vesicle Systems

Resolution of questions of the adequacy of the gradient in bacterial systems as reviewed by BOOS (1974) and SIMONI and POSTMA (1975), for example, seem now to be within reach on the basis of recent experiments by KABACK and his colleagues (RAMOS et al., 1976) with *E. coli* membrane vesicles. Some of the background for these studies is reviewed in the RAMOS et al. paper and other contributions may be found in HAROLD (1974). The questions have been 1. whether the transmembrane electrochemical potential gradient, $\Delta\bar{\mu}H^+$, provides enough driving force for observed accumulation and 2. what is the distribution of this driving force between the membrane potential, $\Delta\Psi$ and the chemical gradient, ΔpH.

Using in part the well-known filtration method and in part an adaptation of the flow dialysis method of COLOWICK and WOMACK (1969), ΔpH was measured

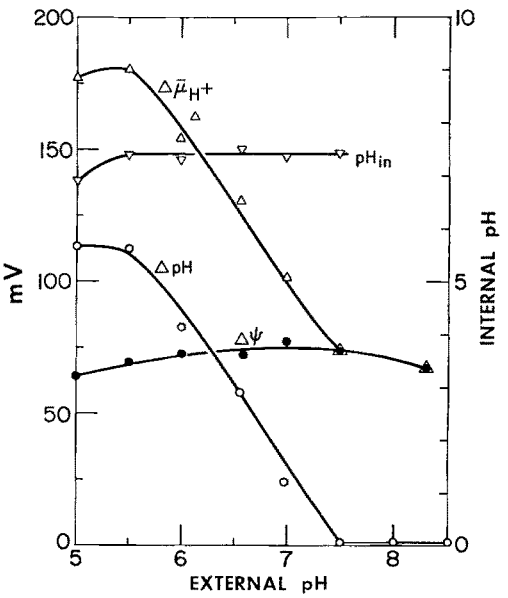

Fig. 22. Electrochemical potential gradient, $\Delta\bar{\mu}H^+$, in *E. coli* membrane vesicles and its distribution between electrical forces, $\Delta\Psi$, and chemical forces, ΔpH, as a function of external pH. (From RAMOS et al., 1976)

by the distribution of weak acids such as acetate, and $\Delta\Psi$ was measured by the distribution of the lipophilic cation, triphenylmethyl phosphonium ($TPMP^+$). Various electron donors were used as energy sources. Figure 22 shows the changes found in the total driving force and its distribution between chemical and electrical components over the pH range 5–8.5 in the presence of ascorbate and phenazine methosulfate. When other electron donors were screened, their ability to drive transport was found to correlate with their ability to generate $\Delta\bar{\mu}H^+$.

Titration of the system with graded concentrations of the K^+-ionophore, valinomycin, decreased $\Delta\Psi$ strongly but increased ΔpH so that the loss in total driving force was only about 20%. Using nigericin, ΔpH was the more severely affected and the total driving force dropped about 45%. Using $TPMP^+$, both components were reduced and the total driving force was reduced to 40% of the control value. The expected difference between pH 5.5 and 7.5 were found.

This system provides the possibility to discriminate transport systems according to their driving forces. Applying it in this way, RAMOS et al. report that vesicular transport systems fall into three groups; those that are driven by $\Delta\bar{\mu}H^+$, those that are driven primarily by $\Delta\Psi$ and those that are driven primarily by ΔpH. What is made clear by this important work is that the gradient is, overall, adequate as a driving force but it cannot be reliably assessed unless the distribution of the driving force in a particular experiment is known and the responsiveness of a particular system to the components of the driving force can be demonstrated.

VIII. The Coupled Carrier as an Entry Mechanism: the "Valve" Effects

Although the energy-utilizing characteristics of gradient-coupled carriers in support of active transport has tended to capture the greatest attention (MITCHELL, 1973) the "valve" effects of this kind of system could turn out to be just as important.

