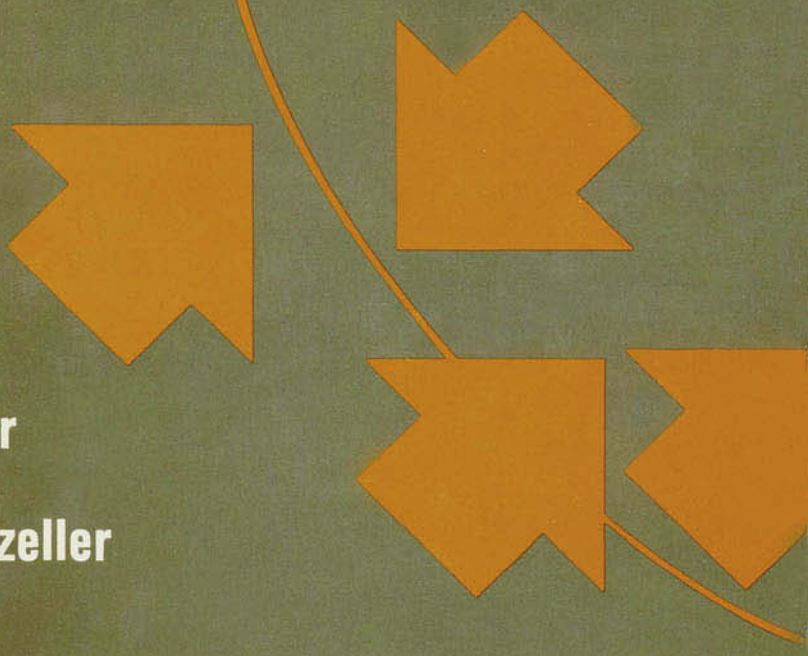
VOLUME 2



Edited by
Felix Bronner
and
Arnost Kleinzeller

current topics in membranes and transport

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Contributors

B. Chance
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Alexander Tzagoloff

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Felix Bronner

Department of Oral Biology
School of Dental Medicine
University of Connecticut
Storrs, Connecticut

and

Arnost Kleinzeller

Graduate Division of Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

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List of Contributors

B. Chance, Department of Biophysics and Physical Biochemistry, Johnson Research Foundation, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Robert E. Forster, Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

W. R. Lieb,* Institute of Life Sciences, The Hebrew University, Jerusalem, Israel

M. Montal,† Department of Biophysics and Physical Biochemistry, Johnson Research Foundation, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

W. D. Stein, Institute of Life Sciences, The Hebrew University, Jerusalem, Israel

Henry Tedeschi, Department of Biological Sciences, State University of New York at Albany, Albany, New York

Alexander Tzagoloff, The Public Health Research Institute of the City of New York, Inc., New York, New York

* Present address: MRC Biophysics Unit, King's College, London, England.

† Present address: Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York.

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Preface

Biological transport of solutes generally involves three major steps: recognition, translocation, and release. Of necessity, recognition must occur at the system boundary, most frequently the membrane boundary of cells or organelles. Translocation through the boundary, release at the other end, and ultimately movement across the entire cell or system complete the transport process, the first and often rate-limiting event in the chain of cellular reaction pathways.

Of the three major steps, recognition has perhaps been most widely studied, as it forms the basis of much current work in molecular structure and function. Of equal interest, but perhaps less readily studied, is the relationship of the recognition site to the remainder of the boundary. Consequently, membranes and their synthetic models have attracted the interest not only of those intrinsically concerned with them, but also of investigators interested in metabolic regulation, biosynthesis, and such physiological processes as transepithelial absorption and secretion, impulse conduction, and muscular contraction. Indeed, few biologists do not claim at least a cursory interest in biological transport. Recent years have, therefore, seen a marked increase in membrane research without, however, a concomitant rise in understanding of the rationale underlying transport processes. Often, indeed, similar experiments have led to contradictory conclusions. Consequently, it seemed appropriate to develop a forum where pertinent work could be discussed critically, assumptions reappraised, and results evaluated. Our colleagues of the Advisory Board of Editors concurred in this view and encouraged our undertaking. We are, therefore, pleased to present the second volume of a series intended to appear approximately annually and to summarize current work in membranes and transport.

Ion transport in organelles, specifically mitochondria, has been an active area of research and it is not surprising that three of this volume's articles deal with one or another aspect of transport in mitochondria. Water transport is a topic with a long and controversial history and we are

pleased to be able to present an authoritative review on this complicated subject. This volume also includes an analytical discussion of diffusion. It is our hope that future volumes will continue to feature theoretical analyses of various aspects of transport.

As editors, we think our primary function is to stimulate thought and experiment. We therefore, do not shun controversy, but hope that the marshaling of evidence in support of a view has also included contrary observations.

We thank the Advisory Board and numerous colleagues for aid, counsel, and help in manuscript review and the publishers for their careful attention to detail.

FELIX BRONNER
ARNOST KLEINZELLER

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The Molecular Basis of Simple Diffusion within Biological Membranes

W. R. LIEB and W. D. STEIN*

Institute of Life Sciences,
The Hebrew University,
Jerusalem, Israel

I. INTRODUCTION

The primary function of the cell membrane is to separate the interior of the cell from its external environment. Thus the membrane is in essence a barrier, and it is this facet of its behavior that is considered here. This aspect has been studied many times before, and one might wonder why it seems necessary to discuss it once again. The reason is that the rapid progress made in the last decade in the analysis of the diffusional process in synthetic polymers has made it possible to understand in some detail

* Present address: MRC Biophysics Unit, King's College, London, England.

the corresponding diffusional process within biological membranes. By considering the membrane as a hydrophobic but polymeric phase, most of the observed properties of this barrier can be accounted for, as far as nonelectrolytes are concerned, in a particularly simple and attractive manner. In particular, it is not necessary to assume that the membrane possesses an additional pathway for the diffusion of small nonelectrolyte molecules, as was necessary previously.

II. DIFFUSION ACROSS SYNTHETIC NONPOROUS POLYMER MEMBRANES

Because biological membranes are composed mainly of high-molecular-weight substances—lipids and proteins—it is reasonable to suppose that diffusion within such membranes might resemble diffusion within membranes of synthetic polymers. The fact that all of the lipid and perhaps much of the protein is largely nonpolar leads one to consider the hydrophobic synthetic polymers as likely analogs of biological membranes. In spite of the strength of this line of reasoning, it is perhaps surprising that the field of diffusion in such nonporous polymers has been almost completely neglected by biologists. We therefore think it worthwhile to review some of the progress made in this field before considering the application of these new concepts to the problem of the permeability of natural membranes.

We consider permeation across synthetic membranes made of such materials as natural rubber and polyisobutylene. Since these membranes can be formed into any desired shape or size and in addition possess defined and homogeneous compositions, they are much more amenable to experimental study and theoretical interpretation than are biological membranes.

A. Experimental Measurements

There are two classic procedures for the determination of diffusion coefficients in synthetic membranes. In the first of these—the permeation method—the rate of movement of permeant across the membrane from one external phase to another is studied. In the second procedure—the sorption–desorption method—the rate of movement of diffusant between a single external phase and the polymer is studied.

We first consider the permeation procedure. In a typical experiment the polymer is first shaped into a sheet of uniform thickness which is then used to form a seal between two compartments. Before the start of the

experiment, both compartments are evacuated. At zero time one compartment is filled to a defined pressure (i.e., concentration) with a given gas or vapor. The rate of appearance of diffusant in the second compartment is then measured as a function of time. In most cases the experiment is so arranged that back-diffusion is negligible. Figure 1 shows the results of a typical permeation experiment. Note that there are two regions of interest—an initial presteady-state period and a subsequent steady-state region, each of which gives somewhat different information about the permeation process.

We discuss the phenomena in terms of parameters and units familiar to the biologist although, as we show later, they differ somewhat from those used by the polymer scientist. In addition, we simplify somewhat by considering only the case in which the diffusion coefficients are independent of the concentrations of the diffusants; this is the usual situation in biological studies. Let us first consider the steady-state region as shown

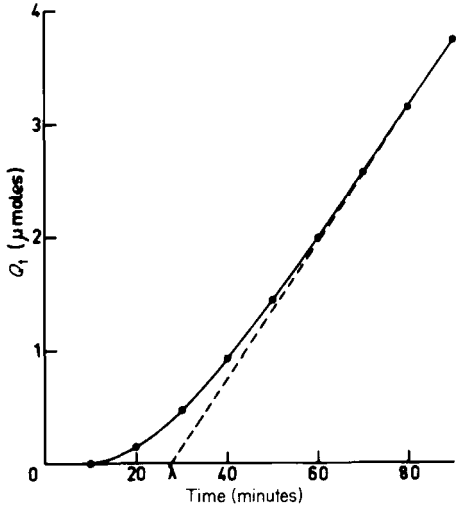

FIG. 1. Typical results for a permeation experiment with synthetic membranes. Abscissa: time (minutes) from beginning of experiment. Ordinate: cumulative amount (micromoles) of permeant having crossed the membrane. The curve was calculated using Eq. (4.25) of Crank (1957) for a membrane of thickness $l = 0.1$ cm and of area 1 cm^2 and for a permeant having a diffusion coefficient within the membrane of $D_{mem} = 10^{-6}$ cm^2 sec^{-1} and an equilibrium distribution coefficient between membrane and external phases of $K = 1$. The permeant concentration was kept constant throughout the experiment at 10^{-4} moles cm^{-3} in the first compartment and at approximately zero in the second compartment. The dashed line is the linear extrapolation of the steady-state region of the curve; the slope of this straight line gives the steady-state flux J, while the intercept on the time axis gives the time lag λ.

in Fig. 1. The slope of the straight line here divided by the membrane area gives directly the steady-state flux J, the amount of diffusant crossing a unit area of membrane per a unit time interval. Since the flux at any point within or on the surface of the membrane must be the same in the steady state, we can express this flux in two different but equivalent ways. The equation most familiar to workers in biological transport is

$$J = P(c_{\text{ext}}^{\text{I}} - c_{\text{ext}}^{\text{II}}) \tag{1}$$

where J is the net flux from compartment I to compartment II, P is the overall membrane permeability coefficient, and $c_{\text{ext}}^{\text{I}}$ and $c_{\text{ext}}^{\text{II}}$ are the concentrations of diffusant molecules in the external compartments I and II, respectively. Within the membrane, however, this same flux can be expressed as

$$J = P_{\text{mem}}(c_{\text{mem}}^{\text{I}} - c_{\text{mem}}^{\text{II}}) \tag{2}$$

where P_{mem} is the permeability coefficient characterizing movement across but within the membrane, and $c_{\text{mem}}^{\text{I}}$ and $c_{\text{mem}}^{\text{II}}$ are the concentrations of diffusant molecules just inside the membrane facing compartments I and II, respectively. Note that if the thickness of the membrane is l and if the diffusion coefficient within the membrane is D_{mem}, then

$$P_{\text{mem}} = \frac{D_{\text{mem}}}{l} \tag{3}$$

It is usually possible to set

$$\frac{c_{\text{mem}}^{\text{I}}}{c_{\text{ext}}^{\text{I}}} = \frac{c_{\text{mem}}^{\text{II}}}{c_{\text{ext}}^{\text{II}}} = K \tag{4}$$

where K is the equilibrium distribution coefficient between membrane and external phases. [In fact, for the lattice model of Zwolinski, Eyring, and Reese (1949), equilibrium exists provided that $mk_{\text{ms}} \gg 2k_{\text{m}}$, where m is the number of lattice sites on a straight line drawn from one face of the membrane to the other, k_{ms} is the rate constant for diffusion out of the membrane across a membrane face, and k_{m} is the rate constant for diffusion within the membrane from one lattice site to an adjacent site (see their Equation 31). For macroscopic synthetic membranes, the large value of m usually assures that this condition is fulfilled. For biological membranes, where m may be only of the order of 10, it is necessary that the rate constant for movement out of the membrane be at least as great as that for movement through the membrane. We see later that this is a reasonable assumption for most molecules used in membrane permeation studies but may not be valid for small hydrophobic diffusants, which in any case move too rapidly to be measured in most permeability studies involving biological membranes.]

Substituting from Eq. (4) into Eq. (2) yields

$$J = KP_{\text{mem}}(c_{\text{ext}}^{\text{I}} - c_{\text{ext}}^{\text{II}}) \tag{5}$$

By comparing Eq. (5) with Eq. (1), we see that

$$P = KP_{\text{mem}} \tag{6}$$

Finally, by using Eqs. (3) and (6) we obtain the diffusion coefficient for movement within the membrane:

$$D_{\text{mem}} = \frac{Pl}{K} \tag{7}$$

Of the terms in Eq. (7), l is determined by direct measurement, P is obtained from a measurement of the steady-state flux J at known external diffusant concentrations using Eq. (1), while K must be determined from a separate experiment in which the distribution of diffusant is determined at different concentrations under equilibrium conditions.

To facilitate the comparison of the experimental results reported for biological and for synthetic membranes, it is worth pointing out how the parameters and units used can be interconverted. The diffusion coefficient D_{mem} in Eq. (7) is generally reported in the same units ($\text{cm}^2\ \text{sec}^{-1}$) in both fields of study. The term K is the membrane/water partition coefficient for biological membranes and is the Ostwald solubility coefficient for synthetic membranes. The experimental data for synthetic membranes are usually presented as Bunsen solubility coefficients, however, which are easily converted into K by multiplying by the factor $T/273$, where T is the absolute temperature of the experiment. Finally, the permeability coefficient P of Eq. (7) is best expressed in the units cm sec^{-1}. In the biological field P is often reported in these units; when not so reported, it can be easily converted to these units by use of Table 2.1 of Stein (1967). In the synthetic polymer field, the situation is more complex. Here the permeability coefficient B (in the nomenclature of Meares, 1965) is, in contrast to the biological situation, normalized to a standard thickness h. B is reported as the number of cubic centimeters of the gas at STP passing each second through 1 cm^2 of polymer of thickness h when the pressure difference across the membrane is 1 cm of mercury. To interconvert P (in cm sec^{-1}) and B, the following formula must be used:

$$P = 76\left(\frac{T}{273}\right)\left(\frac{h}{l}\right)B \tag{8}$$

Note that l is the actual thickness of the membrane under consideration, while h is the standard membrane thickness used by the investigator

concerned to normalize polymer permeability data, l and h being expressed in the same units.

We can avoid having to determine K in order to calculate D_{mem} as in Eq. (7) if we use instead an analysis of the presteady-state region as shown in Fig. 1. If a linear extrapolation of the steady-state line is made back to zero transport, as indicated by the dashed line in Fig. 1, the intercept λ on the time axis is called the *time lag*. It can be shown (Crank, 1957) that the solution of the diffusion equation here yields

$$D_{\text{mem}} = \frac{l^2}{6\lambda} \tag{9}$$

provided the compartment into which diffusion is proceeding is kept at zero concentration. In essence, λ is a measure of the time required to reach a steady-state distribution of diffusant molecules within the membrane and is for this reason independent of K. We see from Eq. (9) that the time to the steady state varies directly as the square of the specimen thickness and so is particularly easy to measure when the membrane can be made to be reasonably thick. Unfortunately, as we show later, the thinness of biological membranes leads to values of λ in the microsecond range, which are impossible to measure by conventional techniques.

The permeation method is generally used for substances that have low distribution coefficients but high diffusion coefficients within the polymer. The simpler sorption–desorption technique is usually used when distribution coefficients are large. Here the amount of diffusant within the polymer is measured as a function of time. In the sorption mode the polymer is initially free of diffusant and at zero time is exposed to a phase containing the diffusant at a concentration maintained constant throughout the remainder of the experiment. In the desorption mode the polymer, initially equilibrated with diffusant to a given concentration, is suddenly exposed to a phase maintained at zero diffusant concentration throughout the experiment. If the diffusion coefficient is independent of concentration, then the half-time $t_{1/2}$ for both modes will be identical and the solution of the diffusion equation for a planar sheet polymer of thickness l is given by (Crank, 1957):

$$D_{\text{mem}} = \frac{l^2}{20.4t_{1/2}} \tag{10}$$

A major advantage of the sorption–desorption technique is that the results are relatively insensitive to the presence of small leaks in the polymer (Crank and Park, 1968). (Although such a leak would greatly affect the permeation technique by providing a very low-resistance parallel

pathway, the only effect on the sorption–desorption technique would be to alter slightly the geometry of the polymer sample.) If we now look at Eqs. (9) and (10), we see that for the same specimen thickness, the times that must be measured experimentally are of the same order of magnitude for both the time-lag permeation method and the sorption–desorption method. Thus the sorption–desorption method is also difficult to apply to biological membranes when using conventional techniques.

B. Experimental Results

1. Size Dependence of Diffusion Coefficients

If the diffusion of spheres is considered, it is always found that the larger the diffusing particle the smaller the diffusion coefficient D. For the case of a sphere of radius r diffusing in a continuous liquid medium of viscosity η, it is well known that

$$D = \frac{kT}{6\pi\eta r} \tag{11}$$

where k is the Boltzmann constant and T is the absolute temperature. This is known as the Stokes–Einstein relationship. If we make the simplifying approximation that the densities of the diffusant molecules are equal and in addition confine our attention to those molecules that do not depart too drastically from the shape of a sphere, the Stokes–Einstein relationship reduces at constant temperature to

$$DM^{1/3} \approx \text{constant} \tag{12}$$

where M is the molecular weight of the diffusant molecule. That Eq. (12) is a reasonable approximation at high molecular weights is shown in Fig. 2 for molecules diffusing in water.

It is also clear from Fig. 2 that for small molecules diffusing in water a mass dependence steeper than that predicted by Eq. (12) is found. In fact, for these smaller molecules a better approximation is

$$DM^{1/2} \approx \text{constant} \tag{13}$$

The reason for this departure from the Stokes–Einstein prediction may well be that for these small molecules the aqueous phase can no longer be considered a continuous medium.

It is often considered that either Eq. (12) or (13) is also obeyed for diffusion in more complex phases, for example, biological membranes. We can easily test this assumption for diffusion in polymeric phases with a

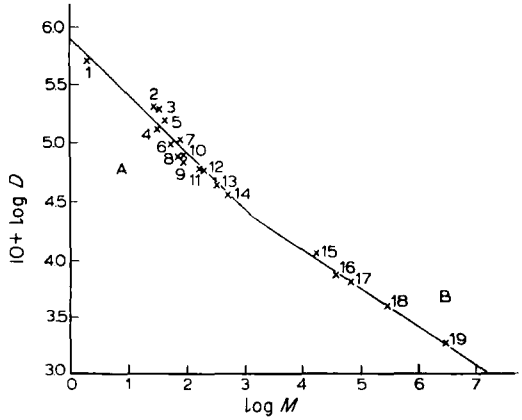

FIG. 2. Diffusion in water as a function of molecular weight. Abscissa: $\log_{10} M$, where M is the molecular weight of the diffusant. Ordinate: $10 + \log_{10} D$, where D (cm^2 sec^{-1}) is the diffusion coefficient in water at approximately 20°. Diffusing molecules are: 1, hydrogen; 2, nitrogen; 3, oxygen; 4, methanol; 5, carbon dioxide; 6, acetamide; 7, urea; 8, *n*-butanol; 9, *n*-amyl alcohol; 10, glycerol; 11, chloral hydrate; 12, glucose; 13, lactose; 14, raffinose; 15, myoglobin; 16, lactoglobulin; 17, hemoglobin; 18, edestin; 19, erythrocruorin. (A) The relation $DM^{1/2}$ constant; (B) the relation $DM^{1/3}$ constant. Taken from Stein (1962).

plot analogous to that in Fig. 2. We have done this in Fig. 3. Instead of the slopes being in the range $-\frac{1}{3}$ to $-\frac{1}{2}$ as predicted by Eqs. (12) and (13), we find a slope of -3.8 for diffusion in polymethylacrylate and of -1.1 for diffusion in natural rubber. Thus we find that the mass dependence for diffusion in polymers may be much steeper than that found for diffusion in simple liquids. We see later that such steep mass dependencies are also characteristic of diffusion within biological membranes.

2. The Importance of the Shape of the Diffusing Molecule

For the diffusion of both large and small molecules in water, it is always found that departure from a spherical shape results in a reduction in the diffusion coefficient (Tanford, 1961; Soll, 1967). This is thought to be attributable to an increase in the effective hydrodynamic volume of the diffusing molecule as it tumbles in the liquid.

The situation is quite different for diffusion in isotropic polymers. In Table I are listed the diffusion coefficients for a number of hydrocarbons in polyisobutylene. Consider the three isomeric pentanes listed in this table. The most spherical of these, neopentane, has only one-quarter of the value of the diffusion coefficient for the straight-chain isomer *n*-pentane, while the intermediate-shaped isopentane has a diffusion coefficient falling

between these. A similar result can be seen for the butanes. Indeed, this type of result is quite general for diffusion in polymers (Meares, 1965).

It has been shown that for diffusion in natural rubber there exists a quantitative relationship between the magnitude of the diffusion coefficient and the least cross-sectional area of the diffusing molecule (Aitken and Barrer, 1955). Thus it might be predicted that, in an homologous series of straight-chain diffusants, a size will be reached when further increases in chain length will produce no increase in least cross-sectional area, and hence no further decrease in diffusion coefficient. This effect is shown in Table I for the *n*-alkanes; increasing the chain length from five to eight carbon atoms has little effect upon D_{mem} for polyisobutylene.

It is quite clear therefore that departures from the spherical shape have quite different effects for diffusion in isotropic polymers and for diffusion in simple liquids. We see later that for biological membranes there are suggestions that the effect of molecular shape is that found for diffusion in isotropic polymers rather than that found for simple liquids.

3. The Effects of Temperature

The temperature dependence of the diffusion coefficients of large spherical molecules in a continuous liquid medium can be seen directly from the Stokes–Einstein relationship [Eq. (11)] to be determined predominantly by that of the viscosity of the medium. This means that the activation

TABLE I

The Effect of the Shape of a Diffusing Molecule on Diffusion in a Polymer[a]

Diffusant	$D_{\text{mem}} \times 10^9$ (cm^2 sec^{-1})	Reference
Propane	4.81	*b*
n-Butane	3.24	*b*
n-Pentane	2.64	*b*
n-Heptane	3.04	*c*
n-Octane	3.16	*c*
Isobutane	1.45	*b*
Isopentane	1.32	*b*
Neopentane	0.62	*b*

[a] Diffusion coefficients (D_{mem}) for diffusion in polyisobutylene at 35°, extrapolated to zero concentration of diffusant.

[b] Prager and Long (1951).

[c] Blyholder and Prager (1960).

energies for diffusion and for viscous flow should be quite similar in such a situation. This expectation is borne out for large molecules diffusing in water and furthermore holds even for small molecules (Table II).

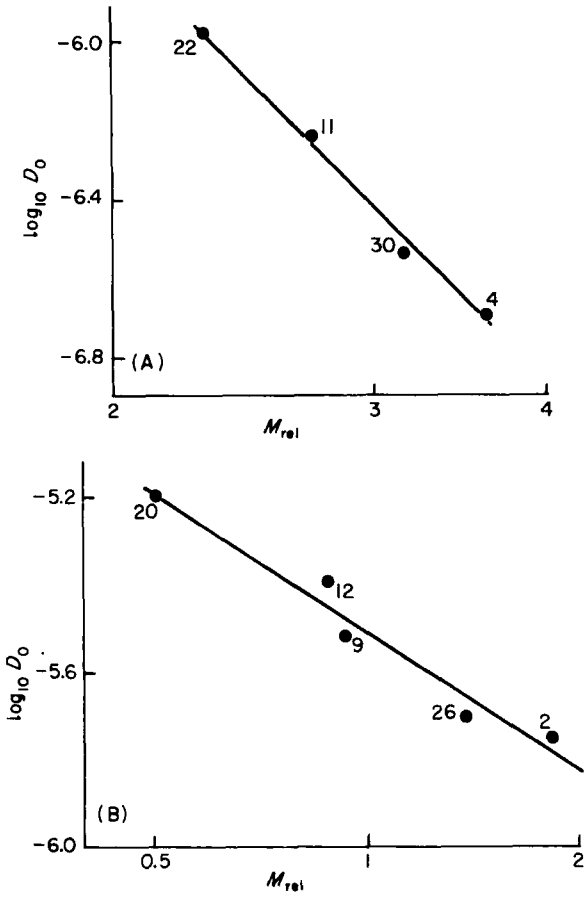

FIG. 3. Diffusion within polymers as a function of molecular weight. D_0 is the diffusion coefficient D_{mem} (cm^2 sec^{-1}) for diffusion within the polymer extrapolated to zero concentration of diffusant. M_{rel} is the molecular weight of the diffusing molecule relative to methanol. The polymers were maintained far above their glass transition temperatures. The straight lines in this and succeeding figures are linear regression lines calculated by the method of least squares (Brownlee, 1960). The polymer in (A) is polymethylacrylate at 65°. The negative of the slope (±S.E.) of the regression line is $s_m = 3.8 \pm 0.2$. The polymer in (B) is slightly cross-linked natural rubber at 80°. The negative of the slope (±S.E.) of the regression line is 1.1 ± 0.1. Diffusing molecules are: 2, butane; 4, butyl acetate; 9, ethane; 11, ethyl acetate; 12, ethylene; 20, methane; 22, methyl acetate; 26, propane; 30, propyl acetate. Where applicable, the *n* isomer is to be understood. Taken from Lieb and Stein (1969).

TABLE II

ACTIVATION ENERGIES FOR DIFFUSION AND VISCOUS FLOW IN WATER

Process	Diffusant	Molecular weight	E_{act} (kcal mole^{-1})	Reference
Diffusion	Water	19–20	4.6	*b*
Diffusion	Urea	60	4.5	*a*
Diffusion	Glycine	75	4.6	*a*
Diffusion	Alanine	131	4.8	*a*
Diffusion	Glucose	180	5.0	*a*
Diffusion	Cyclohepta-amylose	1,370	4.9	*a*
Diffusion	Bovine serum albumin	66,500	5.0	*a*
Viscous flow	—	—	4.2	*c*

[a] Longsworth (1954) at 19°.
[b] Wang *et al.* (1953) for diffusion of HH^2O and HH^3O at 25°.
[c] Computed for 19° from data collected by Dorsey (1929).

In striking contrast are the results for diffusion in polymers. Here the activation energies for diffusion vary dramatically from diffusant to diffusant (Table III). We see from Table III that there is a general increase in the magnitude of the activation energy as the size of the diffusing molecule increases. There is a suggestion that the activation energy reaches a ceiling for large diffusants (Meares, 1965).

In a later section when we consider the effect of temperature upon the permeability of biological membranes, we find that activation energies for permeation are generally high and increase with the size of the permeant. To the extent that this temperature effect is primarily upon the diffusion and not the partitioning process, diffusion in biological membranes thus resembles diffusion in polymers, not simple fluids.

4. THE INFLUENCE OF PLASTICIZERS

If one introduces into a pure polymeric phase sufficient quantities (usually 1% or more) of a soluble low-molecular-weight additive, it is almost always found that diffusion coefficients increase. If this additive is a substance different from the species whose diffusion coefficient is being measured, the additive is termed a *plasticizer*. The diffusant itself can often be considered such an additive, however, and it is therefore not surprising that diffusion coefficients can be increased substantially at high concentrations of diffusants.