A. Substrate Entry in the Absence of an Ion Gradient

The signal difference between covalently energized and non-covalently energized transport systems is the mechanistic demand for energy expenditure in the former and the lack of it in the latter. The transmembrane portal of a covalently energized system such as the bacterial phosphotransferase system (ROSEMAN, 1972) is not significantly open in the absence of the chemical reaction of phosphate transfer from phosphopyruvate to sugar. The covalently energized ion pumps cannot maintain the ion gradients required for cell maintenance without the expenditure of ATP. The gradient-coupled systems, however, provide a portal for entry of substrate which can be just as rapid in the absence of a metabolically sustained gradient as in its presence. As I have pointed out previously (CRANE, 1965) "the rate of inward movement of substrate is not directly controlled by the asymmetry of the system and the cell is able to obtain essential nutrient at an adequate rate irrespective of the state of its available energy supplies". Also the special characteristics of affinity systems can be energy-conservative under certain conditions. When X^+ enters with the substrate in an energy-replete cell, it will be pumped out with the expenditure of metabolic energy to maintain the intracellular environment as described above. This always happens in a velocity system. However, in an affinity system, substrate can enter without X^+ when $[S]_o$ is high and $[X]_o$ is low as was demonstrated in Figure 20. In this case, substrate entry does not lead to energy expenditure. Thus, an affinity gradient-coupled type system, given the right conditions, is energy-conservative as compared to both a velocity system and a covalently energized system. Take the case of the kidney and the intestine. In the kidney, reabsorption of sugars against a concentration gradient is usually considered to represent the essential work of the epithelial cells; the sugar gradient may always be uphill into the blood. However, this need not be the case; a faster reabsorption of salt and water from the tubule would make the sugar gradient downhill into the blood stream and an affinity system, as is found (see above) could be energy-conservative. The situation is perhaps even clearer in the intestine. Absorption against a concentration gradient is required to be performed only during the later stages of the digestive-absorptive process; downhill entry probably accounts for most of the passage of the digestive products of a meal from the lumen of the gut into the blood stream (CRANE, 1975; CRANE, 1976). For this reason, an affinity system with its varying stoichiometry and its ability to transport sugar downhill

without Na^+ would be more advantageous in the jejunum whereas the ileum, which is required to take up the last bits, might profit better from a velocity system. Hamster jejunum has an affinity system; rabbit ileum has has a velocity system. In other cells, animal cells as well as microorganisms, the energy transduction properties of a gradient-coupled carrier would be expected to be of great value only when external substrate is in very short supply and/or low in concentration. (Contrast here *E. coli* living in the animal gut to a similar organism in the open sea). From a biochemists point of view, there would be little value in energy expenditure to maintain an intracellular concentration of substrate above a reasonably saturating concentration for the first enzyme in the metabolic pathway that utilizes it, unless the energy is expended for collection and storage.

B. The "Valve" Effects

1. Effects of External Ions

Sometime ago, (CRANE, 1967), I discussed briefly what may be called the "valve" effects of the gradient-coupled carriers. Although "valve" effects may be predicted by making certain assumptions in the kinetic equations, they are not necessarily obvious. The "valve" effect of $[X^+]_o$ is apparent in the substrate entry process. In the absence of external X^+ or when its concentration is very low, the inward rate of substrate flux is low. At high $[X^+]_o$, the inward rate of substrate flux is high, irrespective of whether the system is an affinity-type, a velocity-type, or mixed. $[X^+]_o$ acts as though it turns on a valve (see Fig. 23).

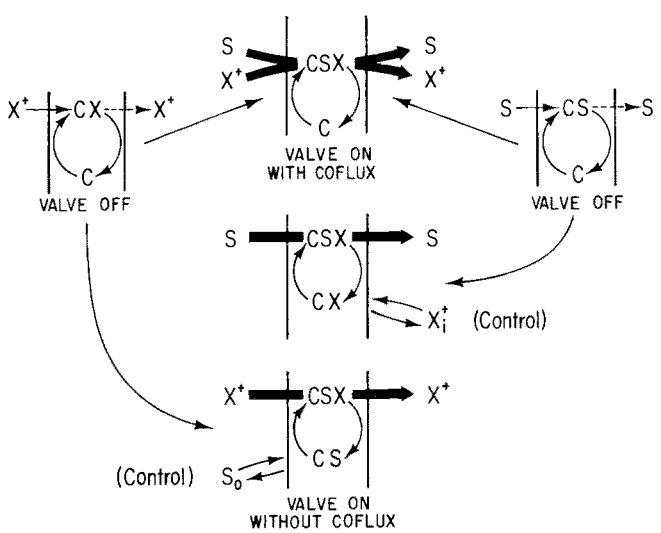

Fig. 23. "Valve" effects predicted for a gradient-coupled carrier system. For description see text

2. Effects of Internal Ions

It is also possible, or so it has seemed to me for a long time without experimental evidence, that $[X^+]_i$ may also act to turn on the valve for substrate entry. To do this would only require that the ion binding site of the two-sited carrier be exposed continuously to the internal space without translocating or if translocating that the attached ion not debind. The carrier would then no longer be gradient-coupled but it would be activated or deactivated by changes in $[X^+]_i$. This effect seems not to have been seriously looked for over the years. However, invoking it would raise the possibility of making the internal ion effects, which appear to regulate glucose entry into a variety of cells (ELBRINK and BIHLER, 1975; CLAUSEN et al., 1975) and perhaps also the effects of other cell alterations (KLETZIEN and PERDUE, 1974a, b) a good deal less mysterious (see Fig. 23).

3. Effects of Substrate

Looking at the "valve" effects the other way around is also interesting. Substrate enhances X^+ entry just as X^+ enhances substrate entry. Inasmuch as the entry of X^+ is electrogenic, the "valve" effect of substrate is to induce an inward electric current along with the influx of X^+. In fact, I suggested (CRANE, 1967) that this "valve" effect of substrate might be the basis of the action of chemical neurotransmitters. Whatever value this particular suggestion may or may not have, the "valve" effect of substrate has now been clearly demonstrated by TOKUDA and KABACK (Personal communication) in a way that would make it worthwhile to scrutinize systems that are activated by an electrogenic ion flux to determine the basis for it in this context.

To understand fully the important contribution which TOKUDA and KABACK have made, it is worth considering briefly the assumed effect of non-penetrating inhibitors on carrier systems. Non-penetrating inhibitors are compounds which are substrate competitive and which do not appear to translocate. The reason they do not translocate, however, is not always the same. As a Widdas model carrier is formulated, interaction of substrate with carrier should lead to translocation unless steric or additional non-specific binding characteristics of the interacting compound can be identified (CASPARY et al., 1969). WILBRANDT and ROSENBERG (1961) had foreseen the possibility that a non-penetrating inhibitor might actually penetrate but debind so slightly that it was left to circulate with the carrier, inhibiting its interaction with substrate, but without perceptible entry. This possibility was specifically ruled out in the case of the non-penetrating inhibitor, phlorizin, by ALVARADO's (1967) analysis of the binding characteristics of phlorizin as compared to phloretin. Phlorizin not only binds to the carrier sugar site it also binds to a second, non-carrier site and thereby immobilizes the carrier. The non-penetrating substrate-competitive inhibitor, L-fucose, is different (CASPARY et al., 1969; CRANE and CASPARY, 1971). L-fucose also binds to the carrier and also stops transport but additional binding interactions like those of phlorizin cannot be predicted from the structure of L-fucose. The carrier-L-fucose-Na^+ complex should theoretically translocate. In view of this, we consid-

ered WILBRANDT and ROSENBERG'S proposal and looked for a "valve" effect of L-fucose binding on Na^+ flux. None was found. It seemed clear that L-fucose prevented translocation of the carrier but it also seemed clear that it does not immobilize it by additional binding interactions. We were thus led to propose that there must be a "second-step" in translocation; that is, a step in addition to substrate and Na^+ binding which does not appear in the simple formulations of a Widdas model carrier. This second step could, we thought, be the conformational changes presumed to underly binding site reversal.