TABLE III

ACTIVATION ENERGIES FOR DIFFUSION WITHIN POLYMERS[a]

Diffusant	E_{act} (kcal mole^{-1})	
	Natural rubber	Polyisobutylene
Hydrogen	5.9	7.6
Oxygen	7.5	10.8
Nitrogen	8.7	11.7
Carbon dioxide	8.9	11.7
Methane	8.5	—
Ethane	9.0	—
Propane	9.0	—
n-Butane	10.8	16.7
Isobutane	11.1	17.5
n-Pentane	12.5	16.0
Isopentane	11.3	18.1
Neopentane	12.1	18

[a] From data collected by Meares (1965) in the range 40–60°.

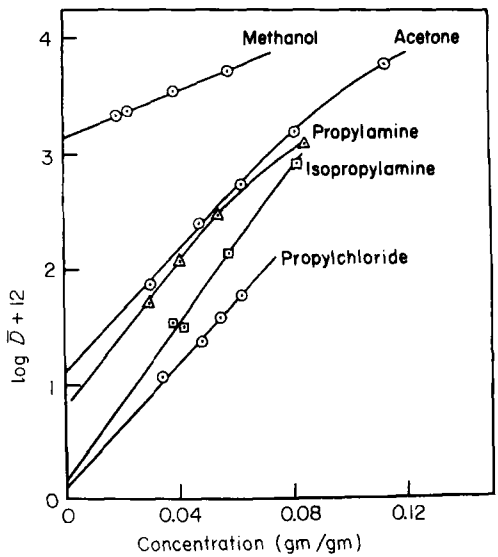

FIG. 4. Concentration dependence of diffusion within a synthetic polymer membrane. Abscissa: concentration (weight fraction) of diffusant in the polymer. Ordinate: $12 + \log_{10} D$, where D is an average value of D_{mem} (cm^2 sec^{-1}) obtained from both modes of a sorption–desorption experiment. The results are for polyvinyl acetate at 40°. Taken with kind permission from Kokes and Long (1953).

Let us consider some examples of the effects of plasticizers. Thus the addition of 2% water to a sample of polyvinyl acetate at 40° resulted in a 25-fold increase in the diffusion coefficient for acetone and in a 75-fold increase for carbon tetrachloride (Long and Thompson, 1954). Again, the addition of 25% tricresyl phosphate or of mixed glycol esters to a polyvinyl chloride–acetate copolymer at 10° resulted in a 2-fold and 40-fold increase, respectively, in the diffusion coefficient for water vapor (Doty, 1946). We suggest in a later section that the action of nonspecific general anesthetics in increasing the permeability of biological membranes to a wide variety of substances may be attributable to their role as plasticizers.

As an example of the "plasticizing" effect of the diffusant itself, Fig. 4 shows some results from a study by Kokes and Long (1953) on the concentration dependence of the diffusion of various organic substances in polyvinyl acetate. This phenomenon is very general in the polymer field for diffusants that are reasonably soluble in the polymer. This effect is usually not seen for permeation across biological membranes, however, presumably because the permeants do not reach plasticizing concentrations within the membranes.

5. The Interplay of Diffusion and Partitioning in the Permeation Process

We have shown that the diffusion process in polymers is governed by a few simple general rules. Unfortunately, such simplicity is not attainable for the overall process of permeation since it, unlike diffusion, is a composite of two independent events. Rewriting Eq. (7) as

$$P = \frac{KD_{\text{mem}}}{l} \tag{14}$$

shows these two events to be (1) partitioning of the diffusant between the external and membrane phases and (2) diffusion across the membrane. Nevertheless, we are forced by practical considerations to take up this more complex study. This is because in any real situation involving the use of membranes it is the overall process of permeation that is of importance. Furthermore, for biological membranes we have data only on permeation, no reliable diffusion coefficients having been reported at the time of writing.

The point we wish to stress in this section is that if one is forced to rely on permeability data alone the underlying simplicity of the rules governing this process can be easily obscured. This can be seen very clearly in Fig. 5 where K, D_{mem}, and P for a homologous series of alkanes and membranes of cross-linked natural rubber have been plotted separately. Whereas the

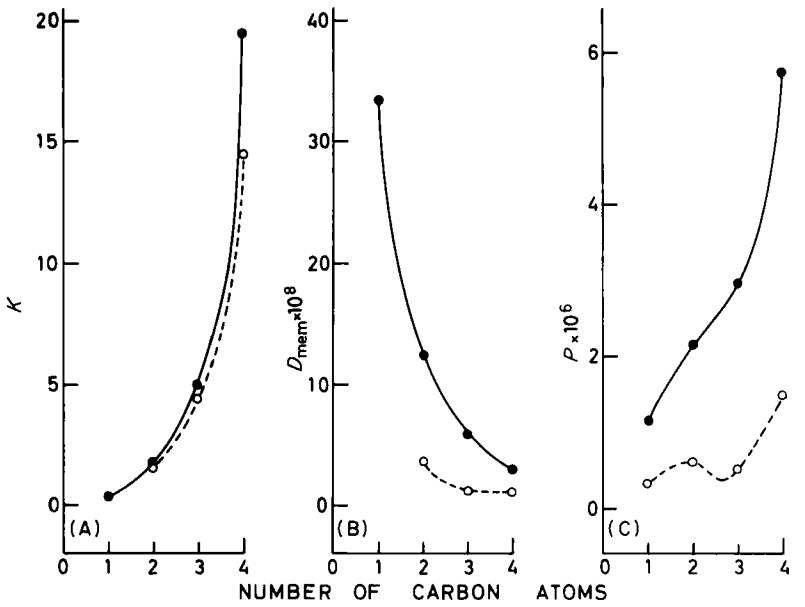

FIG. 5. Distribution, diffusion, and permeability coefficients of an homologous series of *n*-alkanes for membranes of cross-linked natural rubber. Abscissa: number of carbon atoms in the permeant molecules (methane, ethane, propane, and *n*-butane). Ordinate: (A) equilibrium distribution coefficient K between membrane and gas phase; (B) diffusion coefficient D_{mem} ($\times 10^8$, $cm^2\ sec^{-1}$); (C) permeability coefficient P ($\times 10^6$, $cm\ sec^{-1}$). The membranes were of thickness $l = 0.1$ cm and were vulcanized with 7.15% sulfur (solid curves) or with 12.3% sulfur (dashed curves). All results are for 40° and were calculated from the data of Barrer and Skirrow (1948a,b) according to the methods of Section II,A.

curves for K and D_{mem} are, respectively, smoothly increasing and decreasing functions of chain length, the curve for P is seen to be complex. A very similar situation is often found for permeation across biological membranes (Section III,D).

C. The Molecular Basis of Diffusion in Polymers

The results listed in the preceding sections can be explained in a general way by using current theories of diffusion in polymers. Although there is at present no one fully accepted theory in this field (see Kumins and Kwei, 1968), there are certain common underlying concepts that we can use.

It is generally agreed that the random thermal motion of polymer chains results in the formation of transient pockets of free volume or "holes" into

which the diffusant molecule can enter. For simplicity, let us first consider the diffusion of spherical molecules. At a given time one of these molecules is located in one of these holes. In order for it to move out of this hole, there must appear adjacent to it a second hole of the proper size to which the molecule has access. Thus the rate of diffusion is determined by the rate of appearance of holes of the requisite size. This in turn is governed by two factors: (1) the rate of appearance and disappearance of holes, that is, the formation frequency of holes in general and (2) the probability distribution of hole sizes in the polymer. Since the first of these factors should be a property of the polymer itself, being influenced by the temperature and the presence of plasticizers but not by the size of the diffusing molecule, it is the second of these factors that is thought to determine the size selectivity of the diffusion process.

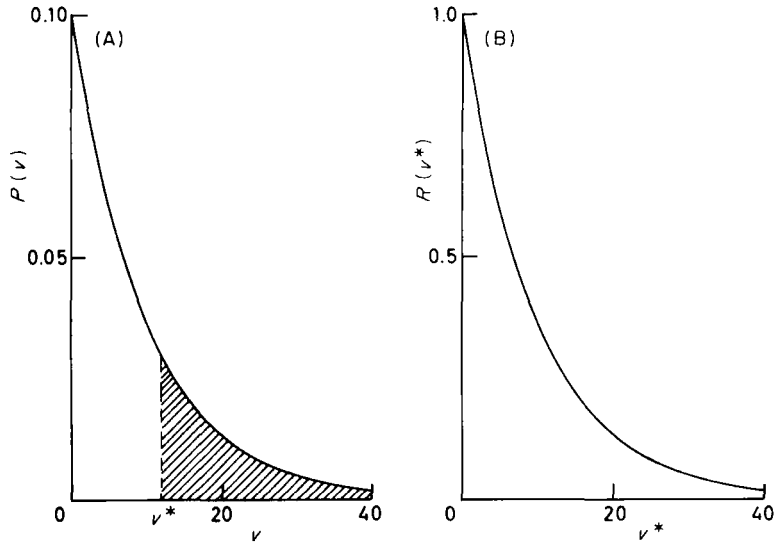

FIG. 6. (A) Probability distribution of hole sizes. Abscissa: volume v (cubic ängströms) of hole. Ordinate: $P(v)$ (Å^{-3}), where $P(v)\,dv$ gives the probability that a given hole will have a volume in the range v to $v + dv$. The curve was calculated from Eq. (12) of Cohen and Turnbull (1959) for the situation in which the average free volume is 10 Å^3 and the geometric factor γ is unity. (B) Probability that a hole adjacent to a given diffusant molecule will be of sufficient size for the diffusant to enter. Abscissa: volume v^* (cubic ängströms) of the smallest hole into which the diffusant can enter. Ordinate: probability $R(v^*)$ that a hole adjacent to the diffusant molecule will be of volume v^* or greater. $R(v^*)$ is simply the ratio of the cross-hatched area to the total area under the curve in (A).

Note that both curves and also the cross-hatched area should extend to infinite values of v and v^*.

From general statistical mechanical considerations, it can be argued (Cohen and Turnbull, 1959) that the probability distribution of hole sizes has the form given in Fig. 6A. (The detailed form of this curve does not affect our present discussion.) We see that there are many more small holes than large holes, but that there is no sharp cutoff. The basis for the shape of Fig. 6A is that it takes much more energy to form a large hole than a small hole. Let us now consider a molecule that requires that a hole of minimum volume v^* be available adjacent to it for diffusion to occur. Then we see from Fig. 6A that the fraction of holes that have the requisite size is given by the ratio of the cross-hatched area to the total area under the curve. This ratio, $R(v^*)$, is a steeply decreasing function of the volume v^* (Fig. 6B), hence of the size of the diffusing molecule. Thus a small molecule has available to it a much larger number of holes of requisite size than does a large molecule. This is the basis for the steep size selectivity of the diffusion process in polymers.

The argument is somewhat more complex for molecules that are not spherical. Consider, for example, a long, cigar-shaped molecule diffusing in an isotropic polymer having the hole size distribution of Fig. 6A. Then it can be seen that it is most unlikely that a hole will be available of a size sufficient to accommodate the long dimension of the molecule, but that many holes able to accommodate the short dimension of the "cigar" will be present. Thus it is the short dimension that will mainly determine the rate of diffusion. This explains the experimental observation that it is the least cross-sectional area of a molecule that seems to be best correlated with the diffusion coefficient.

Let us now consider the effects of temperature. One effect of increased thermal motion is to increase the rate of appearance and disappearance of holes, hence to increase the rate at which holes appear adjacent to a given diffusant molecule. The second effect of a rise in temperature is to increase the total free volume of the polymer (as evidenced by thermal expansion of the polymer as a whole). Again from statistical mechanical considerations (Cohen and Turnbull, 1959), it can be argued that this has the effect of transforming curves I of Fig. 7 (redrawn from Fig. 6) into curves II. From Fig. 7A we see that the size selectivity of the polymer is reduced with increasing temperature. From Fig. 7B we see that the effect of temperature on increasing the number of suitable holes is much greater for large molecules than for small molecules. Thus one can account for the experimental finding (Table III) that there is an increase in the activation energy for diffusion as the size of the diffusant molecule increases. A less rigorous but more intuitive way of looking at this same phenomenon is to recall that it takes much more energy to form a large

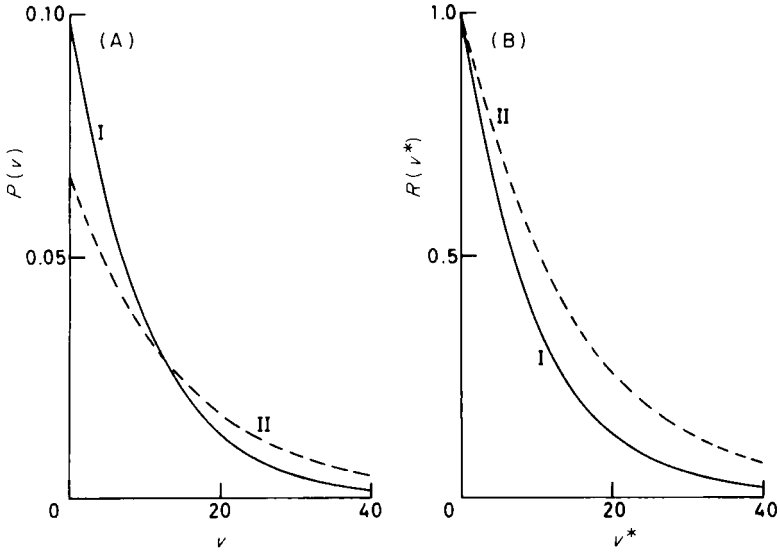

FIG. 7. (A) Effect of temperature upon the probability distribution of hole sizes. (B) Effect of temperature upon the probability that a hole adjacent to a given diffusant molecule will be of size sufficient for the diffusant to enter.

Curves I are identical to those in Fig. 6. Curves II are for a higher temperature, at which the average free volume has increased by 50%. Coordinates are the same as for Fig. 6.

hole than to form a small hole; hence temperature has a greater effect upon the diffusion of large molecules than of small molecules.

We finally come to the molecular basis of the effects of plasticizers. They appear to act by interspersing themselves between polymer chains, thus reducing the opportunities for interchain bonding. This leads to an increase in the mobility of the chains, hence to an increase in diffusion rates.

III. PERMEATION ACROSS BIOLOGICAL MEMBRANES

We would have liked at this stage to present a direct comparison between the data on diffusion in polymer membranes and in natural membranes. Unfortunately, data on biological membranes are not yet available directly in the form of diffusion coefficients but only as permeability coefficients. We have seen previously that the permeability coefficient, being a composite of partition and diffusion parameters [Eq. (14)], is not easy to

interpret (see Section II,B,5 and Fig. 5). Since only diffusion coefficients can be used to explore the molecular basis of movement within biological membranes, we have developed methods for estimating these, as described in Section III,B. It should be pointed out that the two methods that have been used so successfully to obtain diffusion coefficients in polymers directly—the time-lag permeation method and the sorption–desorption method—would require a time resolution in the microsecond range if applied to biological membranes (see Section III,A). It is to be hoped that new methods, perhaps involving the use of nuclear magnetic resonance (NMR), will be developed to overcome these difficulties.

A. Estimation of Diffusion Coefficients for *Chara*

In this section we outline a method for estimating diffusion coefficients within biological membranes from permeability and partition coefficients by using Eq. (7). The method is given in detail in a previous publication (Lieb and Stein, 1969). The problem here is to obtain reliable estimates for the membrane/water partition coefficients. We do this by choosing a particular hydrophobic solvent as a model for the partitioning properties of the membrane and then checking the validity of this choice by a statistical test. We show that a single solvent, for example, olive oil, cannot be used a priori as a model for all membranes but that a range of solvents is required to approximate the differing solvent powers of biological membranes.

We recall Eq. (14):

$$P = \frac{KD_{\text{mem}}}{l}.$$

Of the terms in Eq. (14), it is possible to show (Lieb and Stein, 1969) that one can obtain a good empirical fit for data of a number of different systems by writing

$$D_{\text{mem}} = AM_{\text{rel}}^{-s_m} \tag{15}$$

where M_{rel} is the diffusant molecular weight relative to that of methanol and A and s_m are constants characteristic of a given membrane at a given temperature. For example, the differential mass selectivity coefficient s_m has the value $\frac{1}{3}$–$\frac{1}{2}$ for water, 1.1 for natural rubber, 3.8 for polymethylacrylate, and 3.5 for *Chara ceratophylla* (an algal cell). We must caution that an equation of this form may hold only over a restricted range of molecular sizes and for molecules that do not depart too drastically from the shape of a sphere.

Combining Eqs. (14) and (15) gives

$$P = P_0 K M_{\text{rel}}^{-s_m} \tag{16}$$

where $P_0 = A/l$ is a constant. This can be written in the equivalent form:

$$\log_{10} P = \log_{10} P_0 + \log_{10} K - s_m \log_{10} M_{\text{rel}} \tag{17}$$

The equation in this form cannot be used directly since we do not have experimental values for the membrane/water partition coefficient K. What we do have for each permeant are values K_{est} for various nonaqueous–aqueous two-phase systems which might be used as possible estimates of K. In order to test the reasonableness of a particular choice of hydrophobic solvent, we cast Eq. (17) into the generalized form:

$$\log_{10} P = \log_{10} P_0 + s_k \log_{10} K_{\text{est}} - s_m \log_{10} M_{\text{rel}} \tag{18}$$

It is clear from a comparison of Eqs. (17) and (18) that for a correct choice of hydrophobic solvent the validity index s_k must be unity.

Our problem now is to determine s_k from sets of values of P, M_{rel}, and K_{est}. This can be done using the method of least squares (Brownlee, 1960). When this analysis was applied to permeability data for leaf cells of *C. ceratophylla* (obtained under conditions of zero bulk flow) with olive oil as the model hydrophobic solvent, the least squares estimate (±S.E.) of s_k was found to be 1.1 ± 0.1 (Lieb and Stein, 1969). Thus the validity index here is not significantly different from unity, so that olive oil/water partition coefficients are good estimates to within a constant multiplying factor of the membrane/water partition coefficients. (The least squares analysis would not reveal a constant multiplying factor, since if present it would be buried in the term $\log_{10} P_0$.) We therefore have:

$$K = CK_{\text{est}} \tag{19}$$

where C is the constant multiplying factor. We show below that C is probably of the order of unity for *Chara*.

Since we now have estimates of K good to within a constant multiplying factor, we can use Eq. (7) to calculate from the measured values of P the values of D_{mem}/l, that is, P_{mem}, again to within the constant multiplying factor. Figure 8 is a plot of these relative diffusion coefficients as a function of the relative molecular weights of the diffusants. Note that this plot is almost an exact analog of Fig. 3 for diffusion in polymers. The negative of the slope of Fig. 8 gives the differential mass selectivity coefficient s_m (±S.E.) directly for the *Chara* system as 3.5 ± 0.3. Although this value is quite similar to that obtained for diffusion in polymethylacrylate (Fig. 3), it is far removed from the values of $\frac{1}{3}$–$\frac{1}{2}$ found for diffusion in simple

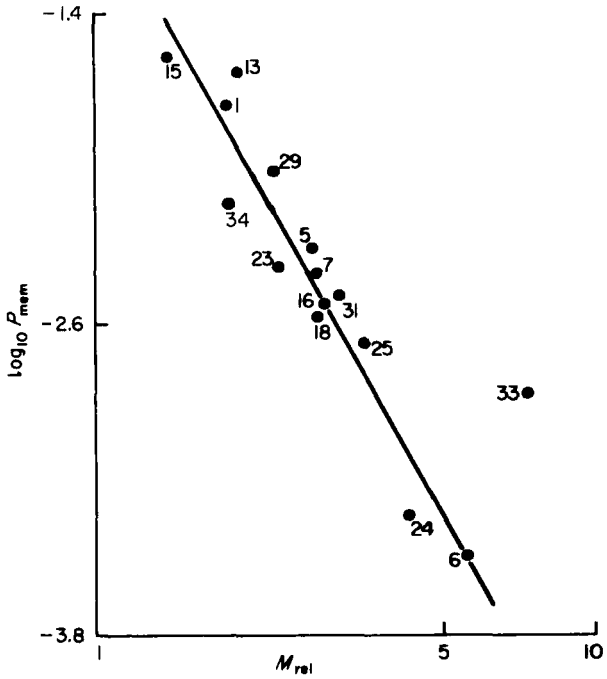

FIG. 8. Diffusion within a biological membrane (*C. ceratophylla*) as a function of molecular weight. P_{mem} is the relative diffusion coefficient within the membrane at about 20°, estimated as the ratio of the measured permeability coefficient P (cm sec^{-1}) to the olive oil/water partition coefficient. M_{rel} is the molecular weight of the diffusing molecule relative to methanol. The regression line was calculated with the exclusion of trimethylcitrate; the negative of the slope (±S.E.) of this line is $s_m = 3.5 \pm 0.3$. Diffusing molecules are: 1, acetamide; 5, *n*-butyramide; 6, diacetin; 7, *N,N*-dimethyl urea; 13, ethylene glycol; 15, formamide; 16, glycerol; 18, lactamide; 23, methyl urea; 24, monacetin; 25, α-monochlorohydrin; 29, propionamide; 31, succinimide; 33, trimethylcitrate; 34, urea. Taken from Lieb and Stein (1969).

liquids (Fig. 2). Thus the process of diffusion in this biological membrane seems to resemble that in an isotropic polymer, not that in a simple liquid.

In order to obtain absolute values for the diffusion coefficients D_{mem} within the membrane system of *Chara* it is necessary to know the values of both the effective membrane thickness l and the constant multiplying factor C. Unfortunately, at present we can only estimate these. For l we take a representative value of 50 Å. For C we have done the following. We are aware of only one substance for which a membrane/water partition coefficient has been determined in a biological system. The partition coefficient for the distribution of benzyl alcohol between the human

erythrocyte membrane and a saline buffer was determined in a study by Metcalfe, Seeman, and Burgen (1968). They found that for aqueous concentrations of benzyl alcohol in the range 2–10 mM the partition coefficient was constant at about 1.5 for a temperature of 23°. In a more recent study of the same effect by one of these investigators (Seeman, 1969), the partition coefficient was found to be 2.5 at 23°. We have measured (unpublished experiments) the partition coefficient of benzyl alcohol between olive oil and a saline buffer at the same temperature and found a value of about 2.5. Thus it seems that olive oil is a good model for the solvent power of the human red cell membrane, at least for benzyl alcohol. (In Section III,C we point out that olive oil is not a good model for the solvent power of the erythrocyte membrane toward basic permeants. Benzyl alcohol, however, is nonbasic.) It is technically impossible at present to measure the relevant partition coefficients for *Chara* membranes. To the extent (and only to the extent) that the solvent powers of these membranes for benzyl alcohol are similar, however, we can deduce that the constant multiplying factor C is of order unity.

Thus from Eqs. (7) and (19) we have for *Chara*

$$D_{\text{mem}} \approx (5 \times 10^{-7}) \frac{P}{K_o} \tag{20}$$

where D_{mem} is in cm^2 sec^{-1}, P is in cm sec^{-1}, and K_o is the olive oil/water partition coefficient. Selected values calculated from Eq. (20) are listed in Table IV. Note that these values span a range of over 50-fold in *Chara* but only 2-fold in water, as expected from the much higher mass selectivity of the membrane. In addition, the membrane values are roughly 1000–40,000

TABLE IV

SELECTED DIFFUSION COEFFICIENTS IN WATER AND IN MEMBRANES OF *Chara*

Diffusant	Molecular weight	Diffusion coefficient × 10^9 (cm^2 sec^{-1})	
		Water[a]	*Chara*[b]
Formamide	45	16,100	13.8
Acetamide	59	12,500	9.0
Propionamide	73	10,900	5.0
Glycerol	92	9,400	1.5
Erythritol	122	8,000	0.2

[a] Longsworth (1963) at 25°.

[b] Calculated from Eq. (20), using data of Collander and Bärlund (1933) for about 20°.

times lower than the corresponding diffusion coefficients in water for the compounds studied. The membrane values for *Chara*, however, are much the same as for diffusion in some polymers (see Table I).

We can now calculate the order of magnitude of the time resolution necessary if diffusion coefficients were to be measured directly for this biological membrane using the time-lag permeation [Eq. (9)] or the sorption–desorption [Eq. (10)] technique. By taking the extreme values in Table IV, it can be calculated that a resolution of at least 100 μsec would be required in the case of the slowest diffusant and of at least 1 μsec in the case of the fastest diffusant listed. It is apparent that such time resolution is not available with present methods. [There has been one study, however, in which the use of the time-lag permeation method was attempted with a biological membrane (Tenforde and Macey, 1968). Here, an apparent time lag in the 10-second range was found for the uptake of glycerol by the beef erythrocyte membrane by using a constant-volume feedback procedure. It seems that the long time lag was attributable to a secondary process, presumably the net leakage of salt from the cells during the initial stage of the experiment. This interpretation is supported by the finding of Macey and Tolberg (1966) that the time lag increased regularly (up to 700%) as the nonpenetrating solute mannitol was added to the external solution, whereas little change was noted in the permeability coefficient for glycerol.]

To summarize, for the membrane system of *C. ceratophylla*, we have obtained accurate estimates of the relative diffusion coefficients and demonstrated their steep size dependence. We have also shown that olive oil serves as a good model for the partitioning behavior of this membrane system. From these findings we conclude that the membrane system of *C. ceratophylla* behaves as a hydrophobic polymer with respect to the diffusion of nonelectrolytes.

B. Analysis of the Permeation Data for Certain Other Biological Membranes

The data on *Chara* provide us with a fortunate instance of a situation in which we have both very accurate permeability data and partition coefficients for a hydrophobic solvent that can be shown to be a good model for the partitioning properties of a biological membrane. There is only one other case in which an extensive listing of accurate permeability coefficients is available; this is the data on *Nitella mucronata* (Collander, 1954). We have analyzed these data in exactly the same way as we did in Section III, A for *Chara*. The results are given in Table V. Unfortunately, as can be seen

TABLE V

LEAST SQUARES ESTIMATES FOR PARAMETERS OF EQ. (18)[a]

Cell	s_k	s_m	$\log_{10} P_0$	σ	N
Chara	1.1 ± 0.1	2.9 ± 0.6	−0.9 ± 0.6	0.29	15
Nitella	1.4 ± 0.1	3.7 ± 0.5	−0.3 ± 0.5	0.28	13
Phascolosoma	1.0 ± 0.4	5.1 ± 1.7	−1.4 ± 1.6	0.44	9
Bovine red cell	1.4 ± 0.3	6.0 ± 1.6	0.7 ± 1.4	0.64	10
Arbacia	1.1 ± 0.2	4.2 ± 1.7	−0.4 ± 0.8	0.30	6

[a] N is the number of permeants used in each analysis and σ is the square root of the variance estimate for $\log_{10}$ P (a useful index of the goodness of fit). The values of K_{est} used in all analyses were those of partition coefficients between olive oil and water. All data (±S.E.) are for about 20°. From Lieb and Stein (1969).

from this table, the validity index s_k (±S.E.) was found to be 1.4 ± 0.1, significantly different from unity. Thus olive oil is not a good model for the partitioning properties of the *Nitella* membrane system, and we therefore cannot use olive oil/water partition coefficients to derive diffusion coefficients. The fact that s_k is greater than unity indicates that a solvent more hydrophobic than olive oil is needed to approximate the solvent properties of this membrane system. We have not, however, found a sufficiently extensive listing of partition coefficients for such solvents. It is to be hoped that such partition information will become available so that the very excellent data of Collander on *Nitella* can be used.