The finding by TOKUDA and KABACK is that a non-penetrating inhibitor of the Na^+-dependent melibiose system in *Salmonella typhimurium* (STOCK and ROSEMAN, 1971) does, in fact, act to turn on the "valve" for Na^+ flux. This finding confirms ROSENBERG and WILBRANDT'S early insight and supports our conclusions about the "second-step". However, much more importantly, it generalizes an experimental basis on which the existence of "valve" effects can be discriminated. The experiments of TOKUDA and KABACK can be summarized as follows: 1. p-nitrophenyl α-galactoside (PNPG) increased influx of Na^+ into membrane vesicles without itself being taken up as measured by radioactivity. 2. When the same experiment was done with vesicles preloaded with cold substrate, radioactive PNPG was taken up into the vesicle indicating that PNPG attached to the substrate binding site and translocated along with Na^+ on the Na^+ site but required help to debind at a significant rate (see Fig. 23).

As a guide to experimentation in other systems and other cells, it is worth emphasizing: Substrate may act to turn on the valve for Na^+ influx and transmembrane current generation *without* itself being seen to translocate. Recalling my proposal for the action of neurotransmitters, it may be recommended that, if not yet done, experiment 2. above be carried out with the acetylcholine receptor (KARLIN, 1974; MICHAELSON and RAFTERY, 1974; MICHAELSON et al., 1974; RANG, 1975). A related experiment testing exit of internal radioactive ion into a medium high in that ion might reveal whether or not internal ions can make a coupled carrier act as a valve for glucose entry as suggested above.

IX. Conclusion

The distinction I have drawn between covalently energized and non-covalently energized transport mechanisms is equally a distinction between those that involve an enzyme and those that do not and between those that may involve vectorially oriented systems without carrier function and those that are clearly based on carrier function. Because of the historical perspective of this review I have lightly treated the Na^+ pump as basically a covalently energized Widdas model carrier system because that is the way it was formulated at the time the development of the Gradient Hypothesis was influenced by it. However, Na^+K^+-ATPase and the Na^+K^+ pump are no longer viewed as functioning in this simple way (GLYNN and KARLISH, 1975). In fact, I cannot bring to mind any example of a covalently energized transport system which is currently regarded as fundamentally a Widdas carrier system. Taking this at face value, it may be useful to sharpen the distinction

between covalently energized transports which are chemiosmotic and non-covalently energized transport which are osmotic. In the broadest sense of meaning, there are only these two kinds.

The term chemiosomotic was originally coined (MITCHELL, 1959) as well as more recently defined (MITCHELL, 1970) specifically to indicate that "a chemical reaction and a translocation reaction are mutually dependent". The concept of chemiosmotic coupling began by "breaking with the tradition that has allowed extensive studies of electrolyte movement complicated by bioelectric potentials to dominate the field of membrane transport" (MITCHELL, 1957) and developed along orthodox biochemical lines by proposing that osmotic work is carried out by covalent energy transduction exactly as in enzyme-catalyzed group transfer reactions. The Gradient Hypothesis, to the contrary, developed strictly out of biophysical tradition and its terminology has been loosely put together by common usage. For example, CHRISTENSEN, as discussed above, proposed that the energy in the K^+ gradient across the membrane could drive amino acid transport in Ehrlich ascites cells and I proposed, as described above, that solute active transport was powered by the Na^+ pump through coupling with the sodium ion circuit. The simple term, Gradient Hypothesis, was finally adopted from HEINZ (1974).

These two concepts; the one at the first devoid of influence by electrolyte movements and potentials, the other, from the first, fully dependent upon them, have been brought together over the years because 1. some chemiosmotic processes have been proposed to be driven by the power in the proton ion circuit (MITCHELL, 1961d, 1970) and 2. the complex of membrane transports in mitochondrial and microbial membranes seem to some (e.g. HAROLD, 1974; HAMILTON, 1975) to be reasonably, if not entirely, explainable on one or the other basis. However, "chemiosmotic" and "osmotic" still remain concepts. It is from studies in purified and defined reconstituted systems that we may hope to learn how the processes underlying these phenomena actually work.

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