Table V also includes the results of our analysis for three animal cell membranes, which are included for general interest. It should be stressed that the permeability coefficients for these animal cells are only approximate, as they were determined by methods involving large volume changes (see for example the analysis of Kedem and Katchalsky, 1958). The uncertainty of these data prevents further analysis. Nonetheless it is quite clear from Table V that all membranes appear to be at least as hydrophobic as olive oil and to have size selectivities of the kind found for isotropic polymers but not for simple liquids. Thus to a first approximation it appears that all these membranes behave as hydrophobic polymers.

We summarize at this point the steps that must be taken in order to apply this analysis to any future investigation.

(1) Permeability coefficients must be obtained for a large number of diverse yet not highly nonspherical compounds by accurate methods not involving bulk flows, for example, by tracer equilibrium techniques.

(2) By using the presently available extensive listings of olive oil/water partition coefficients (Collander, 1954; Macy, 1948), the validity index s_k must be calculated, for example, by applying the procedure of least squares to Eq. (18) as was done for Table V.

(3) If s_k is found to be unity, then one can proceed directly to step (4). If not, it is necessary to obtain partition coefficients for another solvent, either from the literature or by direct experimental determination. As a guide to the proper choice of solvent, we suggest that if s_k is greater than unity the new solvent should be more hydrophobic; the converse is true if s_k is less than unity. Using these new partition coefficients in place of those for olive oil, one must now repeat step (2). Steps (2) and (3) should be repeated until a solvent is found such that s_k is equal to unity.

(4) When s_k is found to be unity, one can then use the relevant partition coefficients to find the relative diffusion coefficients for movement within the biological membrane by use of Eqs. (7) and (19), where l and C are unknown but constant.

(5) By plotting these relative diffusion coefficients against diffusant size in a plot similar to that shown in Fig. 8, one obtains a measure of the size selectivity of the membrane.

(6) If possible, one should try to determine directly the partition coefficients of a few substances (preferably of differing polarities) between the biological membrane and water. If these coefficients are found to be nearly identical to those of the final hydrophobic solvent used as a membrane analog, then the factor C of Eq. (19) is of the order of unity. If the membrane thickness can be estimated, then by the use of Eq. (7) approximate values of D_{mem} can be calculated.

To the extent that the complete procedure just described can be applied to a given membrane, three indices that characterize the membrane will be obtained: (1) the solvent power of the membrane, as reflected by the model solvent that gives s_k equal to unity, (2) the size selectivity of the membrane, as shown by the slope of a graph such as that in Fig. 8, and (3) the absolute value of the diffusion coefficient D_{mem} for a standard reference molecule. It will be of great importance to see how the chemical and physical nature of biological membranes determines these parameters.

C. Qualitative Considerations for Some Special Cases

We wish to consider now two special situations, the human erythrocyte and the rabbit gallbladder. Although permeability data do not lend themselves to a quantitative analysis here, they do bring out what appears at

present to be a novel feature of the solvent power of some membranes. This feature is a selective increase in the partitioning of certain classes of substances (in this case amides) into the membrane. Thus in addition to our earlier distinction between membranes of differing hydrophobicities, we must now also consider the possibility of other types of selectivities.

An extensive listing of accurate permeability data for the human erythrocyte plasma membrane is not yet available, but workers in the field are eagerly awaiting the forthcoming publication of such a study from the laboratory of Solomon (see Solomon, 1968a). In the meantime we must use earlier data determined by methods involving substantial bulk flow. The largest set of such permeability data obtained in a single study is that of Höber and Ørskov (1933). These data are reported not as permeability coefficients but as relative hemolysis times t'. Jacobs (1952) later developed the view that such numbers should be inversely proportional to permeability coefficients, although we know now that this is only an approximation as it neglects solute–water interactions (Kedem and Katchalsky, 1958). Following Eq. (7) we estimate the relative diffusion coefficient as the reciprocal of $t'K_{\text{est}}$ for each diffusant.

In Fig. 9A the relative diffusion coefficients have been plotted against molecular weight, as in Fig. 8 for *Chara*, by using olive oil/water partition coefficients as values for K_{est}. [We have omitted points for glycerol, ethylene glycol, and urea, since these compounds are thought to enter the human erythrocyte by facilitated diffusion systems (Hunter *et al.*, 1965).] It is at once apparent that no single straight line gives a satisfactory fit to these data. Upon closer examination, however, it becomes apparent that the data might be fitted by two straight lines, one connecting the amides (filled circles) and the other connecting all the other diffusants. The fact that all the amides appear to diffuse faster than other substances of similar molecular weight suggests that with olive oil we are not taking into account an enhanced partitioning into the membrane of these somewhat basic compounds. In Fig. 9B this factor is taken into account by using as the model solvent a mixture of olive oil and oleic acid (20%), for which solvent system basic substances specifically have enhanced partition coefficients K_{est}. [Partition coefficients for this solvent system are provided by Collander and Bärlund (1933). Collander (1937) noted that the concept of an acidic membrane could account for anomolous permeation data in certain plant cells.] It can be seen from Fig. 9 that with this mixed solvent a better fit to a single straight line is obtained. The fact that most of the amides are still a little above the common line of Fig. 9B suggests that this particular mixture of olive oil and oleic acid may not be acidic enough to serve as an adequate model for the partitioning behavior of the human erythrocyte membrane. Nonetheless, it is

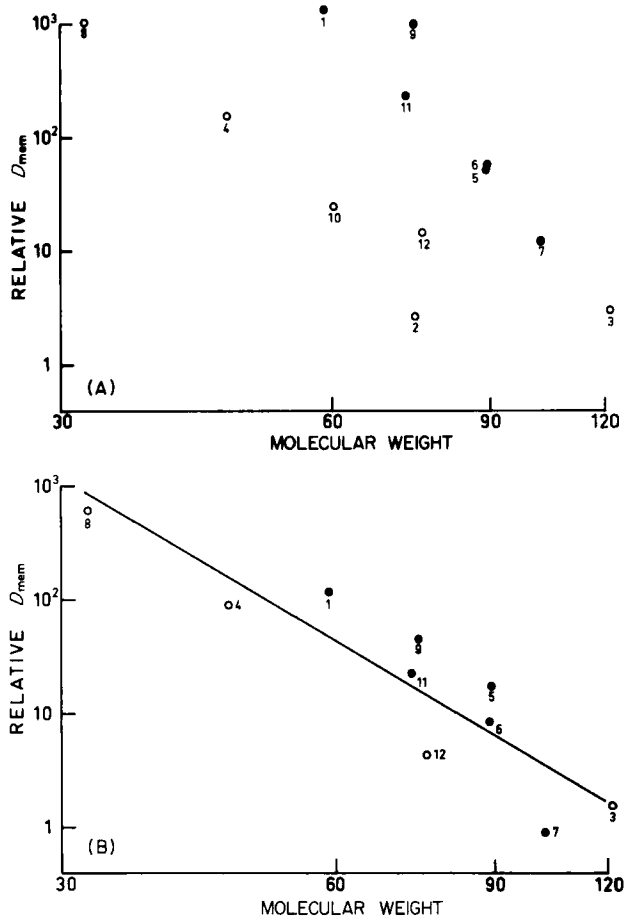

FIG. 9. Diffusion within the cell membrane of the human erythrocyte as a function of molecular weight. Ordinate: relative diffusion coefficients calculated as the reciprocal of $t' K_{est}$, where t' is the relative hemolysis time (as listed in Table 5 of Höber and Ørskov, 1933) and K_{est} is the partition coefficient between a hydrophobic solvent and water. In (A) this hydrophobic solvent is olive oil; in (B) it is a mixture of olive oil and oleic acid (20% v/v). Values of the olive oil/water partition coefficients for butanol and propanol were obtained from Macy (1948); all other partition coefficients for both solvents were taken from Collander and Bärlund (1933). Abscissa: molecular weight of the diffusing molecule. Because ethylene glycol, glycerol, and urea are thought to enter the human red cell by facilitated diffusion (Hunter *et al.*, 1965), these three compounds were omitted. Also, in (B) butanol and propanol had to be omitted, since there were no available values for their partition coefficients in the relevant solvent system. The negative of the slope (±S.E.) of the regression line of (B) is $s_m = 4.7 \pm 0.8$. Diffusing molecules are: 1, acetamide; 2, *n*-butanol; 3, erythritol; 4, ethanol; 5, ethyl carbamate; 6, lactamide; 7, malonamide; 8, methanol; 9, methyl urea; 10, *n*-propanol; 11, propionamide; 12, thiourea. Filled circles are amides.

clearly important to take into account the acidic character of this membrane. Note that if we were to calculate the differential mass selectivity coefficient s_m from this line, we would obtain a value ($\pm$S.E.) of 4.7 ± 0.8, once again in the range found for polymers but not for simple liquids.

Recently, Diamond and Wright have very significantly contributed to the experimental data on the permeation process across biological membranes (Wright and Diamond, 1969a,b; Diamond and Wright, 1969a,b). They studied the movement of a large number of substances across the epithelium of the rabbit gallbladder by an ingenious new technique in which the volume flow induced by the presence of solute was rapidly monitored by electrical means. The parameter measured was the reflection coefficient σ (Staverman, 1951), which is the ratio of the osmotic pressure generated by a given solute to the osmotic pressure generated by an equal concentration of a wholly impermeable solute. σ is a measure of membrane selectivity, but its relation to the permeability coefficient P we have used thus far can be established only if a particular model for for the transport process is assumed. This disadvantage is balanced by the fact that with this method reflection coefficients for many compounds can be measured on a single gallbladder in a short time. Thus one can distinguish between the effects of subtle differences in the structure of permeant molecules.

The results of these experiments could be presented in many ways. The form chosen by Wright and Diamond is reproduced here as Fig. 10A and is a plot of σ versus the olive oil/water partition coefficient. It is apparent from the figure that there is a strong dependence of σ upon the partition coefficient, although there is considerable scatter. Wright and Diamond (1969b) pointed out that most of the permeants lying below the shaded area, hence of penetrability higher than expected, are compounds with relatively low molecular weights. For this reason they assigned these small permeants to a separate category of permeants postulated as being able to cross the membrane through pores. On the basis of the above results for the human erythrocyte, however, we can suggest a simpler interpretation.

We note that the deviating permeants of Fig. 10A are not only of low molecular weight but are also mostly amides. The fact that these amides penetrate faster than expected suggests that the rabbit gallbladder, similar to the human erythrocyte, might possess an amidophilic membrane.

To test this idea we might try a treatment analogous to that used for the human erythrocyte. Unfortunately, there are considerable obstacles to such an analysis. First, as Wright and Diamond (1969a) point out, their values of σ may be in some error because of unstirred layer problems. Second, even if the data were not in error, the derivation of permeability coefficients P from these reflection coefficients σ requires the choice of an

arbitrary model. Nonetheless, let us have the courage at least to attempt such an analysis.

On the basis of a model involving frictional interactions among solute, water, and membrane, Kedem and Katchalsky (1961) derived the following expression:

$$\sigma = 1 - \frac{\omega \bar{V}_s}{L_p} - \frac{K f_{sw}}{\phi_w (f_{sw} + f_{sm})} \tag{21}$$

where ω is directly proportional to P, $\bar{V}_s$ is the partial molar volume of solute, L_p is the hydraulic permeability coefficient, K is the usual membrane/water partition coefficient, ϕ_w is the volume fraction of water in the membrane, while f_{sw} and f_{sm} are, respectively, solute–water and solute–membrane frictional coefficients. If the major pathway for solute is through the hydrophobic phase of the membrane, we can assume that the concentrations of solute and water within this phase are sufficiently low to allow solute–water interactions to be neglected; hence $f_{sw} \approx 0$, and Eq. (21) simplifies to a form that can be rewritten as*

$$\omega = (1 - \sigma) \frac{L_p}{\bar{V}_s} \tag{22}$$

Using Eq. (7) and the relation $P = RT\omega$ (Kedem and Katchalsky, 1958), we obtain

$$P_{mem} = \frac{P}{K} = \frac{(1 - \sigma)}{K \bar{V}_s} L_p RT \tag{23}$$

Since the factors L_p, R (the gas constant), and T (the absolute temperature) are constant for a given membrane and temperature, we can estimate relative diffusion coefficients as $(1 - \sigma)/(K\bar{V}_s)$. By analogy with Fig. 9B, we estimate K from values of partition coefficients between a mixture of olive oil with oleic acid (20%) and water. For $\bar{V}_s$ we use molar volumes in water calculated by the method of Traube as given by Partington (1951). In Fig. 10B these approximate relative diffusion coefficients are plotted versus molecular weight for all the solutes studied by Wright

* Equation (22) brings out some major drawbacks to the use of reflection coefficients. First, it is not possible to calculate satisfactorily permeability coefficients for compounds whose σ values are close to unity. For example, if $\sigma = 0.95$ and is measured with an accuracy as good as only 5%, the error in the calculated value of the permeability coefficient could be as great as 20-fold. If the error in σ were 6% or greater, it would be actually impossible to establish a lower limit (other than zero) for the permeability coefficient. Second, for slowly penetrating solutes it is possible for permeability coefficients to vary over many orders of magnitude, whereas the corresponding variation in σ might easily be compressed into an extremely narrow range, for example, from 0.9 to 1.0. Both of these points also show that it is essential, for an intuitive grasp of the permeation process, to think in terms of $1 - \sigma$ rather than σ.

and Diamond (1969b) for which olive oil + oleic acid/water partition coefficients are available, omitting only those solutes for which $\sigma \geq 0.95$ (see footnote). The data clearly do not fall exactly on the common straight line, a finding not unexpected in the view of (1) the approximate nature of the data, (2) the simplifying assumption of no solute–water interactions, and (3) the necessity of using a rather arbitrary solvent system as a model for the partitioning properties of the membrane.

Nevertheless, even so approximate a treatment is seen to allow the rabbit gallbladder data to be interpreted on the basis of only a single mechanism being operative for permeants of all sizes. The negative of the slope ($\pm$S.E.) of the regression line in Fig. 10B is 2.8 ± 0.5, which once again gives a differential mass selectivity coefficient s_m of the order found for diffusion in polymers.

D. Effects of Permeant Shape, Temperature, pH, and Anesthetics

We consider here the effect of certain variables upon the process of diffusion within biological membranes. Unfortunately, for the most part we have data only on the overall permeation process, and we have seen before the dangers in trying to infer characteristics of the diffusion process from these data (see Section II,B,5, especially Fig. 5). We can illustrate this point directly for the one biological membrane for which we do have good estimates of diffusion coefficients. Thus in Fig. 11 we have in C the permeability coefficient and in B the diffusion coefficient for a homologous series of straight-chain amides in *Chara* versus chain length, in strict analogy with Fig. 5 for a polymer. We see that although the curve for the permeability coefficients is complex, that for the diffusion coefficients is simple. Thus although results such as those shown in Fig. 11C are often interpreted as indicating that the lowest (hence smallest) member of a homologous series penetrates through pores (see, among others, Diamond and Wright, 1969b), we see from Figs. 5 and 11 that this need not be the case.

1. Permeant Shape

It has been found in numerous studies (summarized by Diamond and Wright, 1969b) that the overall permeabilities of branched compounds are lower than those of their more elongated isomers. This effect is often attributed to possible anisotropic structure in the cell membrane, for example, narrow aqueous pores (Soll, 1967), or orientated lipid chains (Wartiovaara and Collander, 1960). As has been rightly stressed by Diamond and Wright (1969a), however, such branched compounds also have partition coefficients lower than those of their more asymmetric analogs, largely as a result of reduced van der Waals contact areas. For

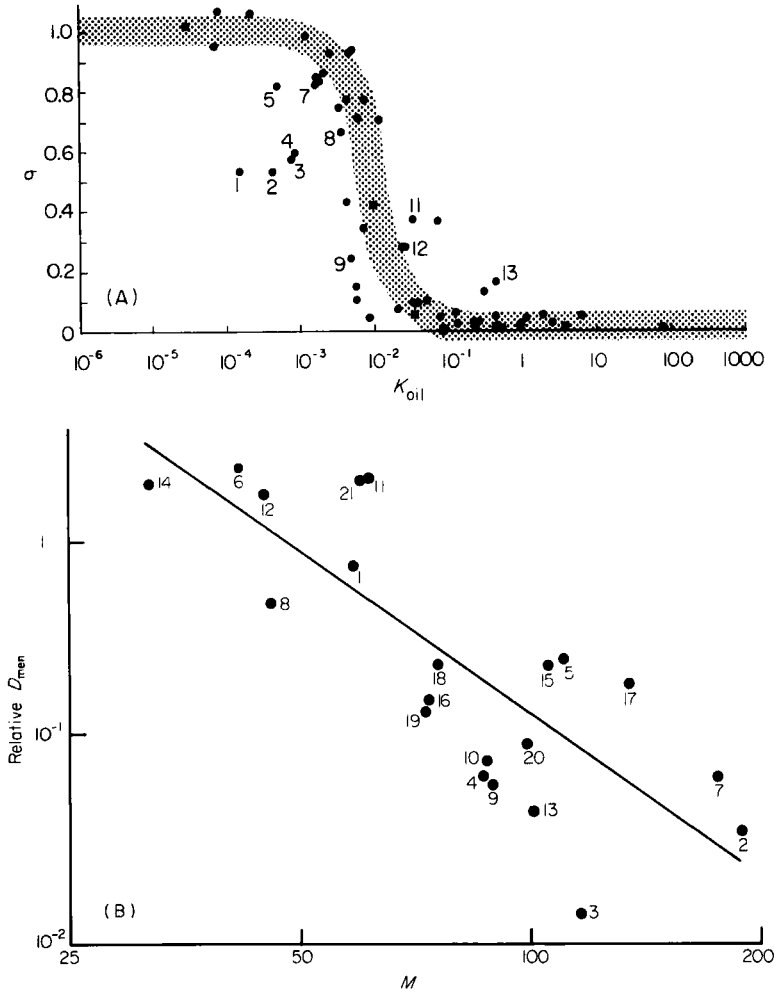

FIG 10. Two different representations of the same permeation data for epithelial cells of the rabbit gallbladder. (A) Ordinate: average value of the reflection coefficient σ determined at 17–20°. Abscissa: partition coefficient between olive oil and water. Certain small and branched solutes are: 1, urea; 2, methyl urea; 3, formamide; 4, acetamide; 5, ethylene glycol; 7, ethyl urea; 8, propionamide; 9, dimethyl formamide; 10, pinacol; 11, isovaleramide; 12, 2-methyl-2,4-pentane diol; 13, triacetin. The shaded line is drawn to indicate the general pattern of the other points and has no theoretical significance. Taken with kind permission from Wright and Diamond (1969b). (B) Ordinate: relative diffusion coefficient within the membrane estimated according to

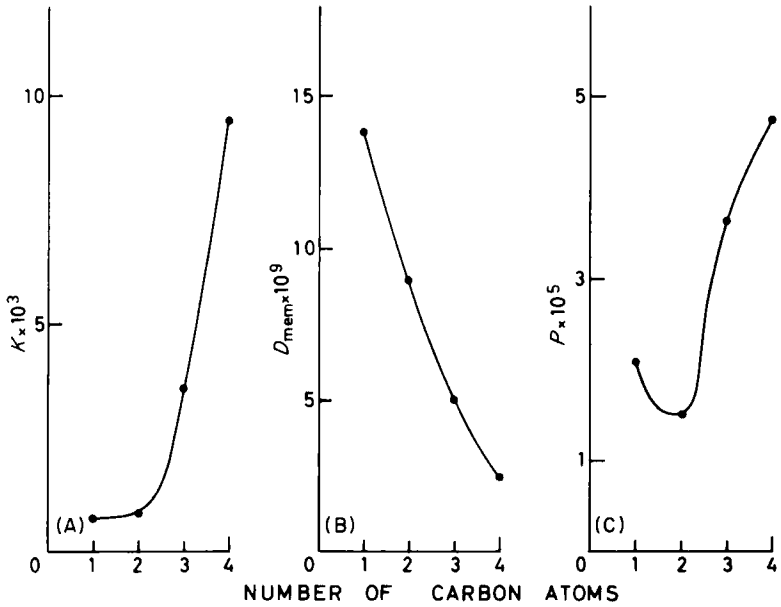

FIG. 11. Influence of length of hydrocarbon chain on the distribution, diffusion and permeability coefficients of an homologous series of *n*-alkylamides for a biological membrane (*C. ceratophylla*). Abscissa: number of carbon atoms in the permeant molecules (formamide, acetamide, propionamide, and *n*-butyramide). Ordinate: (A) equilibrium distribution coefficient K ($\times 10^3$) between membrane and water; (B) diffusion coefficient D_{mem} ($\times 10^9$, cm^2 sec^{-1}); (C) overall permeability coefficient ($P \times 10^5$, cm sec^{-1}). All results are for approximately 20°. Values of K and P were obtained directly from Collander and Bärlund (1933) and were used with Eq. (20) to calculate values of D_{mem}.

Eq. (23) as $(1 - \sigma)/(K\bar{V}_s)$. The reflection coefficient σ was taken from Wright and Diamond (1969b), the partition coefficient K was estimated as that between a mixture of olive oil with oleic acid (20% v/v) and water (Collander and Bärlund, 1933), and $\bar{V}_s$ was calculated as the molar volume in water using the method of Traube as given by Partington (1951). Abscissa: molecular weight of the diffusing molecule. Only those diffusants for which $\sigma \geq 0.95$ (see footnote to page 30) or for which values of the partition coefficient in the above solvent system are unavailable were omitted. The negative of the slope ($\pm$S.E.) of the regression line is $s_m = 2.8 \pm 0.5$. Diffusing molecules are [note that numbering system differs from that in (A)]: 1, acetamide; 2, antipyrine; 3, *asym*-diethyl urea; 4, *n*-butyramide; 5, 3-chloro-1,2-propanediol; 6, cyanamide; 7, diacetin; 8, ethanol; 9, ethyl carbamate; 10, ethyl urea; 11, ethylene glycol; 12, formamide; 13, isovaleramide; 14, methanol; 15, 3-methoxy-1,2-propanediol; 16, methyl urea; 17, monacetin; 18, 1,2-propanediol; 19, propionamide; 20, succinimide; 21, urea.

this reason it is difficult at present to decide whether the effect of branching is primarily upon the partitioning or the diffusion process. If indeed there is a significant effect of diffusant shape upon the diffusion process, this is exactly what is to be expected if the membrane behaves as a polymer. We note that such effects of shape are found in isotropic polymers (Section II,B,2), so that even though the membrane is anisotropic it need not be anisotropy that is responsible for the possible effects of diffusant shape.

2. Temperature and pH

We come now to the effect of temperature. It is generally found that permeation rates increase with increasing temperatures. The magnitude of this increase is almost always much larger for biological membranes than it is for diffusion in simple liquids such as water. In addition, it is generally found that the effect of temperature is greater for larger permeants (Jacobs *et al.*, 1935; Wartiovaara, 1956; Bunch and Edwards, 1969; Wright and Diamond, 1969b). Once again we must be careful to distinguish the effects of temperature upon the partitioning and the diffusion steps of the overall permeation process. This problem can be approached by considering some of the available data on the temperature dependence of partition coefficients. Thus it has been found experimentally that the partition coefficient of benzyl alcohol for the human erythrocyte membrane increases by less than 20% when the temperature rises from 23° to 40° (Metcalfe *et al.*, 1968). Of three compounds (chloroform, ether, and *n*-butanol) whose partition coefficients in a decane/water system were measured as a function of temperature by Johnson and Bangham (1969), butanol was most influenced by temperature, showing a Q_{10} of about 1.5. In a comprehensive study of a number of compounds and solvent systems, Hantzsch and Vagt (1901) found that for a number of these there was almost no variation in the partition coefficient with temperature, while for the others the maximum Q_{10} was once again about 1.5. Conversion of values of Q_{10} into activation energies such as used in Section II,B,3 yields a maximum Q_{10} equivalent to a maximum activation energy of about 7 kcal mole^{-1} at room temperature.

Some representative data on the effect of temperature upon the overall permeation process in biological membranes are recorded in Table VI. It is apparent that the activation energies for permeation are very much higher than those for diffusion in water (Table II). Even when we allow for a possible 7 kcal mole^{-1} activation energy for the partitioning step, we are left with a residual activation energy for the diffusion step of the order found for diffusion in polymers (Table III) but not for diffusion in water. It is conceivable that the increase in activation energy with

TABLE VI

ACTIVATION ENERGIES FOR PERMEATION ACROSS BIOLOGICAL MEMBRANES

Permeant	Molecular weight	E_{act} (kcal mole^{-1})[a]				
		Elodea	*Gentiana*	*Lamium*	*Rheo*	*Spirogyra*
Urea	60	10.6	13.0	13.8	13.8	13.8
Thiourea	76	10.6	13.0	14.4	13.8	14.4
Dimethyl urea	88	21.1	21.1	20.2	21.1	22.0
Methyl thiourea	90	20.2	19.7	19.2	20.2	21.1

[a] Activation energy (E_{act}) values for these plant cells were calculated from Q_{10} values of Wartiovaara (1956), assuming a temperature interval 10°–20°.

permeant size is attributable to a differential effect of temperature upon the partition step. There appears, however, to be no such differential effect in the partition data for simple solvent systems (Hantzsch and Vagt, 1901). Thus it seems likely that the effect of temperature upon diffusion in biological membranes is greater for larger molecules than for smaller molecules. This is just the behavior found for diffusion in polymers. There is thus no need from this data to postulate (Diamond and Wright, 1969b) that there are different pathways for the permeation of large and small molecules across biological membranes.

It seems worthwhile at this stage to make a general comment. We have just seen that temperature has a greater effect on large diffusants than on small diffusants. It might be expected from concepts of diffusion in polymers that any agent that affects diffusion rates would produce such a differential effect. This follows directly from the free volume arguments of Section II,C, or more intuitively from the following argument. To form a large hole it is necessary to move apart a large number of polymer chain segments. If the effect of an external agent is to modify the interaction between, for example, a unit pair of chain segments, then this agent will have a greater effect the greater the number of units involved. We are not aware of many other studies in which the effect of an external agent upon the permeation rates for a large range of permeant sizes has been measured. An interesting example of such a study, however, is that of Wright and Diamond (1969b) on the effect of pH upon large and small permeants. In accordance with our expectations, changes in pH were found to have a more pronounced effect on the permeation of large molecules than of small molecules. [There is reason to believe in this case that the differential effect of pH is probably not upon the partitioning step (Wright and Diamond, 1969b) but rather upon the diffusion process.]

3. General Anesthetics

We finally come to consider the effects of general, nonspecific anesthetics on the permeability of natural membranes. These anesthetics are of major interest insofar as they block nervous conduction by inhibiting the flows of sodium and potassium ions responsible for the action potential (Moore *et al.*, 1964). There are a number of other cases in which an inhibition of membrane permeability is found, for example, that for glycerol in red cells of man and certain other species (Jacobs and Parpart, 1937) and that for sodium ions in the cat red cell (Davson, 1940). In most of these cases, it has been shown that a specialized (i.e., mediated) transport system is involved. For the purposes of the present article, we cannot take up the interesting question of how this particular effect of anesthetics is brought about. Rather we focus our attention upon simple (i.e., nonmediated) permeation, where almost invariably the effect of general anesthetics is to increase transport rates (Davson, 1964). Examples of this are glycerol transport in red cells of the ox, sheep, and several other species possessing low permeabilities to glycerol (Jacobs and Parpart, 1937); thiourea transport in red cells of all species studied (Jacobs and Parpart, 1937); the transport of a number of nonelectrolytes in *C. ceratophylla* (Bärlund, 1938); and the transport of potassium ions in the cat red blood cell (Davson, 1940). Also, in a model membrane system, Johnson and Bangham (1969) showed that three general anesthetics all increase the movement of potassium ions.

Again we attempt to separate the effects of anesthetics upon the partitioning and diffusion steps. We have not found any direct data on the effect of anesthetics upon the partitioning of permeants. The best we can do is, as in Section II,B,4, to consider the additive (here the anesthetic) itself as the permeant; then, studies of the dependence of the partition coefficient of the anesthetic upon its concentration give us an indication of the desired information. As in the case of polymers, the range of interest is where the concentration of the additive within the membrane is of the order of 1%. [This is somewhat higher than the concentration of about $\frac{1}{3}$ of 1% necessary to cause substantial diminution of nervous activity (Mayer, 1937).] Of the common anesthetics studied by Macy (1948), in no case did the partition coefficient between olive oil and water increase significantly in the concentration range in which we are interested. A comprehensive study of the solubility of various anesthetic substances in polyethylene (Rogers *et al.*, 1962) also showed very little variation with concentration in the range of interest here. [There is one case, however, in which an increase in partition coefficient with concentration was found. This is for the partitioning of benzyl alcohol between erythrocyte mem-

branes and water (Metcalfe *et al.*, 1968). The increased partition coefficient here seems to be associated with the exposure of hydrophobic binding sites on proteins; it seems most unlikely that such hydrophobic sites could have any effect upon the partitioning of the hydrophilic substances used in the permeability studies on biological membranes described above.] Thus the limited amount of information available suggests that the primary action of anesthetics is on the diffusion process.

There thus seems to be a close analogy between the effect of plasticizers upon diffusion in polymers and the effect of anesthetics upon nonmediated diffusion within biological membranes. That anesthetics, like plasticizers, increase diffusion rates by increasing chain mobilities is indicated by the NMR studies of Metcalfe *et al.* (1968), in which increasing the concentration of the anesthetic benzyl alcohol in erythrocyte membranes greatly increased the tumbling of this probe within the membrane.

E. Evidence Concerning the Porous Nature of Membranes

Although we have seen that most of the data on permeation of nonelectrolytes across biological membranes can be explained on the basis of the membrane behaving as a continuous hydrophobic phase, there has been much discussion of the possibility that a fraction of the membrane may be composed of aqueous channels continuous across the membrane (see, among others, Solomon, 1968b; Diamond and Wright, 1969b; Passow, 1969). This is the view that there are "pores" in the membrane.

Such pores would offer a pathway parallel to the dissolution-diffusion pathway with which we have been concerned. Movement via such a parallel pathway appears to play an insignificant role in the permeation of nonelectrolytes under most conditions, for instance, in the absence of bulk flow. In some situations, however, it seems likely that a porous pathway may be important. This is especially true for the movement of ions, which have such exceedingly low partition coefficients in simple hydrophobic phases (Parsegian, 1969) that the dissolution-diffusion route would be of minor importance. Certainly, the low electrical resistance of natural membranes as compared with synthetic lipid membranes suggests that either ions move through hydrophilic pores or else on specialized "carriers," of which valinomycin may be a model. A second situation in which a porous route may become important in some membranes is under conditions of bulk flow. Thus it is often found that the bulk movement of water under a hydrostatic force is greater than the exchange flow of water under an equivalent chemical gradient of labeled water (Dick, 1966). This has usually been interpreted to mean that water is passing through pores,

although the same behavior has been observed in nonporous synthetic membranes (Thau *et al.*, 1966). Similarly, measurements of the reflection coefficient σ (see Section III,C), a parameter that characterizes the movement of solute during bulk flow, suggest for erythrocyte membranes that certain nonelectrolyte substances may permeate through pores (Goldstein and Solomon, 1960; Rich *et al.*, 1967). We have seen (Section III,C), however, that reflection coefficient data for the rabbit gallbladder do not require the assumption of pores. Similarly, Dainty and Ginzburg (1964) have concluded that there is nothing in their reflection coefficient data for the algal cells *Chara australis* and *Nitella translucens* that makes it necessary to assume pores. It thus seems that only for erythrocyte membranes do substances of low permeability in the absence of bulk flow have unexpectedly large values of $1 - \sigma$ (Sherwood and Solomon, 1969). It would therefore be of much interest to check by an independent method these red cell reflection coefficients. For example, it would be interesting to see whether or not substantial quantities of the normally quite impermeable substance malonamide are dragged across the human red cell membrane during bulk flow of water as predicted by its high value of $1 - \sigma$.*

Thus in no case has the existence of pores been demonstrated unequivocally for biological membranes. Nonetheless, we tend toward the view that water and ions may often find a significant alternative pathway through pores provided by proteins embedded in the otherwise completely hydrophobic lipid phase of the membrane.

IV. SUMMARY AND CONCLUSIONS

We have shown that diffusion of nonelectrolytes within biological membranes closely resembles diffusion in polymers, not diffusion in simple liquids. This resemblance extends to all of the situations we have investigated. Thus diffusion coefficients in both biological membranes and polymers show very similar dependencies upon diffusant size, diffusant shape, temperature, and the presence of low-molecular-weight additives.

The overall process of permeation across both biological and synthetic membranes is the resultant of two processes—partitioning into and out of the membrane and diffusing within the membrane. The overall process is therefore characterized by two parameters—the equilibrium distribution coefficient K between membrane and external phases and the diffusion coefficient D_{mem} for movement inside the membrane.

* See and compare Forster, this volume.

A formalism is described for deciding whether or not a particular hydrophobic solvent is a good model for the partitioning behavior of a given biological membrane. Although olive oil is a good model for some membranes, for other membranes a more hydrophobic model solvent must be chosen, while for still other membranes the model solvent must have other selectivities. When the proper model solvent has been chosen for a biological membrane, it is then possible to compute relative values of the membrane diffusion coefficient D_{mem}. In favorable circumstances one can calculate in addition the diffusion coefficients absolutely, and the range is within that found for common polymer systems.

Viewing the biological membrane as a hydrophobic polymeric phase has a number of advantages. It allows one to describe the permeation of solute molecules of all sizes in a simple and natural manner, without the additional postulate of porous pathways. It provides a basis for classifying biological membranes according to their partitioning and size selectivity properties. Last, and perhaps most importantly, it provides insights into the behavior of biological membranes by linking this behavior to the behavior of polymers, for which the diffusion process and the factors influencing it are much better understood.

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The Transport of Water in Erythrocytes

ROBERT E. FORSTER

Department of Physiology, School of Medicine
University of Pennsylvania,
Philadelphia, Pennsylvania

I. INTRODUCTION

Investigations of the water permeability of the red blood cell not only have intrinsic value but have made substantial contributions to our understanding of the function and even the structure of the plasma membrane (Jacobs, 1952; Solomon, 1960; Passow, 1969). These cells are also a convenient biological material, easily available, and can be maintained in essentially *in vivo* conditions *in vitro*. Red blood cells have a small volume in relation to their water permeability, however, so that water exchanges are complete in several seconds at physiological temperatures. Therefore special rapid-reaction apparatus is required to follow the volume changes of the cells. The beautifully simple lytic technique of Jacobs (1930) can give only a lower limit of water permeability because lysis is nearly complete in seconds, the limit of resolution of the mixing and observation systems.

Conclusions derived from measurements of water movements in tissue layers, such as capillary walls (Landis and Pappenheimer, 1963), frog skin (Koefoed-Johnsen and Ussing, 1953), and even *Arbacia* eggs (Lucké *et al.*, 1931), do not necessarily apply to water movements across the red blood cell membrane because it is thinner by orders of magnitude, includes only one plasma membrane, and is homogeneous, at least down to the microscopic level. These differences may lead to qualitative as well as quantitative differences in flux mechanism.

We have assumed that there is no active transport of water, being unaware of any good evidence for it.

This chapter is restricted to considerations of water movements of red blood cells, particularly to the explanation of existing experimental results. Only those other aspects of the general problem of red blood cell permeability are mentioned that touch directly on this subject. For a more general discussion of membrane transport in the red blood cell, or even a broader theoretical discussion of water exchanges, the reader is referred to the excellent monographs of Stein (1967), Dick (1966), and Harris (1960), and the thesis of Blum (1968), to all of whom I express my great debt.

II. MEASUREMENT OF THE WATER PERMEABILITY OF RED BLOOD CELLS

Measurement of the water permeability of red blood cells requires a measurement either of changes in volume as a function of time when there is net flow, or of the movement of labeled water in the absence of significant volume change. L_p, the symbol adopted for hydraulic permeability (Kedem

and Katchalsky, 1961) is used for the former, and P_w is used for the latter, both in units of centimeters per second for ease of comparison. This means the chemical potential gradient for L_p is converted to osmols per cubic centimeter rather than pressure [L_p can be expressed in $cm^4/(osmols \times seconds)$ if it is multiplied by the partial molal volume of water, approximately 18 $cm^3/osmol$]. In irreversible thermodynamic discussions of water permeability of membranes, L_p and the analogous osmotic coefficient, L_{pD}, are defined such that if flux and concentration and pressure increments are considered positive in the direction of increasing x, the normal convention, L_p and L_{pD} are negative numbers (Katchalsky and Curran, 1965, p. 119). L_p and L_{pD} are defined herein as positive numbers under the normal conventions in order to maintain consistency with the physical laws of mass transport. The reader should be warned that this has altered the signs of several symbols and terms in comparison with published treatments of the irreversible thermodynamics of membranes.

A. Measurement of L_p, the Hydraulic Coefficient

Because it is not technically possible to produce a pressure difference across the cell membrane, volume changes are produced by creating a step change in the external osmolality of impermeable solutes as rapidly as possible. While there has been controversy in the past as to the equivalence of osmotic and hydrostatic pressures, there now appears to be general agreement on this point (Chinard, 1952; Stein, 1967).

The instantaneous flux of water across the cell membrane is described by Ficks' law of diffusion (Jost, 1952; Stein, 1967; Sidel and Solomon, 1957).

$$\frac{dV_w}{dt} = L_p A \bar{V}_w \left(\frac{C_0 V_{w,0}}{V_w} - C_e \right) \tag{1}$$

where V_w is the osmotically active water volume of the cell at any time and $V_{w,0}$ is its value in the original equilibrium state; $\bar{V}_w$ is the partial molal volume of water; C_0 is the original osmolality of the water in the cell equal to that in its suspending fluid and C_e that in the external medium after the step change, assumed constant, both in osmols per cubic centimeter; A is the area of the red blood cell in square centimeters; t is time in seconds.

Integration of Eq. (1) and rearranging give

$$tL_p = \frac{1}{AC_e^2} \left[C_0 V_{w,0} \ln \left(\frac{C_0 V_{w,0} - C_e V_{w,0}}{C_0 V_{w,0} - C_e V_w} \right) - C_e (V_w - V_{w,0}) \right] \tag{2}$$

Because the experimental measurements are made of total cell volume and not just of the water volume, this equation is more useful if it is transformed with the relationships that follow, based on the experimental finding (Ponder, 1948) that a constant amount of intracellular water appears to be inactive osmotically, that is,

$$V_w = WV_{T_{w,0}} + V - V_0 \tag{3}$$

where W is the fraction of the initial cell water volume $V_{T_{w,0}}$ that is effective osmotically (Ponder, 1948), V is the total cell volume, and V_0 its initial value. From the assumption of a constant quantity of intracellular osmotically active solute molecules

$$V_w C = C_0 V_{w,0} \tag{4}$$

where C is the osmolality of the impermeable solutes in the cell water at any time. The subscript 0 refers to the initial value.

These substitutions into Eq. (2) produce the relationship

$$tL_p = \frac{WV_{T_{w,0}} + V_\infty - V_0}{C_0 A W V_{T_{w,0}}} \times \left[V_0 - V - (WV_{w,0} + V_\infty - V_0) \ln \left(\frac{V_\infty - V}{V_\infty - V_0} \right) \right] \tag{5}$$

where V_∞ is the cell volume after equilibration with C_e. Human red blood cells under isotonic conditions contain about 710 gm water per liter (Savitz *et al.*, 1964). The value of W is obtained experimentally from the slope of a graph of cell volume versus the reciprocal of osmolality (Farmer and Macey, 1970; Blum and Forster, 1970; Rich *et al.*, 1967)and is about 80%. The estimate of cell volume depends primarily on the hematocrit which in turn is a function of the trapped fluid volume. Solomon and his associates (Rich *et al.*, 1967) determined that WV_w/V at isosmolar conditions is 0.57 for human and dog, 0.61 for cat, and 0.52 for beef. It is assumed that the activity of the water inside and outside the cell is equal at equilibrium (Williams *et al.*, 1959), so that the osmolality of the suspending solution, which can be measured conveniently, can be substituted for the initial intracellular osmolality. Since Ponder (1948) it has been assumed that a portion of the intracellular water was rendered osmotically ineffective possibly by binding with the hemoglobin. Recently work by Gary-Bobo and Solomon (1971) indicates that at least in part W is an index of the change in negative charge on the hemoglobin molecule which occurs as the intracellular protein concentration falls with increasing cell volume. This also means that the number of intracellular osmotically active counter

ions must decrease; in other words Eq. (4) is not wholly true. However, as a practical matter, W can be considered an empirical constant describing the experimental relationship between cell volume and external osmolality without diminishing the usefulness of Eq. (5).

It is assumed that the surface area of the cell membrane is constant and that no osmotically active particles cross the membrane during the pertinent swelling or shrinking process. Under these conditions Eq. (5) can be used to calculate L_p from measurements of the change in cell volume with time following a change in the concentration of impermeable solutes in the suspending fluid. A plot of the right-hand side of Eq. (5) against time usually gives a straight line, compatible with the fact that the assumptions are reasonable.

Farmer and Macey (1970) obtained a simple exponential solution for Eq. (1) for conditions under which the change in extracellular osmolality is small (less than 10%). If $V_0 - V$ in Eq. (5) approaches zero, the relation becomes exponential, rendering treatment of the data simpler.

The initial slope of the curve of V as $f(t)$ can also be used to calculate L_p since

$$\left(\frac{dV}{dt}\right)_{t=0} = -L_p A \bar{V}_w (C_e - C_0) \tag{6}$$

where C_e is the external osmolality, considered constant, and C_0 is the intracellular osmolality at zero time, that is, the concentration of the fluid with which the cells were equilibrated prior to mixing (Rich *et al.*, 1967; Blum and Forster, 1970; Sha'afi *et al.*, 1970). It is often difficult, however, to draw a correct tangent to the start of the volume curve, particularly when scattering artifacts (Sha'afi *et al.*, 1967; Blum and Forster, 1970) distort it, and the first observation point is at 0.045 seconds (Sidel and Solomon, 1957).

The experimental conditions for obtaining L_p consist of (1) producing a sudden stepwise change in the mixed extracellular osmolality and (2) measuring the resulting rapid changes in cell volume.

1. Critique of Mixing Technique

The initial equilibrated red blood cell suspension must be mixed with the reactant solution of differing osmolality within a time period considerably less than the half-time of the volume exchanges. The extracellular solution must also be so well mixed that osmolal gradients are not produced around the cell (see later discussion of the stagnant layer in Section II,D,1). Seventy-five percent lysis of human red blood cells occurs in 2–4 seconds (Jacobs, 1930), and the half-times of swelling and shrinking can be of

the order of several tenths of a second (Blum and Forster, 1970). Jacobs' (1927) original technique of mixing cells with reactant solution in a cylinder was not fast enough to resolve the water movements in red cells, as he well realized. Parpart (1935) speeded up the mixing process to 0.05 seconds by forcefully injecting 0.4 ml of reactant fluid into a small chamber containing only 0.013 ml of blood. The volume changes are rapid enough, however, to require for their precise study a Hartridge–Roughton-type reaction apparatus with mixing times of around 0.001 second (Blum and Forster, 1970; Sidel and Solomon, 1957). The large amount of energy expended in forcing the reactant solutions through the mixing chamber may alter or damage the cells. Light-scattering artifacts are produced at relatively low flow velocities (Blum and Forster, 1970; Sha'afi *et al.*, 1967), and by using a Gibson–Milnes-type mixing chamber (Gibson and Milnes, 1964) severe lysis can be produced within several milliseconds or less as the cells pass through. The stop time of a Gibson-type instrument is less than 1 msec. The instrument of Sha'afi *et al.* (1967) had a stop time of around 25 msec and a dead time of 10 msec. These characteristics are adequate for following swelling and shrinking curves but not for defining their start or early parts. Farmer and Macey (1970) injected about 0.5 ml of a perturbing tonicity into 5 ml of cell suspension and mixed with a magnetically driven stirring rod. Clearly, this apparatus does not have the time resolution or mixing characteristics of a stopped-flow rapid-reaction instrument.

2. Critique of Methods of Following Cell Volume Changes with Time

Because of the rapidity of the volume changes, it is necessary to have a technique that is rapid in response and nondestructive. Several phenomena have been taken advantage of, but perhaps the most widely used is the change in light scattering of a cell suspension on swelling or shrinking (Parpart, 1935; Brooks, 1935; Ørskov, 1935; Widdas, 1953; Wilbrandt, 1938). In general, an increase in cell volume produces a decrease in light scattering. No precise theory is available to explain the phenomenon in quantitative terms (Anderson, 1966). The optical characteristics of the system are critical. Either increased transmission (Parpart, 1935, Blum and Forster, 1970) or decreased lateral scattering (Sidel and Solomon, 1957) can be determined as the cells swell. The change in light detected is approximately linear with the changes in cell volume (Sjölin, 1954; Sidel and Solomon, 1957; Blum and Forster, 1970). The instrumentation is relatively simple: a white light source and photodetector. The light need

not be monochromatic nor is the color critical (Blum and Forster, 1970). Light-scattering detection has been incorporated into continuous-flow (Sidel and Solomon, 1957) and stopped-flow rapid-reaction apparatus (Blum and Forster, 1970; Sha'afi *et al.*, 1967; Farmer and Macey, 1970), the latter requiring an oscilloscope to record the changes in photodetector output. Sha'afi *et al.* (1967) stored a point on a curve every 5 msec in a computer, which was more convenient for averaging several records and for subsequent computations but does not alter the fundamental instrumentation. The sensitivity of light scattering, or transmission, to changes in average cell volume varies widely with the optical system. Rich *et al.* (1967) were unable to detect the effect of a change of 10 mosmols/liter out of about 300 mosmols/liter, while Farmer and Macey (1970) followed volume changes versus time when the total change in extracellular osmolality was less than 30 mosmols/liter.

Light scattering by red blood cell suspensions should depend upon the index of refraction of the cell surface compared to that of the medium. Sha'afi *et al.* (1970) found that large changes in extracellular fluid composition (0.397 osmol/liter of urea) produced a marked increase in light scattering. Blum and Forster (1970) found no significant changes in light scattering accompanying changes in sodium chloride concentration from 0.15 to 0.45 *M*, however. These results may differ because of the different optical systems employed. An increase in steady fluid velocity, the flow of which is already turbulent, produces a decrease in light transmission through a red blood cell suspension, probably as a result of changes in the orientation of domains of the discoid particles along flow lines rather than changes in scattering by individual cells. A complicated but reproducible series of changes in light scattering (artifact) is initiated by stoppage of the net forward flow in a stopped-flow rapid-reaction apparatus (Blum and Forster, 1970; Sha'afi *et al.*, 1970). These artifacts are presumably canceled out by subtracting control records, made with mixing but no change in extracellular osmolality, from the experimental records. Sidel and Solomon (1957) found that the zero time intercepts of experiments with different water flux rates, and therefore different extracellular osmolalities, varied widely. They suggested that this represented differences in light scattering produced by the different osmolalities of the solutions. While this may be true, there is still the possibility of a time lag, even if it is residual. A Coulter particulate counter can be adapted to sample the flowing reacting mixture of a continuous-flow rapid-reaction apparatus and provide a measure of change in cell volume (Steen and Forster, unpublished observations). This instrument detects the small changes in electrical current through a fine pore in a glass plate produced by the passage of each particle, the height of the pulse being related to

the volume of the red blood cell. A Millipore filter continuous-flow apparatus (see Section II,B) can be used to separate the suspending fluid from the cells during the exchange process, and this fluid can then be analyzed at leisure and the movements of water calculated from the changes in concentration of impermeable ions or molecules. Temperature changes should occur accompanying water movements, produced by net thermodynamic factors in reactions and dilutions. These changes can be measured in a continuous-flow (Roughton, 1930) or a stopped-flow (Berger, 1963) rapid-reaction apparatus but would probably be small and difficult to interpret.

If hemolysis is assumed to occur at a constant cell volume, it can be used as an index of water movements in the red blood cell. The clever, simple, and historically important *lytic method* of measuring red blood cell permeability to solutes devised by Jacobs (1934) can be used for the measurement of water permeability, although the estimation is admittedly approximate. If it is assumed that the concentration of extracellular impermeable solute C_e is negligible, and this can be easily achieved by mixing a large volume of water with a small volume of cell suspension, then Eq. (1) can be integrated and rearranged to give

$$tL_p = \frac{V_w^2 - V_{w,0}^2}{2AC_0V_{w,0}} \tag{7}$$

Jacobs devised a simple arrangement by means of which he could follow, with a time lag of only 1–2 seconds, the depth of the suspension that would just transmit a clear image of an incandescent light filament. He further determined that this depth was inversely proportional to the concentration of unhemolyzed cells remaining. Later he recorded the transmitted light (Parpart, 1935), which showed a marked increase on hemolysis, as well as the relatively small changes with alterations in the volume of individual intact cells. The value of 75% hemolysis was chosen as a convenient end point, and V_{75h}/V_0, the ratio of the average volume of a red blood cell when 75% have hemolyzed to the average initial cell volume, was determined under equilibrium conditions when the cells had been in the test medium for as long as 1 hour; for human cells this value was 1.7. The difference between total cell volume (V) and osmotically active cell water volume (V_w) ratio was not considered significant. These conditions may not be the same as those obtaining during rapid changes in volume. Jacobs (1955) has discussed the problems and has concluded that he probably underestimated L_p. Unfortunately, the time required for 75% hemolysis is about 1 second for most cells, so that the precision of the technique is limited. Parpart (1935) used his apparatus with its more rapid mixing

time to compare the speed of hemolysis in water with that in D_sO but apparently did not apply it to extended studies of L_p.

The lytic method has another implicit weakness in that it measures only one point in the entire curve of cell volume as a function of time, and it is not possible to check that L_p is constant throughout as can be done when the entire function is recorded (Blum and Forster, 1970; Sidel and Solomon, 1957).

Red blood cells do not all lyse at the same external osmolality but show an S-shaped fragility curve, presumably because of differing initial volumes in the isosmotic fluid (Prankerd, 1961), which means there is in practice a wide distribution of V_h/V_0. Jacobs (1932) chose 75% hemolysis as an end point with the expectation that this value was close to a weighted average. Sjölin (1954) took advantage of this variation in fragility to measure t_h in 85–96 mM sodium chloride and in 78–85 mM sodium chloride. The former provided a measure of the larger L_p in the more fragile (thicker) cells, and the latter the smaller L_p in the less fragile (thinner) cells (Table I).

Last, the lytic method can measure only the rate of *entry* of molecules into the cell.

In view of its inherent inaccuracies, it is gratifying that estimates of L_p by the lytic technique differ from the best available data by as little as a one-half (Table I). The diffusion permeability P_s for several slowly permeating solutes with hemolysis times considerably greater than 1 second have only been measured using the lytic technique, and these data have been included in Table I. It is considered that the technical errors in these permeabilities are much less than those for L_p.

B. Measurement of P_w, the Diffusion Permeability for Water

In order to determine P_w, it is necessary to measure the rate of exchange of labeled water across the red blood cell membrane and calculate its value with the following equation

$$\frac{C^3_{HHO} - C^3_{HHO,0}(1 - \text{fractional } V_{rbc})}{C^3_{HHO} \text{ fractional } V_{rbc}} = \frac{\exp(-P_w A C^3_{HHO,0} t)}{V C^3_{HHO,\infty}} \quad (8)$$

C^3_{HHO} is the concentration of labeled water (in counts) at any time, $C^3_{HHO,0}$ that at zero time, and $C^3_{HHO,\infty}$ that at final equilibrium. Fractional V_{rbc} is the water content of the red blood cells in the mixture expressed as a fraction of the total volume of the suspension. At final equilibrium $t = \infty$,

$$\frac{C^3_{HHO,\infty}}{C^3_{HHO,0}} = (1 - \text{fractional } V_{rbc}) \quad (9)$$

TABLE I

WATER PERMEABILITY OF RED BLOOD CELLS AT ROOM TEMPERATURE (19–25°)

Species	Method[a]	Extracellular osmolality (osmols/liter)	L_p (cm/second)[a]	Reference
Human	Lytic	~0	0.005	Jacobs (1932)
adult	Lytic	0.04	0.002	Höber and Ørskov (1932)
	Lytic	~0.009	0.008	Parpart (1935)
	Lytic	0.169–0.192	0.022	Sjölin (1954)
	Lytic	0.156–0.169	0.0084	
	Stirred vessel	0.2–0.3	0.016	Sjölin (1954)
	Continuous flow	0.150 0.220 and 0.440	0.013	Sidel and Solomon (1957)
	Stopped flow	0.410	0.012	Sha'afi *et al.* (1967)
	Stopped flow	0.300	0.019	Rich *et al.* (1968)
	Stopped flow	~0.300	0.0178 (swelling); 0.113 (shrinking)	Farmer and Macey (1970)
	Stopped flow	0.300	0.015	Blum and Forster (1970)
newborn	Lytic	0.169–0.192	0.0047	Sjölin (1954)
		0.156–0.169	0.012	
	Stirred vessel	0.2–0.3	0.0066	Sjölin (1954)
Dog[b,c]	Lytic	~0	0.008	Jacobs *et al.* (1935)
	Continuous flow	~0.300	0.040	Villegas *et al.* (1958)
	Stopped flow	0.375–0.490	0.020	Rich *et al.* (1967)
	Stopped flow	0.300	0.026	Rich *et al.* (1968)
Cat[c]	Continuous flow	0.380–0.480	0.034	Rich *et al.* (1967)
Horse	Stopped flow	0.300	0.0098	Blum and Forster (1970)
Cow	Lytic	~0	0.005	Jacobs *et al.* (1935)
	Continuous flow	~0.300	0.016	Villegas *et al.* (1958)

	Stopped flow	0.365–0.445	0.016	Rich *et al.* (1967)
	Stirred vessel	0.300	0.041 (swelling); 0.020 (shrinking)	Farmer and Macey (1970)
Chicken[d]	Stopped flow	0.300	0.0012–0035	Blum and Forster (1970)
	Stirred vessel	~0.300	0.0008	Farmer and Macey (1970)
Eel	Stopped flow	0.300	0.0026	Blum and Forster (1970)

Species	Extracellular osmolality (osmols/liter)	P_w (cm/second)[e]	Reference
Human	~0.30	0.0053	Paganelli and Solomon (1957)
adult	~0.305	0.0038–0.004[f]	Barton and Brown (1964)
	~0.30	0.0033	Vieira *et al.* (1970)
fetus	~0.30	0.0023	Barton and Brown (1964)
Dog	0.216–0.474	0.0065	Villegas *et al.* (1958)
	~0.30	0.0046	Rich *et al.* (1967)
	~0.30	0.0057	Vieira *et al.* (1970)
Cow	0.216–0.474	0.0056	Villegas *et al.* (1958)

[a] L_p was measured either by the lytic method, or by changes in light scattering in continuous-flow, stopped-flow rapid-reaction apparatus, or in a simple stirred vessel, as indicated.

[b] Vieira *et al.* (1970) also measured L_p in human and in dog cells but did not give the absolute figures.

[c] Villegas *et al.* (1958) measured L_p in cat and dog at osmolalities greater than and less than 0.300 osmol/liter but found no difference.

[d] L_p did not appear constant in the measurement on chicken red blood cells; the right-hand side of Eq. (5) was not linear as a function of time. Although L_p was larger in hypotonic and smaller in hypertonic solutions, the data are not considered reliable enough to decide whether L_p really varies with extracellular osmolality.

[e] P_w was measured in a membrane filtration continuous-flow rapid-reaction apparatus using tritiated water.

[f] Barton and Brown (1964) used the same and an improved version of the apparatus of Paganelli and Solomon (1957) but obtained this lower value. The essential difference in technique was the extrapolation of the data points to zero time and the use of this value for the initial tritiated water concentration rather than the value calculated from the hematocrit of the suspension and the relative contributions of the reactants.

$C^3_{HHO,\infty}/C^3_{HHO,0}$ can be obtained from the initial and final isotope counts and the relative flows of the two reactant solutions. Fractional V_{rbc} can be obtained from the latter plus the water content of the red blood cells and the hematocrit in the original cell suspension. The extrapolated intercept at $t = 0$ should be unity, providing a check on errors in the hematocrit, the calculated composition of the mixture, and the presence of a time lag in the apparatus.

The only technique developed that is capable of following the exchange of isotopic water with cells is the continuous-flow filtration method that separates a small fraction of suspending fluid from the cells at different times after mixing (Dirken and Mook, 1931; Paganelli and Solomon, 1957; Roughton and Rupp, 1958; Tosteson, 1959). The volume of sample filtered off is so small (1:5000) that its removal certainly does not affect the course of the remaining reaction. Barton and Brown (1964) improved the apparatus and decreased the volume of the reactants required.

A summary of published values is given in Table I. P_w is in general one-third to one-half L_p. If the volume of osmotically effective red blood cell water were the same as the volume in which isotopic water distributed, P_w would be even smaller. L_p is at least an order of magnitude less for nucleated chicken and eel red blood cells than for the other cells listed.

Barton and Brown (1964) reported that the extrapolated zero time intercept of the logarithm of the left-hand side of Eq. (8) did not agree with the value calculated from the composition of the mixture and the hematocrit. In our laboratory a comparison between the extrapolated intercept of the time course of a reaction and the value calculated from the composition of the mixture is used as the most sensitive indicator of time lag errors in an instrument. We are therefore left with the impression that the filtration-type apparatus may have a time displacement artifact in it.

There is a finite stagnant layer of fluid adjacent to the filter which includes mixture at a wide distribution of reactant times. The weighted reactant time for the collected sample is impossible to predict theoretically, even from tests using mixing processes not including reactions. The time displacement error makes the calculated rate of water movement greater than it actually was, particularly near the start of the process. Its greatest effect is on the zero intercept. An analogous artifact exists for any stationary detecting system in a moving stream, such as a P_{CO_2} or P_{O_2} electrode (Rotman *et al.*, 1970).

Fortunately, the diffusion exchange of water is exponential after the first few milliseconds (Paganelli and Solomon, 1957), and the rate can be calculated without critical dependence on the zero intercept. In any case P_w would presumably be increased by any time lag, if it existed. Thus the fact that P_w is smaller than L_p is probably not caused by the above

type of experimental error. P_w has been increased by 14% to compensate for the lower diffusion coefficient of tritiated water as compared with H_2O.

Barton and Brown (1964) corrected the data of Paganelli and Solomon (1957) for a discrepancy in the zero intercept. On the assumption that this is the most accurate calculation, it is included in Table I.

A compilation of published data on L_p and P_w for different species at room temperature is presented in Table I. There are additional reported values of L_p obtained by the lytic method, but in view of the limited accuracy of the results, a representative rather than an exhaustive review is given. Jacobs *et al.* (1935) reported hemolysis times for rat and guinea pig that are about the same as those for human, so the lytic L_p would also approximate the same value. Höber and Ørskov (1932), using the lytic technique but with 0.04 osmol/liter extracellularly rather than a negligible value found hemolysis times t_h in rat, mouse, pig, cow, codfish, pigeon, and duck similar to those for human cells. Chicken red blood cells had a t_h approximately $\frac{1}{10}$ that of human red blood cells, consistent with the results in Table I. Villegas *et al.* (1958) reported that L_p in dog was about twice the value found later by Rich *et al.* (1967). The former also reported that 100% of the water in dog cells was osmotically active, but subsequent investigators in the same laboratory were not able to duplicate this observation (Rich *et al.*, 1967). Even such a large discrepancy in R does not explain the larger L_p for dog, however, and we are led to the conclusion that the results of Villegas *et al.* (1958) for dog are to be ignored.

There is such a variety of methods that only an approximate estimate of the reproducibility of L_p is warranted. Within one dog the range is about 10%; among dogs it is up to 70% (Rich *et al.*, 1968). Inspection of published figures suggests that a range of $\pm 10\%$ for L_p reproducibility in an individual blood is a reasonable representation.

C. Values of L_p and P_w

1. Variation in Water Permeability with Temperature

The available data on the activation energy and Q_{10} from 20° to 30° for both L_p and P_w for several species are summarized in Table II.

The most extensive study on the effect of temperature on L_p and P_w was carried out by Vieira *et al.* (1970). These investigators calculated L_p in cubic centimeters per dyne × second; this requires conversion of osmolality difference into pressure difference involving RT. While there may be disagreement as to whether one should work in concentration or pressure gradients, clearly if L_p and P_w are to be compared they should

TABLE II

EFFECT OF TEMPERATURE ON WATER PERMEABILITY OF RED BLOOD CELLS

Species	Temperature (degrees)	L_p[a] ΔE (calories/mole)	L_p[a] Q_{10}	P_w[a] ΔE (calories/mole)	P_w[a] Q_{10}	Reference
Dog	7–37	4,300 ± 400	1.28	4,900 ± 300	1.28	Vieira *et al.* (1970)
Human	7–37	3,900 ± 400	1.18	6,000 ± 200	1.34	Vieira *et al.* (1970)
	0–50	5,700	1.32	—	—	Jacobs *et al.* (1935)
	4–42	2,800	—	—	—	Blum (1968)
Cow	10–40	4,000	1.22	—	—	Farmer and Macey (1970)
Chicken	10–40	11,400	1.64	—	—	Farmer and Macey (1970)

[a] Q_{10} calculated from 20° to 30°.

be in the same units; in this chapter centimeters per second are used. This involves increasing the activation energy computed by Vieira *et al.* (1970) for L_p by 600 cal/mole.

The activation energy for diffusion in lipids (nonpolar) is less than that in water (polar), values ranging from as little as 1 kcal/mole to nearly 10 kcal/mole, respectively (Stein, 1967, Table 3.1). Since we do not know the precise structure of the red blood cell membrane, we cannot easily conclude whether the activation energies in Table II are compatible with theory or not. If water molecules passing through the membrane collide largely with other water molecules, however, we would expect the activation energy to correspond approximately to that of diffusion in free water. This was determined to be 4.9 kcal/mole (Wang *et al.*, 1953), agreeing with the value for P_w in dog but being less than that for human.

2. VARIATION IN WATER PERMEABILITY WITH SPECIES

The most salient fact is the great similarity of L_p among non-nucleated red blood cells of different species, as found earlier by other investigators (Höber and Ørskov, 1932), and of course the large absolute value of red blood cell permeability, larger than other known cells (Jacobs, 1950). In spite of a variety of methods and experimenters, reliable values differ only by a factor of 2 for a given species, and the permeability for the different animal cells can be arranged in the order: cat > dog > human and beef > horse ≫ chicken > eel.

There is a strikingly lower L_p in nucleated cells, as also noted by Höber and Ørskov. Whether this is because they are nucleated and have more complete biochemical equipment or whether it is primarily a species difference, or a matter of size (Dick, 1964) cannot be ascertained at this time.

P_w is consistently less than L_p in the same species but is remarkably similar in human, dog, and beef, the only animals for which values are known.

Water permeability is less in red blood cells of the human newborn and fetus, possibly related to the presence of more immature and biochemically active forms. Barton and Brown (1964) calculated the theoretical pore radius from their measurements of P_w and the measurements of L_p reported by Sjölin (1954), employing the lytic method, and obtained 3.9 Å. Comparing this with the radius of 4.2 Å (Goldstein and Solomon, 1960) for adult cells, they concluded that the adult cell has a thinner membrane and/or more total pore area, that is, a greater number of pores. L_p for fetal sheep cells appears to increase with gestational age (Widdas, 1951).

3. Variation in Water Permeability with Osmolality and Ionic Strength

L_p values in human (Rich *et al.*, 1968; Blum and Forster, 1970), horse (Blum and Forster, 1970; Farmer and Macey, 1970), dog (Rich *et al.*, 1968), and chicken (Blum and Forster, 1970) red blood cells decrease markedly with increased extracellular osmolality. An obvious conclusion is that the altered external conditions change the permeability of the membrane.

This interpretation may be at least partly ambiguous, however, because a decrease in extracellular osmolality produces swelling under generally achievable conditions, and an increase in extracellular osmolality produces shrinking. It is therefore possible that L_p alters with the direction of flow. In order to test this hypothesis, it is necessary to measure L_p at the same extracellular osmolality during shrinking and swelling. This requires a wide variation in the osmolality of the equilibrated cell suspension before reaction, $C_{e,0}$, and the greater the change in C_e with the reaction the greater this variation in $C_{e,0}$ must be. Neither Rich *et al.* (1968) nor Blum and Forster (1970) varied $C_{e,0}$ sufficiently to provide significant overlap of shrinking and swelling at the same C_e to be sure of this point. Farmer and Macey (1970) measured volume changes with time for slight ($\pm 25\%$) changes in C_e and measured L_p during inflow and outflow over a range of C_e. They reported that for beef cells $L_{p,in}/L_{p,out} = 1.52$, for chicken cells 1.07, and for human cells 1.39. L_p was independent of C_e. It was not logical, however, for them to assume that all the effect of changing C_e is

produced by changing the direction of water flux. We calculated from their tabulated results that $L_{p,out}$ for beef cells decreases markedly with increasing C_e; $L_p = 0.035–4.7 \times 10^{-4}$ mosmol/liter. There are not enough data on beef $L_{p,in}$ to make a recalculation, and the data on human cells are not given. We conclude that while their data show rectification, there is also probably a fall in L_p with increasing C_e, as reported by other investigators. Farmer and Macey (1970) changed osmolality in a cell suspension by injecting 0.1 ml of fluid into a 5-ml cuvette containing a suspension of different osmolality. It is extremely doubtful that the mixing in this apparatus was as fast as that in a Hartridge–Roughton-type instrument and therefore a stagnant layer of significant thickness probably existed around the cells. As Dainty (1963) pointed out, such a stagnant layer decreases $L_{p,out}$ as compared to $L_{p,in}$. An argument against a stagnant fluid layer affecting the measurements is that the values for L_p are the same as those of other workers who used rapid-mixing instruments.

Rectification of water flux across membranes has been reported before, for example, by Lucké *et al.* (1931), who found swelling of *Arbacia* eggs to have a greater rate constant than shrinking. Again, Dainty (1963) has suggested that unstirred layers, inside and outside, may provide an explanation for this phenomenon. Patlak *et al.* (1963) and Blum and Forster (1970) have pointed out that, even in the absence of unstirred layers, if the membrane is made up of several homogeneous layers, rectification can result.

Internal osmolality (C) does not appear to affect L_p since it remains constant [right-hand side of Eq. (5) linear with time] in spite of the fact that C changes as much as ± 200 mosmols/liter during water exchanges (Rich *et al.*, 1968; Blum and Forster, 1970). L_p does not vary when C_0 is varied, although the range of C is usually small (Blum and Forster, 1970).

Rich *et al.* (1968), as well as Sha'afi *et al.* (1970), loaded cells in saline with 0.05 to 0.2 M urea or creatinine at 37° and determined L_p at room temperature when the external concentration of electrolyte was increased but that of the non-electrolyte was kept constant. At this temperature urea is highly permeable but creatinine nearly impermeable. Thus the internal as well as external osmolality was increased initially in the two experiments. The value of L_p was the same, from which it may be concluded that neither the internal osmolality nor the reflection coefficient are important in determining L_p, since the reflection coefficients of urea and creatinine were markedly different. Therefore if Farmer and Macey's finding that rectification occurs is verified, we can conclude that red blood cells show a decreased L_p with increasing C_e, and L_p is independent of C (internal osmolality) except that there is a discontinuity in L_p when $C = C_e$ and flow reverses.

An attractive hypothesis for the effect of external osmolality on L_p is that a difference in activity between water within the membrane and the external fluid produces pressures changing the spatial relationships of the membrane. The experiments of Rich *et al.* (1968) require that the water molecules be in parts of the membrane into which neither urea nor creatinine penetrate.

Veatch (private communication, in Sha'afi *et al.*, 1970) measured P_w for ^{3}HHO in (presumably) human red blood cells that had been placed in 0.3 *M* urea 15 minutes before the experiment as compared with control cells in isotonic saline. Although it is stated that the experiments were not designed to study the effects of osmolality on diffusion permeability to water, P_w was not significantly different in the two circumstances. Villegas *et al.* (1958) had earlier reported that P_w decreased as cell volume increased, an effect on L_p opposite that found by most investigators. Since this was not confirmed by later investigators in the same laboratory, we have neglected it.

The decrease in L_p with increasing extracellular osmolality must take place within a few milliseconds because L_p appears constant over the duration of a swelling or shrinking curve even though the extracellular osmolality changed on mixing. While this may not be an extremely sensitive index, certainly the gross changes in L_p produced by altering extracellular osmolality are complete in 0.010 seconds (Blum and Forster, 1970) or by 0.050 seconds (Rich *et al.*, 1968).

An important question, particularly in view of the charged membrane theory of anion permeability (Passow, 1969) is whether or not ionic strength has a significant effect on L_p. Rich *et al.* (1968) replaced up to one-quarter of the extracellular sodium chloride with sucrose, and Blum and Forster (1970) replaced over three-quarters with sucrose, but both found no effect on L_p other than that of total osmolality.

4. Variation in Water Permeability with Cell Volume

It is difficult to separate the effect of cell volume from that of the osmolality of the cell before mixing, that is, C_0. There are circumstances in which the relationship found between volume and osmolality in normal human adult cells is altered, or at least presumed to be altered, such as in cells of different ages. Sjölin measured L_p with the lytic technique and found that those cells that were more sensitive to hemolysis had a greater L_p; presumably these cells are larger and thicker. The adult human red blood cells that lysed in 84.5–96 m*M* sodium chloride had an average L_p of 0.022 cm/second, while those that lysed in a concentration of 78–84.5 m*M* had an average L_p of 0.0084 cm/second. Cells from newborn humans

(cord blood) gave analogous results, although the absolute values of L_p were about one-half as great (see Table I). The L_p of fetal blood appeared to be independent of hemolytic concentration of sodium chloride.

5. Variation in Water Permeability with Miscellaneous Factors

Rich *et al.* (1968) found that L_p for human red blood cells was independent of pH in the range 6–8.

Tetrodotoxin, which selectively blocks sodium conductance in nerve and muscle (Narahashi *et al.*, 1960), and valinomycin, which increases K^+ and H^+ exchange in mitochondria and alters K^+ permeability of artificial lipid membranes (Andreoli *et al.*, 1967), does not affect L_p detectably (Rich *et al.*, 1968).

The substitution of potassium chloride for sodium chloride in human red blood cell suspensions did not alter L_p (Sha'afi *et al.*, 1967).

Heparin is widely used to prevent coagulation and being an active surface-active agent might alter the permeability characteristics of the red blood cell. At least its effect on P_s and σ should be the same.

Human red blood cells incubated for 1 hour at room temperature in 20 m*M* fluoride, a potent inhibitor of glycolysis, had an L_p one-third less than control cells incubated under the same conditions but without the fluoride (Blum and Forster, 1970). This is interesting considering the findings of Weed *et al.* (1969) that reducing red blood cell ATP concentration is associated with a stiffened membrane, although Sirs (1969) did not find a decrease in L_p in cells stiffened with formaldehyde. In a preliminary note Macey and Farmer (1970) report that incubation of human red blood cells with *p*-chloromercuribenzoate and *p*-chloromercuribenzene sulfonate reduced L_p to as little as $\frac{1}{10}$ its normal value.

D. Errors in the Measurement of L_p and P_w

Some pertinent technical and instrumental errors have been dealt with already in the discussion of the different methods of measuring water permeability. In this section are included several major experimental defects common to all techniques.

In making measurements of the water permeability of the red blood cell membrane, it is absolutely essential that the increment of water activity across the membrane itself be known. It is technically impossible to collect separate samples over such microscopic distances and one is forced to rely on an assumption of complete mixing in the extra- and intracellular phases, obtaining samples from the former.

1. Stagnant Layer around the Cell

Theoretically, there is a stagnant (unstirred) boundary layer of fluid of some finite thickness on cell surfaces, even during mixing or passage down the observation tube of a continuous-flow rapid-reaction apparatus under turbulent flow conditions. A diffusion barrier of this nature might decrease overall P_w (Teorell, 1937), and its possible presence was an early concern in measurements of rapid exchanges of red blood cells (Holland and Forster, 1966). Dainty (1963) has criticized the measurement of P_w on these grounds and points out that volume flux during osmotic flow is impeded less by a layer of this nature than diffusion of labeled water in the absence of net flow. There are, however, several arguments to the effect that the stagnant layer is not of major importance in rapid-mixing apparatus.

(1) Geometrical considerations of the experimental conditions set a maximum average thickness on the fluid shell around each cell. For example, in a mixture with 5% cells by volume, a usual value, this shell would have to be less than 5 μ thick on the average.

(2) It is possible to calculate the diffusion resistance of the red blood cell "membrane," which includes any stagnant layer of fluid, from measurements of the rate of oxyhemoglobin formation in cell suspensions as compared with that in hemoglobin solutions (Forster, 1964; Blum and Forster, 1970). We obtained a maximal estimate corresponding to a layer of saline 1.4 μ thick.

(3) The exchange of oxygen with red blood cells, which has a half-time of about 0.03 seconds, a much faster process than the exchange of water, has the same rate in a stopped-flow rapid-reaction apparatus as in a spectrophotometric continuous-flow rapid-reaction apparatus (Holland and Forster, 1966; Sirs and Roughton, 1963). In the latter, turbulent flow conditions exist along the observation tube, which while presumably not as efficient in maintaining mixing as a four-jet mixing chamber, should be significantly better than no flow at all. If a stagnant layer were important, one would expect the measured rate in the stopped-flow apparatus to be much less than that in the continuous-flow apparatus.

(4) Dainty (1963) points out that an estimate of the importance of unstirred layers in experimental estimates of permeability can be obtained from a summation of the diffusion resistances:

$$1/P_{w,\text{apparent}} = 1/P_{w,\text{true}} + \text{thickness layer}/D_w. \tag{10}$$

For $P_{w,\text{apparent}}$ of 0.005 cm/second, the measured value in Table I, a layer of 5 μ, and D_w equal to 2.3×10^{-5} cm^2/second, the maximal error is 10%.

Limiting calculations show that water movement through such layers would cause a delay of the volume-versus-time curve of 0.2 to 4.5 msec, and the dead time alone of some instruments is over 10 msec (Sha'afi *et al.*, 1967). Based on this species of argument, we do not believe it is technically possible at the present time to determine the effect of a 1- to 5-μ stagnant layer in distorting or displacing the onset of the curve of volume against time (Blum and Forster, 1970).

2. Diffusion Gradients within the Cell

Paganelli and Solomon (1957) calculated a 90% equilibration time of 0.2 msec for the diffusion of isotopically labeled water with a diffusion coefficient of 2.3×10^{-5} cm^2/second into a semiinfinite layer of protein solution 0.5 μ thick, corresponding to a red blood cell. Since the half-time for the exchange of labeled water with human red blood cells is about 4 msec, these authors argued that diffusion gradients with the cell should not retard the overall exchange or produce an erroneous underestimate of P_w. While this conclusion appears justified, Dick (1964) has pointed out that this does not necessarily mean that intracellular water activity gradients do not limit the rate of water exchange during osmotic swelling or shrinking and lead to an underestimate of L_p. This net movement of water across the cell membrane changes the osmolality of the perimeter of the intracellular water; this activity alteration, if not reduced by diffusive mixing, would lower the osmotic driving gradient, decrease the water flux, and lead to an underestimate of L_p. It is important to note that such diffusive mixing involves the movement of hemoglobin, in addition to water itself and the small electrolyte molecules, and the diffusion coefficient of the protein in 30% solution at 37° is $4.5–6.4 \times 10^{-8}$ cm^2/second (Moll, 1966), three orders of magnitude less that the diffusion coefficient of water itself. In the case of labeled water exchange, as in the measurement of P_w, there is no net water movement and the labeled molecules can diffuse around the hemoglobin molecules and mix with the unlabeled water whether the protein moves or not. Dick (1964) obtained an approximate solution for the diffusion of water into and within a semiinfinite layer of protein solution of given thickness and water diffusion coefficient covered with a membrane of permeability L_p. This represents a reasonable model of the red blood cell. An experimental measurement of L_p does not permit us to distinguish between the circumstance in which the cell membrane is infinitely permeable and the entire process is rate limited by diffusion within the cell and the circumstance in which the flux resistance across the membrane is entirely rate limiting. If reasonable values for the diffusion coefficient of hemoglobin and an experimental estimate of L_p are

inserted into Dick's (1964, Fig. 2) computations, we can obtain the true L_p across the membrane. In the case of the human red blood cell, using the value for D_{Hb} above, which should represent a lower limit for the mutual diffusion coefficient, and the experimental value of L_p, 0.013 cm/second (Sidel and Solomon, 1957), there appears to be little limitation of osmotic exchange by intracellular water activity gradients. The diffusion coefficient of water would have to be about an order of magnitude less for intracellular diffusion to be partly rate limiting, and this seems unlikely. Moreover, more than 80% of the osmolality of the intracellular contents is produced by small mobile electrolytes, so that a large portion of any water activity gradients arising within the cell dissipates by their diffusion and the pertinent diffusion coefficients would be of the order of 10^{-6} cm^2/second or greater. This fraction of the osmolality could diffuse around the protein molecules just as the labeled water presumably does.

There is an additional argument against the existence of major water activity gradients inside the cell. Klocke *et al.* (1968) obtained a value of 0.012 cm/second for the ammonia permeability of the human red blood cell using a continuous-flow apparatus and a pH electrode at 37°. Ammonia is hydrophilic and has interatomic distances comparable to water (Weast and Selby, 1966), so both should pass through the membrane with equal ease. At the same time, there is a very large intracellular sink for ammonia. It would ionize almost completely at the intracellular pH. This reaction is for practical purposes instantaneous (Eigen and De Maeyer, 1963). The hemoglobin would act as a buffer, supply H^+ locally, and the resulting NH_4^+ should diffuse almost as rapidly as ammonia, in contrast to the intracellular reactions of carbon monoxide and oxygen with hemoglobin. Thus intracellular gradients should be minimal during ammonia exchanges, and the fact that P_{NH_3} is approximately the same as P_w and L_p argues against there being a significant underestimation of P_w because of water activity gradients in the red blood cell during osmotic swelling and shrinking.

III. REFLECTION COEFFICIENT, σ

One cannot discuss movements of water in red blood cells without a consideration of osmotic forces because it is not technically possible to produce pressure gradients across the red blood cell membrane. Any discussion of osmotic gradients in turn requires consideration of the reflection coefficient σ, defined by the following (Staverman, 1951):

$$J_w = L_p \sigma \Delta C_s \tag{11}$$

ΔC_s is the concentration difference of solute. J_w is the flux of water in

centimeters per second. As pointed out earlier, there has been discussion in the past about the equivalency of hydrostatic and osmotic pressure across the membrane, but there now appears to be general agreement on this point (Chinard, 1952; Stein, 1967).

A. Methods of Measuring σ

Goldstein and Solomon (1960) devised an ingenious method of measuring the reflection coefficient utilizing a continuous-flow rapid-reaction apparatus and following changes in reflected light. A suspension originally in isotonic saline is mixed with a solution of the test molecule in water. Under these conditions the initial osmotic pressure difference of the impermeable electrolytes $\Delta\pi_i$ is opposed by the initial osmotic pressure difference of the test molecule. By varying the osmolality of the test molecule $\Delta\pi_s$ in the mixture, it is possible to find, by interpolation, the $\Delta\pi_s$ at which there is no volume change. It is most convenient to obtain the rate of volume change at zero time, that is, before there has been any significant alteration in the chemical conditions within the cell, because they can be assumed to be the same as in the previous equilibrium state. The zero time rate of volume change can be obtained by extrapolation or, if the record is free from artifact, by a tangent. Then

$$-\frac{\Delta\pi_i}{\Delta\pi_s} = \sigma \tag{12}$$

A stopped-flow rapid-reaction apparatus can also be used to obtain fast volume changes. Unfortunately, this instrument produces marked output artifacts during this portion of the record. Therefore control records in the absence of volume changes must be obtained and subtracted from the experimental record, and even this may not be sufficient (Blum and Forster, 1970; Sha'afi *et al.*, 1970).

In an experiment of this kind, in which the net osmotic pressure initially outside the cell is greater than that inside, the volume first decreases and then, when sufficient permeable solute has moved into the cell, starts to increase. The minimum volume has been used by Jacobs to obtain the solute permeability P_s (Jacobs, 1934; Sha'afi *et al.*, 1970) and can be used to obtain σ since at this point there is no volume flux and Eq. (12) applies.

B. Results of Measurements of σ

Those measurements of σ that are immediately pertinent to a discussion of water permeability in human red blood cells and for which diffusion

permeability values P_s have been published, are assembled in Table III. It should be noted that even though P_s decreases by four orders of magnitude $1 - \sigma$ does not vanish.

For a number of solutes, particularly those less permeable, the only available estimates of P_s were made using the lytic technique of Jacobs (1934) which neglects any interaction between solute and water fluxes; in effect, σ is assumed equal to 1. This is important when L_p and P_s are of comparable magnitude. The largest P_s in Table III (obtained independently by the lytic method) is two orders of magnitude less than L_p however, and therefore, for practical purposes, instantaneous water equilibrium across the cell membrane can be assumed.

Sha'afi *et al.* (1970) have reported measurements on the changes in red blood cell volume with time in a stopped-flow rapid-reaction apparatus from which estimates of σ at minimum volume could have been calculated, although they did not do so. They did publish, however, a curve of the changes in volume following the mixture of a suspension of human red blood cells with a hypertonic solution of urea. One can estimate intracellular urea concentration at the minimum volume either (1) from a permeability for urea of 0.00037 cm/second (Savitz and Solomon, in Katchalsky and Curran, 1965) and the elapsed time at the minimal volume, 0.015 seconds, or (2) back-calculating from the average intramembrane concentration, $\bar{C}_s$, which Sha'afi *et al.* gave in their article. The value of σ obtained is closer to unity than to 0.55, the value reported elsewhere in the same article resulting from interpolation of initial rates of volume change as a function of extracellular urea concentration. The higher estimate of σ is more consistent with the estimates of P_{urea}, although our calculations are necessarily approximate.

IV. DISCUSSION OF MEASUREMENTS OF σ AND P_s

In order to interpret the experimental measurements of solute permeability and reflection coefficient in the red blood cell, it is necessary to review and compare the theories of the mechanism of osmosis and the kinetic basis of the osmotic behavior of permeable solutes.

A. Theory of Osmosis

A kinetic theory of osmosis is helpful in studying the steady-state volume of the red blood cell but is an absolute necessity in studying transient water exchanges. Irreversible thermodynamics has provided powerful insights

TABLE III

COMPARISON OF σ AND P_s IN HUMAN RED BLOOD CELLS[a]

Molecule	Molecular weight	Diffusion coefficient (cm^2/seconds $\times 10^5$)[b]	Molecular radius (Å)[c]	P_s (cm/second)	$1 - \sigma$	$P_s\bar{V}_s/L_p\bar{V}_w$	A_s/A_w from σ	A_s/A_w from P_s and P_w	A_s/A_w from σ divided by A_s/A_w from P_s and P_w
Water	18	2.3	1.9	3.8×10^{-3}[d]	—	0.30	—	1.0	—
Formic acid	46	1.4	2.1	1.5×10^{-3}[e]	<0[e]	0.18	<.18	0.46	—
Acetamide	59	1.3	2.6	$2.9–4.3 \times 10^{-5}$[f]	0.42[g]	0.0055–0.0081	0.41	0.1	41
Urea	60	1.4	2.6	3.7×10^{-4}[h] 4.4×10^{-4}[i]	0.38[g] 0.45[e]	0.062–0.073	0.35–0.42	0.13	2.7–3.2
Ethylene glycol	62	1.3	2.9	1.9×10^{-4}[g]	0.37[g] 0.30[c]	0.04	0.29–0.36	0.064	4.7–5.6
Propionamide	73	1.2	3.0	2.1×10^{-5}[f]	0.20[g]	0.005	0.20	0.008	25
Methyl urea	74	1.1	2.4	1.1×10^{-5}[f]	0.20[g]	0.0029	0.20	0.0042	47
Propylene glycol	76	1.1	3.1	$2.9–5.7 \times 10^{-6}$[j]	0.15[g]	0.00078–0.0026	0.15	0.0011–0.0022	68–136
Thiourea	76	1.2	2.8	$4.7–12.7 \times 10^{-7}$[f]	0.15[g]	9.5×10^{-5}–2.6×10^{-4}	0.15	$1.8–4.9 \times 10^{-4}$	300–830
Glycerol	92	1.0	3.1	5.3×10^{-6}[i]	0.12[g]	0.0015	0.12	0.00064	187
Malonamide	102	0.97	2.6	2.8×10^{-8}[f]	0.17[g]	1.9×10^{-6}	0.17	10^{-5}	17,000

[a] All data were measured at 20°–25° (or have been corrected to that temperature) using the activation energy of Jacobs *et al.* (1935).

[b] Values for diffusion coefficients were obtained from Wang *et al.* (1953) for water and from Longsworth (1954) for urea; all the rest were calculated as equal to D_{H_2O} (18/molecular weight)$^{1/2}$ according to Stein (1967).

[c] Molecular radii were calculated as the radius of a sphere of equal volume and density, except for methyl urea and malonamide which were taken from the measurements of Goldstein and Solomon (1967) on molecular models. Morgan and Warren (1938) obtained a value of 1.5 Å for water from crystallographic data, but this is lower than most other estimates.

[d] Barton and Brown (1964) state that there were technical errors in the original measurements of P_w by Paganelli and Solomon (1957) and so the latter's figure of 0.0053 cm/second has been replaced by the presumably more reliable figure of 0.0038 cm/second.

[e] Klocke *et al.* (unpublished observations).

[f] Höber and Ørskov (1932).

[g] Goldstein and Solomon (1960).

[h] Savitz and Solomon (quoted in Katchalsky and Curran, 1965).

[i] Jacobs *et al.* (1935).

[j] Stein (1967).

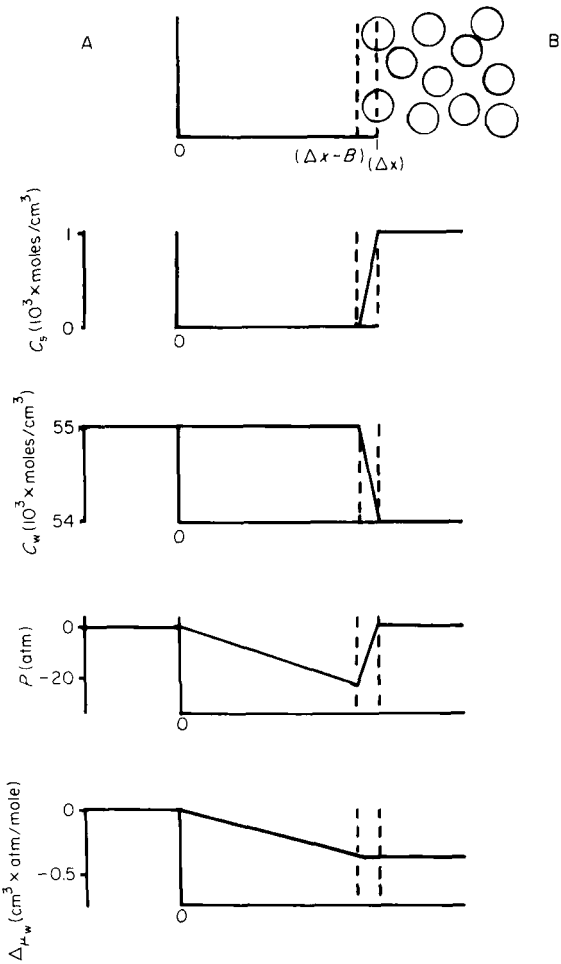

FIG. 1. A diagram of the hypothetical mechanism of osmotic water flux through a membrane produced by an impermeable solute. In the uppermost diagram the solute molecules are shown as circles; the water molecules are not indicated, being much smaller. Solution A is pure water; solution B is the solute in water, both perfectly mixed. The boundary region of the membrane is identified by the dotted lines, and is B centimeters wide. The total thickness of the membrane is Δx. C_s is the concentration of the solute in moles per cubic centimeter assumed for simplicity to be 10^{-3}, that is, 1 M, in B; C_w is the concentration of water in the same units; P is pressure in atmospheres; μ_w is the chemical potential of water in cubic centimeters × atmospheres per mole.

into the overall forces and fluxes during osmotic phenomena but is of less help in uncovering mechanisms or the actual kinetics within the membrane. The molecules of solute and solvent must possess thermal statistical

motion. Over the short distances involved in the membrane, particularly at the surface region, these kinetic energies must produce finite diffusion fluxes, regardless of the existence of possible bulk flow. Since no other forces acting on the molecules over such short distances have been suggested, and since the diffusion theory appears eminently reasonable, we accept it as the basis of the phenomenon. The following discussion owes a great deal to Ray (1960), Mauro (1960, 1965), and Landis and Pappenheimer (1963).

The model mechanism shown in Fig. 1 is presented here for review. The interaction of membrane and solute produces a decrease in solute concentration within the membrane, which must take place over a finite distance, leading to an opposite change in water activity, which in general is accompanied by a decrease in hydrostatic pressure, required to prevent a discontinuity in chemical potential of water. For the special case of an impermeable solute during steady flux at any point through the membrane,

$$J_w = D_{w,m}\left(\frac{dC_s}{dx} - \frac{\bar{V}_w C_w dp}{RTdx}\right) \tag{13}$$

J_w is again the water flux in moles per square centimeter per second; $D_{w,m}$ is the effective diffusion coefficient of water in the membrane; $\bar{V}_w$ is the partial molar volume of water in cubic centimeters per mole; C_w is the concentration of water, C_s that of solute in the membrane water, both in moles per cubic centimeter; R is the gas constant in cubic centimeters $\times$ atmospheres/(degrees Kelvin $\times$ moles); T is temperature in degrees Kelvin; x is the distance through the membrane; p is pressure in atmospheres.

The membrane is considered homogeneous in the y and z planes. No channels or pores are assumed. The first term on the right-hand side represents the water flux produced by the activity gradient of water resulting from the solute concentration gradient; the second term is that produced by the pressure gradient. In this formalism no distinction is made between water diffusion and bulk flow. According to the arguments presented in Section V,B, it appears that bulk flow does not affect the water movements. If this is wrong, the value of $D_{w,m}$ could be altered to take this into account.

If Eq. (13) is integrated from 0 to Δx under steady-state conditions, since there is no difference in p across the membrane,

$$J_w = \frac{C_B - C_A}{\int_0^{\Delta x} \frac{dx}{D_{w,m}}} \tag{14}$$

C_B and C_A are the concentrations of solute on either side of the membrane;

Δx is the total thickness. $D_{w,m}$ is considered a function of x. The mechanism of osmotic flow proposed in Fig. 1 acts to interconvert concentration and pressure forms of chemical potential of water. Note that J_w is independent of the value of p within the membrane and depends on the total concentration gradient across the membrane and the distance weighted average of $D_{w,m}$. Since J_w is constant with x, and C_w is nearly so, Eq. (14) can be substituted into Eq. (13) and the latter solved for dp/dx, giving

$$\frac{dp}{dx} = \frac{RT}{\bar{V}_w C_w}\left(\frac{dC_s}{dx} - \frac{C_B - C_A}{D_w \int_0^{\Delta x} \frac{dx}{D_{w,m}}}\right) \tag{15}$$

If the concentration gradient, which is the first term on the right-hand side, is able to produce the driving force required for the water flux through the membrane, the second term on the right-hand side, then there is no pressure gradient. If dC_s/dx is constant through the membrane, a possible although not highly likely situation, there would be no change in p within the membrane. That is, osmotic water flux does not require that a pressure be developed within the membrane.

The numerical value of $D_{w,m}$ may be greater if there is a pressure gradient than if there is a concentration gradient alone, provided there is a significant possibility of water molecules colliding with each other within the membrane. An extreme example of this is bulk flow.

We are unaware of a comparable expression for the osmotic flow induced by a permeable solute at this time.

The negative hydrostatic pressure within the membrane can become extremely large. For a 1 M difference in water concentration across the total membrane, which is easily achievable experimentally, the negative pressure can reach -25 atm. The internal pressure of water is of the order of 20,000 atm (Moore, 1962), so that the negative pressures expected in the red blood cell membrane should not cause a separation of water molecules, although large forces could be exerted on the membrane structure itself.

The time required for a quasi steady-state flux of solute and water flux through the membrane to be reached should be much less than 1 msec in view of the incompressibility of the system (Sha'afi *et al.*, 1970, Appendix by Mikulecky).

B. Theory of the Reflection Coefficient

When a concentration gradient of permeable solute is produced across the red blood cell membrane, there is a diffusion of solute through the

membrane in the direction opposite the osmotic flow of water. The effect of this solute flux is to decrease the net osmotic flow of water. Goldstein and Solomon (1960) ingeniously determined the reflection coefficient from the osmolality of impermeable solute required to balance instantaneously a given and opposite osmolal gradient of permeable solute. Thus when $dV/dt = 0$, by definition (Staverman, 1951)

$$L_p \Delta C_i \bar{V}_w = L_{pD} \Delta C_s \bar{V}_w \tag{16}$$

where L_p is the hydrodynamic permeability of the membrane or mechanical filtration coefficient and L_{pD} is the osmotic coefficient, both in centimeters per second, and ΔC_i and ΔC_s are the concentrations of impermeable and permeable solute, respectively, in osmols per cubic centimeter, and $\bar{V}_w$ is the partial molal volume of water. The value of σ, which is defined as L_{pD}/L_p by our convention, equals $-\Delta C_i/\Delta C_s$.

The relationship between σ, L_p, and the diffusion permeability of the membrane for the solute, P_s, can be derived on a kinetic basis as follows (see Kedem and Katchalsky, 1961):

$$\begin{matrix}\text{Total volume flux} \\ \text{produced by } \Delta C_s\end{matrix} = \begin{matrix}\text{Volume flux of water} \\ \text{produced by } \Delta C_s\end{matrix} + \begin{matrix}\text{Volume flux of solute} \\ \text{produced by } \Delta C_s\end{matrix} \tag{17}$$

where all fluxes are in cubic centimeters/(seconds $\times$ square centimeters). The total volume flux equals $L_{pD} \Delta C_s \bar{V}_w$ by definition (Katchalsky and Curran, 1965). The osmolal difference across the membrane would produce a flux of water equal to $-L_p \Delta C_s \bar{V}_w$ except that the diffusion of solute through the water in the membrane produces an opposing force on the water, decreasing the net flow. This frictional force on the water, in dynes per cubic centimeter for a solute flux of 1 osmol per square centimeter $\times$ second, equals $RT/D_{s,w}$, where $D_{s,w}$ equals the diffusion coefficient for the solute in water in square centimeters per second. If all of the solute is assumed to pass through the water-filled parts of the membrane, the total frictional force per square centimeter of membrane equals:

$$RTP_s \Delta C_s \Delta x/(D_{s,w} F_w \vartheta) \tag{18}$$

$\Delta x/\vartheta$ is the effective length of the diffusion path through the membrane, Δx being the total thickness and ϑ originally defined as a "tortuosity" factor (Kedem and Katchalsky, 1961). F_w is the proportion of the membrane that is water. If macroscopic homogeneity is assumed, F_w also equals the fraction of the membrane cross section occupied by water. This factor is required because it is assumed that all the solute flux passes through the watery part of the membrane. The back-flux of water produced by solute drag on the water equals the total frictional force [Eq. (18)] times $L_p \bar{V}_w/RT$. The flux of solute equals $-P_s \Delta C_s \bar{V}_s$. Therefore, substituting

in Eq. (17),

$$L_{pD}\Delta C_s\bar{V}_w = L_p\bar{V}_w\left[\Delta C_s - \left(\frac{\Delta C_s P_s \Delta x}{D_{s,w}F_w\vartheta}\right)\right] + \Delta C_s P_s \bar{V}_s \tag{19}$$

Dividing through by $L_p\bar{V}_w\Delta C_s$ and rearranging,

$$1 - \sigma = + \frac{P_s\bar{V}_s}{L_p\bar{V}_w} + \frac{P_s\Delta x}{D_{s,w}F_w\vartheta} \tag{20}$$

This is the classic expression for σ derived by Kedem and Katchalsky [1961, Equation 4-10]. The first term on the right-hand side is the ratio of the permeability of solute to the mechanical filtration coefficient of the membrane, in the same units. This ratio is generally small, and several workers have neglected it with impunity (Landis and Pappenheimer, 1963; Goldstein and Solomon, 1960).

The major reason that the instantaneous water flux for a permeable solute is less than that for an impermeable solute lies in the second term on the right-hand side of Eq. (20); this represents the "drag" of *solute* on *solvent* (see Kedem and Katchalsky, 1961, esp. p. 155). This term equals the permeability of the membrane to solute (assumed to pass only through water in the membrane) divided by the permeability the water in the membrane would have to solute diffusion if there were no friction between solute and membrane; that is, if the hypothetical pores were so large as to impose no restriction to solute movement. It is important to note that $D_{s,w}$ in Eq. (20) is not a restricted coefficient but that in free solution. It appears in the expression as a measure of the frictional force between solute and water independent of the membrane's interactions, at least as a first approximation. The virtual water flux produced by solute drag is in the direction opposite the osmotic flux resulting from the water activity gradient and may be small in absolute terms but is important in relation to $L_p\bar{V}_w\Delta C_s$, about one-half in the case of a urea gradient across the red blood cell membrane.

If the solute drag term is significant, that is, if $1 - \sigma$ is greater than $P_s\bar{V}_s/L_p\bar{V}_w$, this means that an important fraction of solute flux is passing through water in the membrane, but it does not necessarily mean that there is bulk flow of solution or that solute flow increases measurably with solvent flow as stated by Stein (1967), nor even that the watery paths need be continuous through the membrane.

Equation (20) is not correct for the situation in which a significant fraction of solute flux passes through nonwatery, lipophilic regions of the membrane. In these circumstances P_s in the second term on the right-hand side should be that of the solute in the water of the membrane and not that of the membrane as a whole.

In the restricted-diffusion concept of Pappenheimer (Pappenheimer *et al.*, 1951; Renkin, 1954), the difference between solute diffusion in the water of the membrane, as contrasted with diffusion in water in free solution, is represented as a decrease in the effective diffusion area in the pore available for the solute A_s. Thus:

$$P_s = nA_sD_{s,w}/\Delta x \tag{21}$$

where n is the number of pores per square centimeter of membrane. Pappenheimer and associates (Renkin, 1954) provided a semiempirical relation between A_s and the true geometric cross-sectional area of the pore A_p as a function of the radius of the solute molecule r_s and the radius of the pore r_p:

$$\frac{A_s}{A_p} = \left[1 - \left(\frac{r_s}{r_p}\right)\right]^2\left[1 - 2.104\left(\frac{r_s}{r_p}\right) + 2.09\left(\frac{r_s}{r_p}\right)^3 - 0.95\left(\frac{r_s}{r_p}\right)^5\right] \tag{22}$$

This mathematical statement includes both a steric factor, representing a decreased probability of the molecule hitting the available opening, as well as an effective decrease in diffusion coefficient within the membrane because of the probability of hitting the walls.

In an analagous fashion A_w is the restricted pore area available for the diffusion of water through the membrane. A_w is considerably less than the area of the pore A_p; for example, considering water to have a radius of 1.5 Å and r_p = 6.0 Å, which is large, A_w would be only 28% of A_p. The tortuosity factor ϑ is essentially immeasurable but can be defined:

$$A_w = F_w\vartheta \tag{23}$$

Therefore Eq. (20) can be expressed:

$$1 - \sigma = (A_s/A_w) + (P_s\bar{V}_s/L_p\bar{V}_w) \tag{24}$$

The relative retention of a solute, or sieving, when a solution is forced through a membrane by hydrostatic pressure can be (and has been) used to give an estimate of A_s/A_w according to the following relationship (Pappenheimer, 1953; Renkin, 1954).

$$\frac{A_s}{A_w} = \frac{C_D}{C_U}\left[1 + \left(\frac{D_{s,w}}{J_v}\right)\left(\frac{A_s}{\Delta x}\right)\right] - \frac{D_{s,w}}{J_v}\left(\frac{A_s}{\Delta x}\right) \tag{25}$$

where C_D is the downstream concentration of solute and C_U the upstream value. J_v is the total volume flow through the membrane. This equation is derived on the assumption that the gradient of solute concentration through

the membrane is constant,* whereas it is exponential, but the error involved is minor. Experimentally, $A_s/\Delta x$ is determined by measuring the diffusion of labeled water through the membrane under a condition of zero net flux.

Sieving cannot be used to measure σ in the red blood cell because a pressure cannot be produced across the membrane, but the method has been useful in large, and artificial, membrane studies.

C. Discrepancy between σ and P_s

As Stein (1967) has indicated, there appears to be an inconsistency between the solute permeability of the red blood cell P_s and the reflection coefficient σ. In view of this contradiction, it seemed important to examine comparisons of σ and P_s in membranes simpler than that of the red blood cell, namely, in artificial membranes.

1. Measurements in Artificial Membranes

Only a limited number of observations comparing P_s and $1 - \sigma$ on the same membrane are available but, fortunately, Renkin (1954), Durbin (1960), and Ginzburg and Katchalsky (1963) all studied the movements of sucrose in what appears to be the same dialysis tubing. We have recalculated their results and they are presented in Table IV. The consistency of the measurements of P_s and σ can be determined either by comparing the hypothetical pore radii or the ratio A_s/A_w, that is, restricted pore area for sucrose/restricted pore area for water, calculated therefrom.

a. Calculation of Pore Radius r_p. i. Estimation from Measurements of P_s and P_w. If Eqs. (21) and (22) are divided by analogous expressions for A_w, the restricted-diffusion area for water, the following relationship is obtained.

$$\frac{A_s}{A_w} = \frac{P_s D_w}{P_w D_{s,w}} = \frac{\left[1 - \left(\frac{r_s}{r_p}\right)\right]^2\left[1 - 2.104\left(\frac{r_s}{r_p}\right) + 2.09\left(\frac{r_s}{r_p}\right)^3 - 0.95\left(\frac{r_s}{r_p}\right)^5\right]}{\left[1 - \left(\frac{r_w}{r_p}\right)\right]^2\left[1 - 2.104\left(\frac{r_w}{r_p}\right) + 2.09\left(\frac{r_w}{r_p}\right)^3 - 0.95\left(\frac{r_w}{r_p}\right)^5\right]} \tag{27}$$

D_w represents the self-diffusion coefficient of water. If we now substitute physical estimates of r_s, r_w, D_w, and $D_{s,w}$, as well as experimental values

* A more correct relationship is

$$\frac{A_s}{A_w} = \frac{C_D}{C_U}\left[1 - \exp\left(\frac{-J_v A_s}{D_{s,w} A_w}\right)\left(\frac{\Delta x}{A_s}\right)\right] \tag{26}$$

TABLE IV

COMPARISON OF PORE RADII AND RESTRICTED DIFFUSION AREA FOR SUCROSE IN DIALYSIS TUBING[a]

Reference	L_p ($\times 10^2$ cm/second)	P_w ($\times 10^2$ cm/second)	P_w/L_p	P_s ($\times 10^5$ cm/second)	σ	Pore radius[b]			$\frac{P_s\bar{V}_s}{L_p\bar{V}_w}$	A_s/A_w	
						From P_s and P_w	From σ	From L_p and P_w		$\frac{P_s D_w}{P_w D_{s,w}}$	$1-\sigma-\left(\frac{P_s\bar{V}_s}{L_p\bar{V}_w}\right)$
Renkin (1954)	1.32	0.044	0.028	3.6	0.45[c]	18	18	18.9	0.034	0.34	0.52
Durbin (1960)	1.54[d]	0.035	0.022	—	0.37[e]	—	29	23	—	—	~0.63
Ginzburg and Katchalsky (1963)	4.7	0.11	0.024	9.8–8.7	0.163[e]–0.114	19.5–18	>60	23	0.026–0.023	0.38–0.33	0.81-0.86

[a] These data were calculated for the following conditions: radius of water molecule, 1.9 Å, of sucrose, 5.3 Å; temperature, 25°. Diffusion coefficient for H_2O, ^3HHO, and D_2O is 2.3×10^{-5} cm²/second, and for sucrose, 0.55×10^{-5} cm²/second (Renkin, 1954).

[b] Calculations of radii from P_s were made using Renkin's Equation 11, which combines steric hindrance at the entrance of the pore with frictional resistance within the pore. Analogous calculations from reflection coefficients utilized Renkin's Equation 19 with an additional steric correction allowing for a radial gradient of velocity across the pore.

[c] σ was calculated from filtration data, the "sieving coefficient" (Renkin, 1954).

[d] The total area of the membrane was taken to be 1.54 cm² (Ginzburg and Katchalsky, 1963).

[e] The value of σ was calculated from osmotic flow and hydraulic flow.

of P_s and P_w, we can solve for r_p graphically. The two values in Table IV are reasonably consistent.

ii. ESTIMATION FROM MEASUREMENTS OF σ. A modification of Eq. (22) has been derived (Renkin, 1954) for A_s/A_p when there is filtration, in order to take account of the increased probability of a solute molecule entering a pore because of the higher linear velocity of fluid near the axis under viscous flow conditions. This modified equation for A_s/A_p can be divided by a similar expression for A_w/A_p to give an equation for A_s/A_w:

$$\left(\frac{A_s}{A_w}\right)_{filtr} = \frac{\left\{2\left[1-\left(\frac{r_s}{r_p}\right)\right]^2 - \left[1-\left(\frac{r_s}{r_p}\right)\right]^4\right\} \times \left[1 - 2.104\left(\frac{r_s}{r_p}\right) + 2.09\left(\frac{r_s}{r_p}\right)^3 - 0.95\left(\frac{r_s}{r_p}\right)^5\right]}{\left\{2\left[1-\left(\frac{r_w}{r_p}\right)\right]^2 - \left[1-\left(\frac{r_w}{r_p}\right)\right]^4\right\} \times \left[1 - 2.104\left(\frac{r_w}{r_p}\right) + 2.09\left(\frac{r_w}{r_p}\right)^3 - 0.95\left(\frac{r_w}{r_p}\right)^5\right]} \quad (28)$$

An experimental estimate of $(A_s/A_w)_{filtr}$ was obtained from measurements of sieving by the membrane using Eq. (25), which permitted graphical solution of Eq. (28) for r_p because r_w is known. While the estimate of r_p from σ for Renkin's data is consistent with the other estimates of r_p from the same investigator, the estimates of r_p from σ values determined by the other investigators are not.

iii. ESTIMATION FROM MEASUREMENTS OF L_p AND P_w. A third estimate of r_p can be obtained from the hydrodynamic permeability of the membrane L_p, ostensibly assuming viscous flow through a cylindrical pore. From the Poiseuille relationship, it can be determined that

$$r_p = 2\left[\frac{2L_p\eta}{(RTnA_w/\Delta x)}\right]^{1/2} \quad (29)$$

where η is the viscosity of water in free solution in poises (dynes $\times$ seconds per square centimeter), nA_w is again the fractional cross-sectional area of the membrane made up of water, and Δx is the membrane thickness. This relationship, although widely used to estimate pore size in artificial membranes (Ferry, 1936), gives $r_p = 7.5$ Å for Renkin's data, which presumably is grossly in error. A likely fault is the substitution of fractional

water content for the total fraction of surface area in the pores. Pappenheimer (1953) ingeniously used an estimate of $A_w/\Delta x$ obtained from measurements of the diffusion of labeled water through the membrane according to Eq. (21) for water. The estimates of r_p computed in this manner are remarkably consistent in the case of Renkin's results and at least acceptably consistent among the three investigators. r_p for the dialysis tubing is at least 10 times greater than the radius of the water molecule, but even so the theoretical restricted pore area for diffusion of water is 35% less than the geometrical pore area. It should be mentioned that this computation combines data obtained under conditions with (L_p) and without (P_w) hydrodynamic flow but provides a consistent value of r_p. This might be taken as evidence for the existence of Poiseuille-type flow through the membrane (although arguments against the existence of differences in linear velocity as a function of radius are given in Section VI,G), or of no bulk flow during measurement of L_p.

b. Calculation of A_s/A_w. *i.* ESTIMATION FROM P_s AND P_w. A_s/A_w can be calculated as $P_sD_w/P_wD_{s,w}$ [Eq. (27)]. Although the polynomial restricted-diffusion equation is not directly involved, there is an implication that the differences in membrane permeability to sucrose and water can be assigned to differences in their diffusion coefficient in solution and restricted pore areas. The last-mentioned encompasses many factors.

ii. ESTIMATION FROM σ. A_s/A_w can also be calculated from measurements of σ according to Eq. (24). The small term, $P_s\bar{V}/L_p\bar{V}_w$, has been subtracted from $1 - \sigma$, but this does not imply that the value of A_s/A_w is dependent on P_s.

The two values of A_s/A_w are reasonably consistent for the data of Renkin but are certainly different for the data of Ginzburg and Katchalsky, in the last case apparently as a result of the value for σ, which is much less than that of the other two investigators. This discrepancy in the data was not immediately apparent because it was absorbed in the tortuosity factor.

In conclusion, the experimental measurements of σ and P_s for sucrose in dialysis tubing, limited in extent, show a reasonable consistency only for the data of Renkin. In view of the marked discrepancies between the results of P_s and σ in red blood cells, it would be helpful to have more extensive and consistent data on artificial membranes to substantiate experimental techniques and theory in application.

A_s/A_w is a more useful index to compare the experimental measurements of σ and P_s than the hypothetical pore radii because it changes a great deal for very small changes in pore radius and because the empirical equations of restricted diffusion, which may introduce some bias, need not

be invoked. In the calculation of r_p, the value chosen for the effective radius of water is often extremely critical. Solomon and his group generally use 1.5 Å (Rich *et al.*, 1967) but there appear to be equally good and recent arguments for a value of 1.9 Å (Renkin, 1954). Unfortunately, the larger value gives unreasonable calculations of A_s/A_w (for example, P_s for urea is less than $\frac{1}{10}$ that for water in the human red blood cell, but with a molecular radius of 2.03 Å (Rich *et al.*, 1967) and a pore radius of 4 Å, Eq. (22) predicts a difference of only 30%).

2. Measurements in Red Blood Cells

There are only limited data on P_s and σ under comparable conditions in the same red blood cells, and these are assembled in Table III.

According to Eq. (20), $1 - \sigma$ should be proportional to P_s except for slight changes in the diffusion coefficient of the solute in water $D_{s,w}$ which can be predicted as being proportional to $1/(\text{molecular weight})^{1/2}$ (Stein, 1967). Figure 2 is a plot of $\log_{10}(1 - \sigma)$ against $\log_{10} P_s$ for the data in Table III. Logarithms are used only to encompass the large range of P_s. A theoretical line is included in the figure merely to illustrate the slope that would be expected for the range of molecular weights. The absolute $\log_{10} P_s$ of this line has been chosen so that it passes through the point for malonamide as a matter of convenience. Although $\log_{10}(1 - \sigma)$ increases with increasing $\log P_s$, as predicted by Eq. (20), it does so at about 100

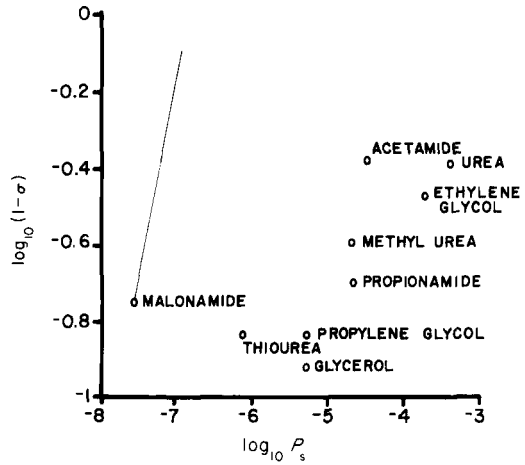

Fig. 2. A graph of $\log_{10}(1 - \sigma)$ plotted against the $\log_{10}$ of P_s for a series of uncharged hydrophilic solutes at room temperature. The basic data are given in Table III. The solid line is the theoretical relationship predicted by Eq. (20), referred to the point for malonamide.

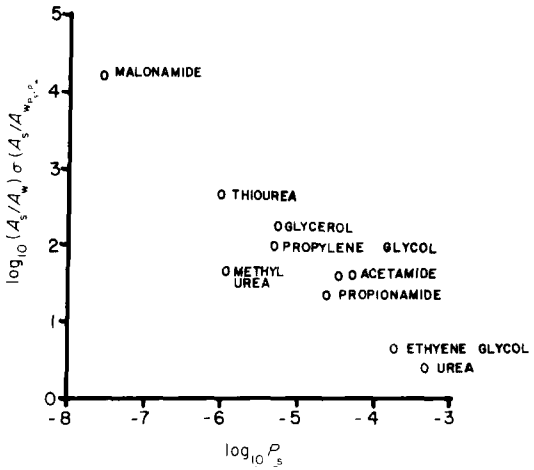

FIG. 3. Graph of $\log_{10}$ (A_s/A_w calculated from $\sigma/A_s/A_w$ calculated from P_s and P_w) versus $\log_{10} P_s$ for a series of molecules in human red blood cells.

times the expected rate. P_s varies over three orders of magnitude, while $1 - \sigma$ varies over less than one order of magnitude.

A comparison of the experimental measurements of P_s and σ can also be made in the form of their A_s/A_w. These values have been computed in Table III. Figure 3 shows $\log_{10}(A_s/A_w$ computed from experimental measurements of σ over the same ratio calculated from experimental measurements of P_s and P_w) plotted against $\log_{10} P_s$. Theoretically of course the ratio should be unity, which it approaches at a P_s of about 0.01 cm/second. It is clear that the discrepancy increases with decreasing permeability of the red blood cell membrane to the solute, reaching more than four orders of magnitude for the least permeable molecule studied. Savitz and Solomon (quoted in Sha'afi *et al.*, 1970) compared P_s for urea measured by the tracer method using a continuous-flow filtration apparatus with the value calculated from volume changes as a function of time according to the method of Jacobs (1930), obtaining the values 0.0004 and 0.00034 cm/second, respectively. This is an instance in which experimental measurements of permeability of a solute give the same results with and without a water flux. Although the value of P_{urea} varied with flux, the extent of this variation is much less than the discrepancy between estimates of A_s/A_w from measurements of σ and of P_s and P_w. As discussed in Section III,A, the values of P_s and σ for urea are inconsistent even though calculated from the same minium volume curve.

Rich *et al.* (1967) have provided limited data on dog red blood cells, sufficient, however, to suggest that the discrepancy between σ and P_s

seen in human red blood cells is also present in this other species. They determined σ by measuring dV/dt as the tangent at the start of swelling and shrinking curves in a light-scattering stopped-flow apparatus (Sha'afi *et al.*, 1967) and obtained a value of 0.38. They also report a preliminary estimate of permeability for urea, $P_s = 0.00062$ cm/second, presumably obtained by a minimal volume method in a stopped-flow apparatus (Sha'afi *et al.*, 1970). P_w was 0.0046 cm/second as measured with an improved filtration continuous-flow apparatus (Barton and Brown, 1964). Calculating A_s/A_w as for Table III, we obtain 0.22. $1 - \sigma - (P_s\bar{V}_s/L_p\bar{V}_w)$ is 0.55. The value of

$$\log_{10}\left[\frac{(A_s/A_w)_\sigma}{(A_s/A_w)_{P_s,P_w}}\right]$$

would fall on the points plotted for urea in Fig. 3, the results for human red blood cells.

The measured values of P_s for different solutes in human red blood cells are not consistent over their entire range with the variations predicted by the restricted diffusion area theory [Eq. (22)]. While this point has been elaborated by Stein (1967), it is worthwhile to mention here the magnitude of this discrepancy. If radius of urea/radius of water = 2.6/1.9, then in order to obtain the measured permeability urea/permeability water = $1/10$, the radius of urea must be greater than 90% of the radius of the pore. In other words, the radius of the pore must be ≤ 2.9 Å. Since the theory does not permit passage of molecules with a radius greater than the pore, all the other hydrophilic molecules that permeate the membrane must have radii between 2.6 and 2.9 Å. Regardless of the theoretical possibility of fitting all the experimental data to predictions, the precision required for the radii of the solute molecules is unreasonable; they would have to be known to better than hundredths of an ängström for the less permeable solutes.

3. Discussion of the Discrepancy between P_s and σ

It is incumbent upon us to provide some explanation for the small value of permeability and/or the high value of $1 - \sigma$ for hydrophilic solutes in the red blood cell membrane. Unfortunately, there appears to be no completely satisfactory reason at this time (Stein, 1967). Several possibilities have been or can be suggested.

a. Experimental Error. The actual measurements are sophisticated and rely on different rapid-reaction techniques. The results of different investigators using different apparatus are surprisingly consistent in supporting the discrepancy, however. A stagnant layer on either side of the cell membrane tends to make diffusion slower, that is, P_s lower, in relation to

osmotic flux (see Section II,D). The effect of this artifact, however, should become magnified as the rapidity of diffusion increases; that is, the discrepancy between P_s and $1 - \sigma$ should become *greater* as permeability increases. Figures 2 and 3 clearly indicate the opposite.

Inspection of Fig. 2 shows that $\log_{10}(1 - \sigma)$ decreases approximately linearly with decreasing $\log_{10} P_s$ but fails to decrease below -0.8 to -0.9 although $\log_{10} P_s$ falls several log units further. The failure of $\log_{10}(1 - \sigma)$ to decrease is probably not a result of random statistical error, because if this were true we would expect some values of $\log_{10}(1 - \sigma)$ less than -1, and these are not seen.

Although there are uncertainties in the techniques, such as the failure of the extrapolated volume-time curves to intercept at zero volume change in the continuous-flow rapid-reaction apparatus (Sidel and Solomon, 1957), it appears unlikely at this time that a technical error produces the discrepancy.

b. Parallel Paths through the Membrane. The solute may diffuse through a lipophilic path in the membrane, as well as through the water in the membrane. In this case the term representing solute drag in Eq. (20), $\Delta x P_s / D_{s,w} F_w \vartheta$, would be decreased, as it applies only to that fraction of total solute flux that passes through the water. If the magnitude of the solute flux is large enough, $\bar{V}_s P_s / L_p \bar{V}_w$ becomes large and increases $1 - \sigma$. For example, the permeability of the human red blood cell for valeric acid is 0.027 cm/second (Klocke *et al.*, 1971). With L_p equal to 0.015 cm/second, $\bar{V}_s P_s / L_p \bar{V}_w = 11.8$, so that $1 - \sigma$ is greater than 1 and σ is less than 0. For the hydrophilic solutes in Table III, however, the $\bar{V}_s P_s / L_p \bar{V}_w$ term is extremely small. Therefore a parallel path through lipid does not explain the apparent overestimate of $1 - \sigma$.

Active transport of solute, in addition to diffusion, might occur. This could be considered another form of parallel pathway and ruled out as a significant factor in producing the discrepancy. A mechanism that carries water in a direction opposite that of the osmotic flux would be required.

c. Solvent Drag. Bulk or hydrodynamic flow of solution through the membrane might "drag" the solute molecules in the direction opposite their diffusion flux. The question of solvent drag is considered in Section V but can be disposed of here by pointing out that measurements of σ are made under conditions of zero net volume flow.

d. Movement of Electrolyte across the Membrane. It is assumed in most considerations of volume changes in red blood cells that the total amount of osmotically active substance inside the cell, mainly electrolyte, is constant. This is not unreasonable for a few seconds but may not be justified

for longer periods, such as are required for hemolysis with substances as impermeable as malonamide. While electrolytes are less permeable than water and nonelectrolytes in general, significant quantities might exchange over tens of seconds (Passow, 1969). In measuring σ there is, at least initially, a normal concentration of electrolytes intracellularly, but much less, usually half this osmolality, extracellularly. This produces at least a diffusion exchange of internal Cl^- for external OH^- (Crandall *et al.*, 1971; Wilbrandt, 1940; Jacobs and Stewart, 1947). Goldstein and Solomon (1960), Rich *et al.* (1967), and Sha'afi *et al.* (1970) all used a control reaction with glucose extracellularly, a molecule considered impermeable, in an attempt to compensate for any volume changes secondary to these electrolyte movements. Rich *et al.* (1968) measured L_p for human red blood cells during shrinking, with 290 mosmols/liter intracellularly and a total of about 385 mosmols/liter extracellularly, in one case entirely made up of sodium chloride and in another case with 100 mosmols/liter sucrose. L_p was the same in both cases, indicating that the osmotic effect of a gradient of impermeable electrolyte and nonelectrolyte on water flux is the same, as expected. Cass and Finkelstein (1967) found, however, that L_p for a thin membrane made from ox brain lipid was greater when the osmotic gradient was produced by sucrose or glucose than when it was produced by sodium chloride.

Rich *et al.* (1967) discussed the problem of measuring σ close to unity, at least in the case of nonelectrolytes, and came to the conclusion that they could discriminate technically between a σ of 1 and a σ of 0.96, but they were unable to detect changes produced by 20 mosmols/liter osmotic imbalance, nor could they find a difference between σ for glucose and that for sucrose. If a 6.0-Å pore radius and 1.5-Å water molecule radius are assumed, according to the restricted pore area theory, σ for sucrose should have been about 3% greater than for glucose. This could also mean that they both were really unity.

e. Error in Theoretical Argument. The discrepancy between the experimental measurements of σ and P_s might lie not in the actual measurements but in the theoretical arguments required to compare them.

The expression for σ as a function of A_s/A_w [Eq. (24)] depends upon the basic expression for σ as a function of the permeability of the membrane for solute and water [Eq. (20)] plus the assumption, or definition, that $nA_w = F_w\vartheta$ [Eq. (23)]. F_w is unambiguously the fraction of the membrane made up of water; homogeneity is consistently assumed throughout. ϑ is more arbitrary but in the kinetic development is specifically a factor to correct the actual thickness of the membrane Δx to the average distance a solute molecule passes through water in traversing the membrane (tortuosity).

We obtain A_s/A_w from P_s and P_w, however, by means of the restricted diffusion arguments in which, by analogy with Eq. (21),

$$nA_w = P_w\Delta x/D_w \tag{30}$$

where D_w is the diffusion coefficient of water in liquid water. A_w in this statement includes a tortuosity factor but in addition contains a factor proportional to the available diffusing area (restricted) for water in the membrane. According to Eqs. (23) and (30),

$$F_w\vartheta = \frac{P_w\Delta x}{D_w} \tag{31}$$

In this case ϑ is a function of the real tortuosity as well as the restricted diffusion area. Therefore ϑ has a different definition when used to describe σ than when used to describe the restricted diffusion area for water [Eq. (22)]. This means that A_w in the restricted diffusion theory is not exactly the same as in the theory of σ. Fortunately, this difference is constant and, since the restricted diffusion theory [Eq. (21)] is used to relate A_s to P_s for the derivation of both Eqs. (24) and (27), this theoretical inconsistency would alter $(A_s/A_w)_\sigma/(A_s/A_w)_{P_s,L_p}$ by a constant factor. Thus the graph in Fig. 3 should be parallel to the abscissa, although not necessarily at a zero value of the ordinate. Clearly, this theoretical error does not provide an explanation for the variation in $\log[(A_s/A_w)_\sigma/(A_s/A_w)_{P_s,P_w}]$ with $\log_{10} P_s$.

Intuitively, as P_s decreases a point should certainly be reached where $1 - \sigma$ becomes practically zero independent of the arguments of Eq. (20), and Fig. 2 demonstrates that this is not true.

V. SOLVENT DRAG

We have seen the effect of the fractional drag of a solute flux on water in the membrane in decreasing σ. Another approach to the study of the interaction of solute and water in the red blood cell membrane is to measure the effect of total solution flux, or water flux, on the transport of solute; in other words, solvent drag (Anderson and Ussing, 1957). We have attempted to demonstrate solvent drag experimentally and have come to the conclusion that solute diffusion through the red blood cell membrane is so rapid that it is at present technically impossible to produce a net volume flow great enough to effect measurable differences in solute movement. In the following section we develop theoretical arguments concerning solvent drag from kinetic and irreversible thermodynamic goints of view and present limited experimental observations.

A. Solvent Drag from a Diffusion Approach

The diffusion of a solute in a stream of flowing solution (Fig. 4) can be described by the equation (Jost, 1952)

$$J_s = -D_{s,w}\left(\frac{dC_s}{dx}\right) + vC_s \tag{32}$$

where J_s is the flux of solute in osmols per square centimeter $\times$ seconds, $D_{s,w}$ is the diffusion coefficient of the solute in the solution in the channel within the membrane in square centimeters per second, and v is the average velocity of the stream in centimeters per second.

The first term on the right-hand side is the diffusion flux of solute J_d in water in the membrane in reference to a fixed x, that is, in reference to the membrane. The second term on the right-hand side is the flux of solute at x as it is carried by the total solution flux, that is, solvent drag. It is implicit in the statement that there is no frictional force between the solute and the membrane and that the diffusion coefficient $D_{s,w}$ is that for the solute in free water. The effect of pressure on diffusion of the solute itself has been neglected as insignificant.

If C_B and C_A are the concentrations of solute in the solutions on the right- and left-hand sides of the membrane, respectively, the solution of

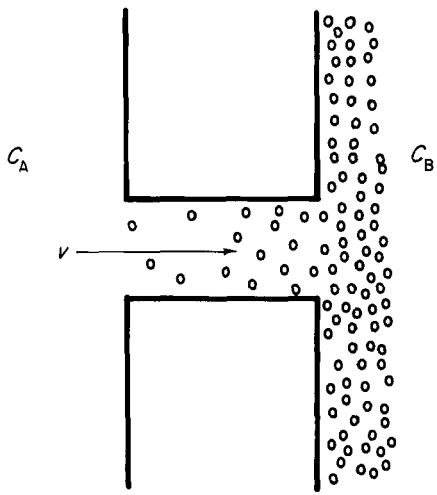

FIG. 4. Diagram illustrating solvent drag in membrane. Solution flows from inside to outside (left to right) with mean velocity v. C_A and C_B are the osmolalities of the solutions inside and outside, respectively. The open circles represent molecules of diffusing solute.

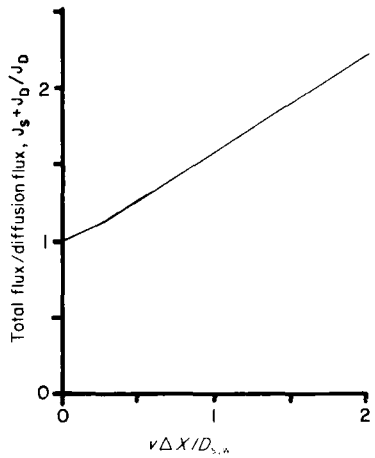

FIG. 5. A graph of the theoretical total flux of solute, that is the diffusion flux plus solute that is "dragged" or convected, divided by the diffusion flux of solute, plotted against the dimensionless constant $v\Delta x/D_{s,w}$.

Eq. (32) is

$$J_s = v\left(\frac{C_B - C_A \exp(+v\Delta x/D_{s,w})}{1 - \exp(v\Delta x/D_{s,w})}\right) \tag{33}$$

where Δx is the total thickness of the membrane. This equation has also been used by Dainty (1963, p. 304) to calculate diffusion flux versus total solution flux outside a shrinking red blood cell.

The mean velocity of the diffusing molecules is equal to the diffusion flux, $-D_{s,w}dC_s/dx$ moles per second $\times$ square centimeters divided by the concentration of a tracer solute C_s, that is to say, $-(D_{s,w}/C_s)(dC_s/dx)$. At the left-hand boundary of the membrane where concentration of solute equals C_A, considering C_s at the right-hand boundary is zero, the mean diffusion velocity approximates $-D_{s,w}/\Delta x$. Thus $vD_{s,w}/\Delta x$ can be thought of as the ratio of the absolute mean velocity of the net *solution* flux, v, divided by the absolute mean velocity of the tracer solute *diffusion* flux at the left-hand boundary.

The diffusion flux of solute through the membrane in the absence of any net flux of solvent is equal to

$$J_d = -D_{s,w}(C_B - C_A)/\Delta x \tag{34}$$

The magnitude of the effect of net solution flux on the total flux of tracer solute, solvent drag, can be assessed by the ratio J_s/J_d, any difference from unity indicating the fraction of solvent drag. A graph of this

ratio as a function of $v\Delta x/D_s$ is given in Fig. 5. $v\Delta x/D_s$ must be more than 1.65 to produce a solvent drag effect doubling the solute flux and must be 0.2 to produce a 10% increase in solute movement.

In an actual red blood cell membrane, there is a significant frictional force between the membrane and the solute in addition to that between the water and the membrane, that is, there is sieving. First, this means that the magnitude of the net diffusion coefficient for solute in the flowing stream of solution is less than $D_{s,w}$, its value in free solution. The proper value becomes the sum of $D_{s,w}$ and $D_{s,m}$ in parallel, namely,

$$D_{s,w}D_{s,m}/(D_{s,w} + D_{s,m})$$

According to the restricted diffusion theory, it also equals

$$D_{s,w}A_s/A_p$$

The diffusion flux at a given point is equal to

$$\frac{-D_{s,w}D_{s,m}}{D_{s,w} + D_{s,m}} (dC_s/dx)$$

reduced by the friction between solute and membrane.

Second, the solvent drag flux is no longer equal to the linear velocity of solution times the solute concentration at each point vC_s, because the additional friction between solute and membrane tends to retard the flow of solute relative to water. The effect of membrane-solute friction on solvent drag can be analyzed as follows.

There is a frictional force on the solute tending to retard it, which is proportional to the difference in velocity between the solute and the membrane wall. Thus

$$\text{Force (solute-membrane) in dynes per mole} = -v_s RT/D_{s,m} \quad (35)$$

where v_s is the velocity of the solute and $D_{s,m}$ the diffusion coefficient of solute in or against the membrane. A similar expression can be obtained for the frictional force between the solute and the water, which is proportional to the difference in velocity between the solute and water and is positive in direction:

$$\text{Force (water-solute) in dynes per mole} = (v - v_s)RT/D_{s,w}. \quad (36)$$

v_s is constant through the membrane, so these two forces should sum to zero. Therefore we can solve for v_s from Eqs. (35) and (36), giving

$$v_s = \frac{v}{1 + (D_{s,w}/D_{s,m})} \quad (37)$$

For the general case in which there is frictional force between the solute

and the membrane as well as between solute and water, Eq. (32) becomes

$$J_s = \frac{-D_{s,w}D_{s,m}}{D_{s,w} + D_{s,m}} \frac{dC_s}{dx} + \frac{vC_sD_{s,m}}{D_{s,m} + D_{s,w}} \tag{38}$$

When this equation is integrated, the exponent in the solution equals $v\Delta x/D_{s,w}$, as in Eq. (33), where it must be emphasized that $D_{s,w}$ is the solute diffusion coefficient in free solution. The frictional force between the solute and membrane reduces the diffusion flux and the solvent drag flux in the same proportion according to the above analysis. Sha'afi *et al.* (1970) concluded that the concentration profile of urea passing through the red blood cell membrane would be distorted by an experimentally attainable volume flux. The discrepancy between their conclusion and ours lies in this point; they used a solute diffusion coefficient in Eq. (32) that had in effect been reduced by the ratio A_s/A_p. Other investigators have concluded that even in larger artificial membranes with much greater volume flux/diffusion flux the effect of solvent drag can be safely neglected (Landis and Pappenheimer, 1963).

The practical problem is to obtain a maximal numerical estimate of $v\Delta x/D_{s,w}$. While reasonable estimates of Δx and $D_{s,w}$ are available, we have none for v. It equals J_w/F_p, where F_p is the fraction of the total cell area made up of pores; this is the geometric area corresponding to A_p. We can calculate J_w because it equals $L_p\bar{V}_w\Delta C_s$, but we have no reliable estimates of F_p, at least they vary by orders of magnitude (Paganelli and Solomon, 1957). We can, however, obtain a maximal value for the exponent by letting $F_p = F_w$, where F_w is the fractional pore area available for water. Thus

$$P_w = F_wD_w/\Delta x \tag{39}$$

Substituting into the expression for the exponent,

$$\frac{v\Delta x}{D_{s,w}} \leq \frac{L_p\bar{V}_w\Delta C_s}{P_w} \frac{D_w}{D_{s,w}} \tag{40}$$

$L_p = 0.015$ cm/second (Table I); $\bar{V}_w = 18$ cm^3/mole; a maximal value of $\Delta C_s = 1$ osmol/liter; $D_w = 2.3 \times 10^{-5}$ cm^2/second; $D_{s,w} = 10^{-5}$ cm^2/second for malonamide, a minimal value; and $P_w = 0.0038$ cm/second (Table III). Therefore

$$\frac{v\Delta x}{D_{s,w}} \leq \frac{0.015 \times 18 \times 10^{-3} \times 2.3 \times 10^{-5}}{0.0038 \times 10^{-5}} = 0.16$$

According to Fig. 5, the effect of solvent drag on the total flux would be less than 5%, which would be undetectable by present experimental

methods. In other words, the mean velocity of diffusion flux is so great over a thin cell membrane that the solvent drag adds little. While ΔC_s could be increased further, the shrinking of cells occurs very rapidly at a 1 M gradient and the process would become more difficult to follow. It should be noted that using different solutes varies the exponent only by (molecular weight)$^{1/2}$.

B. Irreversible Thermodynamic Approach to Solvent Drag

From Kedem and Katchalsky (1961, Equation 3-1),

$$J_s = P_s(C_B - C_A) + \bar{C}_s(1 - \sigma)J_v \tag{41}$$

J_v is the total volume flux in centimeters per second, approximately equal to $\bar{V}_w J_w$. $\bar{C}_s$ is a species of average concentration of C_s through the membrane defined in such a way as to make the total chemical potential of solute across the membrane resolvable into the sum of a pressure term and a concentration term. Equation (41) represents an approximation of Eq. (32). The first term on the right-hand side is the diffusion flux and the second is the solvent drag.

If we consider the case of diffusion of malonamide from outside to inside ($C_A = 0$), while an osmotic gradient of 1 osmol/liter produces a large volume flux,

$$\frac{J_s}{J_d} = 1 + \left(\frac{(\bar{C}_s)\ (1 - \sigma)J_v}{(C_B - C_A)\ P_s}\right) \leq 1 + \frac{10^{-3}\ \bar{V}_w}{2}\left(\frac{\bar{V}_s}{\bar{V}_w} + \frac{L_p D_w}{P_w D_{s,w}}\right) \tag{42}$$

In the inequality $1 - \sigma$ has been eliminated by means of Eq. (20), $F_w\vartheta/\Delta x$ has been replaced by P_w/D_w and $\bar{C}_s/(C_B - C_A)$ substituted for $\bar{C}_s$, both of which would exaggerate the solvent drag effect.

Now if we substitute the numerical values used in evaluating Eq. (40) and a maximal estimate of the molal volume of molonamide of 102 cm^3 per osmole, we obtain

$$\frac{J_s}{J_d} \leq 1 + \frac{18 \times 10^{-3}}{2}\left(\frac{102}{18} + \frac{0.015 \times 2.3 \times 10^{-5}}{0.0038 \times 0.97 \times 10^{-5}}\right) = 1.13 \tag{43}$$

This is similar to the conclusion reached by the diffusion kinetic approach. It must be pointed out that a large solvent drag effect would be predicted if the experimental values of $1 - \sigma$ and P_s in Table III were substituted in Eq. (42). However there appears to be a large discrepancy between the experimental measurements of σ and of P_s as discussed in Section IV,C and it does not appear reasonable to rely on them for this present calculation. Furthermore the computation of maximal solvent drag from a diffusion approach does not depend on experimental measurements of σ and P_s.

C. Attempts to Measure Solvent Drag in Red Blood Cells

Klocke *et al.* (1968) measured the permeability of human red blood cells at 37° in a continuous-flow rapid-reaction apparatus using a pH electrode to indicate the course of the reaction, when water was entering the cells under a 0.075 *M* osmolal gradient and when it was leaving the cells under a 1.17 *M* osmolal gradient. The extra- and intracellular ammonia concentrations are plotted against time in Fig. 6. Clearly, there is no significant effect of the large difference in water flux upon the solute movement. This experiment has the peculiar advantage that the sink for ammonia within the cells is very large and any intracellular diffusion gradients should be minimal in size.

At the very least, this experiment indicates that under available conditions it can be assumed that water flux does not change the flux of a hydrophilic solute significantly.

Although according to theory (see Section V,B) the proportion of the total flux represented by the additional flux produced by solvent drag is relatively independent of P_s, Klocke *et al.* carried out similar studies on formic acid, whose molecular radius is about 2 Å as compared with about

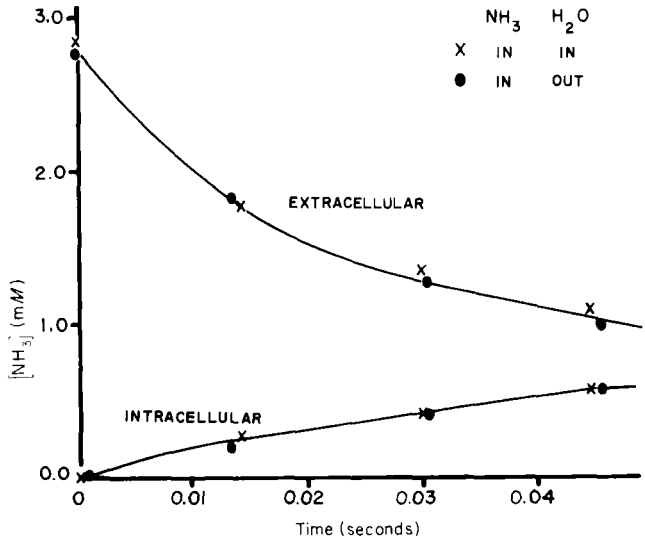

FIG. 6. A graph of the changes in intracellular and extracellular ammonia concentration with time after suddenly mixing a 1:10 dilution of human red blood cells in isotonic saline at 37° with a 2 m*M* solution of ammonia in water (×) and in 2.5 *M* saline (●). The changes in pH were followed with a pH electrode in a continuous-flow rapid-reaction apparatus and the ammonia concentration calculated therefrom.

1 Å for ammonia. Just as in the case of ammonia, no effect of solvent drag could be seen.

Sha'afi *et al.* (1970) found that P_{urea} varied with volume flow when red blood cells were mixed with a hypertonic solution of urea, increasing linearly with increasing volume flux in the opposite direction. This is the opposite of what would be expected from solvent drag. They suggest that the variation in P_{urea} with J_v may be an artifact secondary to the value of urea concentration in the membrane $\bar{C}_s$, which for small differences across the membrane equals the average value. At one point they say that the phenomenological equations from irreversible thermodynamics relating J_d and J_v do not describe correctly the effects of a urea concentration gradient. It seems likely, as they state earlier, that this apparent failure results from the inability to assign the correct numerical values to L_d, I_{pD}, and L_p, since the weakness of the irreversible thermodynamics lies more in its practical application rather than in its logic.

VI. DISCUSSION OF THE MECHANISM OF WATER TRANSPORT ACROSS THE RED BLOOD CELL MEMBRANE

The relative discontinuity in the permeability of the red blood cell membrane as molecular size increases, the remarkably high value of L_p and of P_s for small hydrophilic molecules, gave rise to the concept of water-filled pores in the membrane, a "molecular sieve" (Jacobs, 1952), through which bulk flow, possibly of the laminar type, could take place under an osmotic gradient. Solomon and his associates (see Goldstein and Solomon, 1960) have put this viewpoint forward, strongly buttressed with a long series of elegant experiments. The pertinent available experimental results and arguments are now discussed in relation to the reality of pores.

A. Comparison of L_p and P_w

The reported measurements of P_w are all less than those of L_p (Table I). While this phenomenon has been reported for many kinds of membranes (Hevesy *et al.*, 1935; Lucké *et al.*, 1931), most of these results are subject to the criticism that stirring was inadequate and stagnant layers could have existed on the cell surfaces and interfered with water diffusion more than with osmotic flow. The measurements in Table I are among a few that can be exonerated from this defect. The measurements of P_w have all been made by the same method, however, which is subject to the

noted criticisms, and made in the same laboratory. No other technique has been reported that could measure P_w, except to calculate it from σ and, as discussed, there appears to be an inconsistency between these two types of measurements.

Sjölin (1954) reported that L_p measured by the lytic technique was decreased to one-half of the expected diffusion in free water in the pore, just the opposite of the accepted experimental findings of other investigators later. Although the available pore area was critical to his calculations, he had to estimate it.

The fact that L_p is greater than P_w is evidence for the interchange of momentum among water molecules in the membrane but not necessarily for bulk flow. This is in disagreement with Rich *et al.* (1967). Diffusion flux of labeled water across the membrane is the difference between diffusion fluxes in the two directions; the average molecular momentum is zero and the flux of labeled molecules takes place because there are more on one side than on the other, not because of a difference in momentum of the two species of molecule. Under pressure as is assumed to exist during most osmotic flow, however, a chemical potential gradient is produced for all molecular species and each possesses an average momentum in the direction of flux even if less than the unidirectional diffusion flux. Thus if there is any finite probability of one water molecule colliding with another within the membrane, this collision under a pressure gradient will not result in a net loss of momentum on the average. In the case of diffusion of labeled water, it would, since on the average the molecules of water have no net momentum.

That L_p can be greater than P_w without the presence of pores is shown in the findings of Thau *et al.* (1966) and Sidel and Hoffman (1961). L_p/P_w was >1 in liquid membranes but, as expected from the arguments above, L_p/P_w approached 1 as the water content of the membrane decreased.

A special case of water collision within the membrane is the long-pore effect (Harris, 1960; Austin *et al.*, 1967; Lea, 1963.) If the cross-sectional area of the path through which water passes across the membrane is so narrow that individual water molecules rarely pass each other, a single file is the extreme, water exchange becomes governed by this effect. The probability of a labeled molecule advancing through the whole file, which is the situation for ^3HHO diffusion, is far less than the probability of a water molecule colliding with the tail of the file on one side of the membrane and driving off a molecule at the head of the file on the other side of the membrane into the free solution. Since the effective radius of the pores or passages appears to be of the same order as the radius of the water molecule, the long-pore effect may hold to some degree in the

red blood cell membrane. However, Hirsch (1967) has published theoretical arguments that channels of molecular width and about 100 Å length would rarely contain two or more water molecules and therefore that the long pore effect would not be important in water transport across the red cell membrane.

The fact that L_p is greater than P_w does not require that there be a continuous pore through the membrane. Even an isolated watery layer would present greater diffusion resistance to labeled water exchange than to net water movement. In the latter water molecules dissolve in the surrounding membrane layers and diffuse under a chemical potential gradient produced either by a concentration or a pressure difference. In the watery region the water molecules would exchange momentum and there would be an average forward velocity for every molecule, increasing the effective diffusion coefficient.

B. $1-\sigma$ Greater than $P_s\bar{V}_s/L_p\bar{V}_w$

While the finding of a significant numerical value of $1 - \sigma - (\bar{V}_s P_s/L_p\bar{V}_w)$ indicates that there is measurable solute drag on the water molecules in the membrane and therefore that there must be regions where the probability of water molecules colliding with each other is high, it does not prove the existence of bulk flow or of solvent drag on solute (Stein, 1967) and does not necessarily mean that these watery regions must be continuous through the membrane. Let us assume that the membrane consists of a lipid layer and a layer in which water is present. In the experimental determination of σ we arrange conditions so that the concentration of impermeable solutes in the inside C_i, and that of permeable solute on the outside C_B, result in zero net volume flux. $C_i/C_B = \sigma$. There is a flux of solute through the membrane $-C_B P_s$ which produces a force on the water in the watery layer proportional to its length; this force in turn produces a solute drag flux of water equal to force times $L_p\bar{V}_w/RT$. If the water channel is continuous through the membrane, corresponding to the absence of the lipid layer, this flux will be maximal as discussed in the derivations of Eqs. (19) and (20) which apply to continuous pores. If there is no watery region at all, there will be no solute drag flux and A_s/A_w will disappear. The magnitude of the solute drag flux will depend on the proportion of the total solute path through the membrane occupied by the watery region. Thus $1 - \sigma - (P_s\bar{V}_s/L_p\bar{V}_w)$ can be greater than zero even if the watery channels are not continuous, although its magnitude will be less than if they are, with the same restricted-diffusion area for solute in water. We cannot tell from a given experimental determination of A_s/A_w

whether the channels are continuous or discontinuous but with a larger restricted diffusion area for solute in water.

C. Mass Selectivity of the Red Blood Cell Membrane

The large decrease in P_s as molecular size, weight, and/or radius increase may be explained by the restricted diffusion theory of movement through pores, although not consistently over the entire range (Stein, 1967). Lieb and Stein (elsewhere in this volume) point out, however, that nonporous polymer membranes have much more mass selectivity than diffusion in liquid, P_s decreasing as (molecular weight)$^{-3.8}$ in some instances, instead of as (molecular weight)$^{1/2}$ as required by kinetic theory and Graham's law. Similar large mass selectivities are found in certain cell membranes. Thus it is not a priori necessary to assume a sieving phenomenon to explain the mass selectivity of the red blood cell. The recent findings of Macey and Farmer (1970) that L_p of human red blood cells could be reduced to 1/10 of its initial value by incubation with *p*-chloromercuribenzoate and *p*-chloromercuribenzene sulfonate without significantly changing P_s for such solutes as ethyl urea and diethylene glycol does not seem consistent with simple pore theory.

The value chosen for the radius of the solute molecules, and particularly of water, is extremely critical. For example, there is no significant correlation between solute molecule radius and $1 - \sigma$ in the data of Table II. If, however, the values of Goldstein and Solomon (1960) for solute radii are used, a relationship becomes more apparent. This difference is a matter of fifths of an angstrom.

D. Discrepancy between σ and P_s

This discrepancy may indicate an error in logic or technique at a fundamental level. With this remaining unexplained, one hesitates to concentrate on the magnitude of the pore. "The internal consistency of the various estimates of pore radius in artificial membranes constitutes the chief evidence justifying the application of similar techniques to biological membranes of comparable size" (Landis and Pappenheimer, 1963). The estimates referred to are from P_s and filtration, corresponding to P_s and σ. Without an explanation this discrepancy cannot contribute to our ideas of the mechanism of water transport.

E. Temperature Dependence

Vieira *et al.* (1970) advanced an argument in support of bulk fluid flow

through the membrane based on the temperature dependence of L_p as compared with that of P_w. They found experimentally that L_p is independent of T, as it should be for viscous flow, while P_w/T is independent of T, as it should be for diffusion flow. They argued that this is strong evidence for the existence of viscous flow during the measurement of L_p under an osmotic gradient, and for diffusion flow under a concentration gradient of labeled water during the measurement of P_w. If we have interpreted their experimental results correctly, in order to express L_p in terms of a pressure gradient the concentration difference was converted to a pressure difference. This involved multiplying L_p by $1/RT$. Therefore the experimental results are that P_w/T and L_p/T are independent of T. We find this argument for hydrodynamic flow as against diffusive flow unconvincing. At the same time, the heats of activation of L_p and P_w was remarkably small. If one is to explain the mass selectivity of the red blood cell membrane on the basis of diffusion in a polymer, the activation energy should be 2 to 3 times larger than it is (Lieb and Stein, this volume).

F. Solvent Drag

Our interpretation of the experimental measurements and theory is that the red blood cell membrane is so thin that unidirectional diffusion fluxes through it are much greater than any net osmotic volume fluxes we can produce experimentally. Thus if there were bulk or laminar flow through the membrane, we could not detect its effects on solute movement.

G. Bulk Laminar Flow in Pores

It appears doubtful that viscous flow could exist in channels of such narrow radius. Thermal diffusion equilibrium across the radius of a 6-Å cylinder would require but 1/1000 of the transit time required for osmotic water flux through the membrane assuming the membrane is 100 Å thick, that the pores are but 0.01% of the total cell surface area, and that L_p is 0.02 cm/second. This means that the exchange of momentum among fluid molecules would have been completed in this time period, which would obliterate any differences in longitudinal velocity as a function of radius, making viscous flow impossible.

Brooks (1935) and Parpart (1935) found that cow, rat, and sheep red cells lysed more slowly in D_2O than in H_2O at room temperature. It is difficult to draw precise conclusions as to the mechanism of water transport across the cell membrane from these interesting results because while

the viscosity of D_2O is greater than that of H_2O, its diffusion coefficient is less, as is its fugacity.

As Renkin (1954) pointed out, the unmodified Poiseuille equation does not describe flow through an artificial membrane correctly, and it was for this reason that Pappenheimer had developed the ingenious approach of measuring the effective diffusing area membrane thickness with radioactively labeled water. This is logical when the molecular radius of water is 1/10 (artificial tubing; Renkin, 1954) or 1/30 (Landis and Pappenheimer, 1963) of the radius of the pore, for the diffusing water provides a good index of the geometrical area of the channel, the restriction amounting to 10–30%. In the case of the red blood cell, however, with pore radii as small as 3.5 Å (Goldstein and Solomon, 1960), the restriction by Eq. (22) is 92%, and one can hardly argue that this provides a geometric estimate of the pore area.

The findings that L_p is always less than P_w and that σ is less than 1 for permeable hydrophilic molecules are evidence of exchange of momentum among water and water-soluble solutes within the membrane but do not require the existence of bulk or hydrodynamic flow. One might conceive of this membrane water as a pore, or equally justifiably as merely dissolved in the membrane structure. The hydraulic coefficient for thin films of egg phosphotidylcholine and *n*-decane during water flow produced by concentration differences of sodium chloride, sucrose, and urea averaged 1.9×10^{-3} cm/second (Hanai and Haydon, 1966). Presumably, the flux of water simply dissolves in the hydrocarbon layer, for L_p was consistent with simple diffusion of the water and known estimates of the solubility and diffusion coefficient of water in decane. It would require little water or hydrophilic material in the membrane to increase L_p by a factor of 10 and make it comparable with that of the red blood cell.

In view of the large area of the membrane available for diffusion for hydrophobic molecules in comparison to the apparent water-filled area, the oil solubility/water solubility of a solute must be known in order to draw significant conclusions about the mechanism of transport. It is technically difficult to measure extremely low solute solubilities in lipids because of possible contamination of the lipid with water, among other problems. Furthermore, solute solubilities have not been measured in membranes but in representative lipids. As the permeability of hydrophilic solutes decreases, presumably a point is reached where transport through the lipid, small though the oil solubility/water solubility may be, becomes important because of the very much larger area available. If we must think of water-filled channels through the membrane to account for the permeability of small hydrophilic molecules (less than 3-Å radius), should we not also think of "lipid" pores to account for the permeability of

lipophilic molecules such as un-ionized valeric acid, with a P_s of 2.7 cm/second, about 6 times that of water, and a radius twice as large?

VII. CONCLUSIONS

The hydraulic permeability coefficient for the flow of water across red blood cell membranes L_p is generally greater than the analagous diffusion permeability for labeled water P_w under conditions of zero net volume flow.

The reflection coefficient σ for small, uncharged, hydrophilic molecules is less than unity, which is theoretically explained by frictional drag of solute flux on water in the membrane.

Although there are possibly errors in the experimental techniques used to measure rapid exchanges across the red blood cell membrane, even with proper rapid-reaction techniques the influence of such phenomena as unstirred layers outside or inside the membrane should not be great enough to influence the general conclusions.

The unidirectional diffusion flux across the thin red blood cell membrane is so much greater than any net osmotic volume flux yet produced so as to render experimentally undetectable at present any possible drag of volume flux on diffusing molecules. Our own experimental results bear this out.

There appears to be a marked discrepancy between the experimentally determined values of σ and of P_s, as interpreted by the theory of osmotic flow and of restricted diffusion, in that the more impermeable, uncharged, hydrophilic molecules develop less osmotic flow (lower σ) than expected from their diffusion permeability. No good explanation is offered, but the cause should be sought because of possible implications concerning the accuracy of present theories of osmotic flow.

The overall results indicate that there is an exchange of momentum among water and water-soluble solutes within the membrane. At the same time, there is no unambiguous evidence for bulk flow, hydrodynamic flow, or the necessary presence of continuous water-filled channels in the membrane.

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LIST OF SYMBOLS

A Area in square centimeters. A_p, area of a pore; A_w, restricted area for water diffusion in a pore; A_s, restricted diffusion area for a solute in a pore

C Concentration in osmols per cubic centimeter. Subscripts: $_0$, initial value before a step change in extracellular conditions; $_\infty$, final value at equilibrium; $_A$ and $_B$, values on either side of the membrane; $_U$ and $_D$, values upstream and downstream, respectively; $_s$, for solute; $_w$, for water; $_i$, for impermeable solute; $_e$, for extracellular fluid; $_{^3HHO}$, for tritiated water. C without a subscript is intracellular solute concentration

$\bar{C}_s$ "Average" concentration of solute in the membrane defined as in Kedem and Katchalsky (1961)

D Diffusion coefficient in square centimeters per second. Subscripts: $_{Hb}$, for hemoglobin; $_s$, for solute; $_m$, for membrane; $_w$, for water. Example: $D_{s,w}$ is the diffusion coefficient for solute in water

F Fractional area of the cell membrane made up of pores, $_p$, or of water, $_w$

Fractional V_{rbc} Water content of red blood cells per total cell volume

J Flux. J_v, Volume flux in centimeters per second. The remainder of the fluxes are in osmols per square centimeter $\times$ seconds. Subscripts: $_s$, refers to solute; $_w$, refers to water; $_d$, refers to diffusion flux

L_p Hydraulic coefficient in centimeters per second. Subscript $_{in}$ refers to flux in; subscript $_{out}$ refers to flux out

L_{pD} Osmotic coefficient in centimeters per second

n Number of pores per square centimeter membrane surface

P Permeability in centimeters per second. Subscript $_s$ refers to solute; $_w$ refers to water

p Pressure in atmospheres

R Gas constant in cubic centimeters $\times$ atmospheres per mole $\times$ degrees Kelvin, or in dynes centimeters per mole $\times$ degrees Kelvin

r Radius in centimeters. Subscripts: $_p$ refers to a pore; $_s$, refers to the effective radius for a solute; $_w$, refers to the effective radius for water

T Temperature in degrees Kelvin or degrees centigrade

t Time in seconds t_h. Time required for a specified amount of hemolysis to occur

V Volume in cubic centimeters. Subscripts: $_0$, initial value; $_\infty$, at final equilibrium; $_h$, volume at which a specified amount of hemolysis occurs; $_w$, osmotically active water; T_w, total osmotically active water in the cell. V without subscript refers to total cell volume

v Average linear velocity in centimeters per second

x Distance in centimeters

Δx Thickness of the membrane in centimeters

ϑ Tortuosity factor

π Osmotic pressure in atmospheres. Subscript $_i$ refers to impermeable solutes; $_s$ refers to permeable solutes

η Viscosity in poises

μ Chemical potential in cubic centimeters $\times$ atmospheres per mole. Subscript $_w$ refers to water

σ Reflection coefficient

$\bar{V}$ Molal volume in cubic centimeters per mole. Subscript $_s$ refers to solute; subscript $_w$ refers to water

W Fraction of total cell water that is osmotically active.

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Ion-Translocation in Energy-Conserving Membrane Systems

B. CHANCE AND M. MONTAL*

Department of Biophysics and Physical Biochemistry, Johnson Research Foundation, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Man knows much more than he understands.

A. Adler

* Present address: Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York.

I. INTRODUCTION AND STATEMENT OF PROPOSED MECHANISMS OF ION TRANSPORT AND ENERGY COUPLING

A. Introduction

In this chapter we do not review exhaustively the literature on ion translocation in mitochondria but concentrate on available information pertinent to understanding the mechanism of ion translocation and its relation to the primary event in the energy-coupling process.

A number of excellent recent reviews have covered in detail the ion-translocating properties of mitochondria (Harris *et al.*, 1966b; Lehninger *et al.*, 1967; Pullman and Schatz, 1967; Greville, 1969; Pressman, 1969).

Two periods can be distinguished in mitochondrial ion transport research. The early period (1953–1961) includes the contributions of Davies and co-workers (Bartley and Davies, 1954; Bartley *et al.*, 1954; Fonnesu and Davies, 1956; Price *et al.*, 1956), Spector (1953), and McFarlane and Spencer (1953), who drew attention to the fact that isolated mitochondria can retain cations against a concentration gradient in an energy-dependent fashion.

The present period was initiated in 1961 with the formulation of the chemiosmotic hypothesis of energy coupling in oxidative and photosynthetic phosphorylation (Mitchell, 1961) and the findings of Vasington and Murphy (1962), DeLuca and Engstrom (1961), and Saris (1963) on the energy-dependent massive uptake of Ca^{2+} by isolated mitochondria.

B. Energy Sources

It is now generally accepted that biological membranes behave as reversible transducers of various energy sources; mitochondria, submito-

TABLE I

ENERGY SOURCES IN BIOENERGY-CONSERVING MEMBRANE SYSTEMS

Energy type	Equations[a]
Redox	$\Delta G_e = \Delta G^0{}_e + RT \ln \frac{(BH_2)(A)}{(AH_2)(B)}$
Phosphate bond	$\Delta G_p = \Delta G^0{}_p + RT \ln \frac{(ADP)(P_i)}{(ATP)}$
Electrochemical	$\Delta G_i = z_i F \Delta\psi + RT \ln[a_{i1}/a_{i2}]$

[a] G represents free energy and the subscript indicates the energy type (e, redox, p, phosphate bond, i, electrochemical); R, the gas constant; T, the absolute temperature; F, the Faraday; z, the electrovalency; $\Delta\psi$, the electrical potential difference. Quantities enclosed within parentheses represent electrochemical activities, and a is the ion activity. (Taken from Mueller and Rudin, 1969.)

TABLE II

OBSERVED ENERGY TRANSDUCTIONS IN BIOENERGY-CONSERVING MEMBRANE SYSTEMS[a]

Energy transduction[b]	Mitochondria	SMP	Chloroplasts	Chromatophores
(1) Oxidative (or photosynthetic) phosphorylation	Yes (*1*)	Yes (*1*, *2*)	Yes (*3*)	Yes (*4*, *5*)
(2) Phosphorylative oxidoreduction	Yes (*6*, *7*)	Yes (*8*, *10*)	No[c]	Yes (*11*, *12*)
(3) Redox-dependent ion translocation	Yes (*13*, *14*)	Yes (*15–18*)	Yes (*19–22*)	Yes (*23–27*)
(4) Ionic oxidoreduction	Yes (*28*)	No	Yes (*29*)	No
(5) Phosphorylative ion translocation	Yes (*13*, *14*)	Yes (*15*, *16*)	Yes (*33*, *34*)	Yes (*27*)
(6) Ionic phosphorylation	Yes (*30*, *31*)	Yes (*35*)[d]	Yes (*32*)	No

[a] Italicized numbers in parentheses indicate the following references:
(*1*) Racker (1961). (*2*) Hansen and Smith (1964). (*3*) Arnon *et al.* (1954). (*4*) Frenkel (1954). (*5*) Frenkel (1956). (*6*) Chance and Hollunger (1957). (*7*) Chance and Hollunger (1961). (*8*) Löw *et al.* (1961). (*9*) Löw and Vallin (1963). (*10*) Löw *et al.* (1963). (*11*) Baltscheffsky *et al.* (1967). (*12*) Keister and Yike (1967). (*13*) Lehninger *et al.* (1967). (*14*) Pressman (1969). (*15*) Mitchell and Moyle (1965). (*16*) Chance and Mela (1967). (*17*) Montal *et al.* (1970b). (*18*) Cockrell and Racker (1969). (*19*) Jagendorf and Uribe (1966b). (*20*) Dilley and Vernon (1965). (*21*) Crofts (1967). (*22*) Packer and Crofts (1967). (*23*) Von Stedingk and Baltscheffsky (1966). (*24*) Chance *et al.* (1966b). (*25*) Nishimura *et al.* (1968). (*26*) Jackson *et al.* (1968). (*27*) Scholes *et al.* (1969). (*28*) Cockrell (1968). (*29*) Mayne and Clayton (1966). (*30*) Cockrell *et al.* (1967). (*31*) Reid *et al.* (1966). (*32*) Jagendorf and Uribe (1966a). (*33*) Crofts (1966). (*34*) Carmeli (1970). (*35*) Hatase and Oda (1969).

[b] (1) Phosphorylation driven by redox energy. (2) Reversal of electron transfer (Ernster and Lee, 1964) driven by phosphate bond energy. (3) Ion translocation driven by redox energy. (4) Reversal of electron transfer driven by ion electrochemical energy. (5) Ion translocation driven by phosphate bond energy. (6) Phosphorylation driven by ion electrochemical energy.

[c] The terminal enzyme of photophosphorylation in chloroplasts is not ordinarily active as an ATP hydrolase (ATPase); this explains the lack of energy transduction (2) in intact chloroplasts (Vambutas and Racker, 1965).

[d] An unexpected finding in view of the opposite direction of the pH transition (alkali to acid rather than acid to alkali).

chondrial particles (SMP), chloroplasts, and their bacterial equivalents, the chromatophores, are examples of energy-conserving membrane systems that perform reversible transformations of three equivalent types of energy, oxidoreduction (redox), hydrodehydration (phosphate bond or

ATP), and electrochemical (ion gradient). The generalities and applicability of the thermodynamic equations are presented in Table I (modified from Mueller and Rudin, 1969).

Table II presents the six possible energy transformations that have been observed in mitochondria, SMP, chloroplasts, and bacterial chromatophores.

C. Proposed Mechanisms of Ion Transport and Energy Conservation

Three proposed mechanisms of ion transport are currently being experimentally considered.

Scheme A in Fig. 1 represents the chemiosmotic coupling hypothesis in which the primary event is H^+ migration with consequent development of an electrochemical H^+ gradient or proton motive force (PMF, $\Delta\bar{p}$) consisting of two components: a concentration term attributable to the differential distribution of H^+ across the membrane (a pH gradient), and an electrical term attributable to charge transfer across the membrane (membrane potential or electrical gradient) (Mitchell, 1966a,b, 1968, 1969a,b); cation uptake is a secondary event and is driven mainly by the direction (or sign) of the potential (see Section II,C).

Mitchell and Moyle (1969a) estimated the values of the membrane potential and pH gradient present in rat liver mitochondria during β-hydroxybutyrate oxidation based on the equilibrium distribution of K^+ across a valinomycin-treated membrane (see Section II,A). They also reported estimates of the buffering powers of inner and outer phases and pH changes observed upon addition of the detergent Triton X-100 to mitochondria. Their results are shown in the accompanying tabulation.

Mitochondria	State[a]	$\Delta\psi$[b]	$-59\Delta pH$[b]	$\Delta\bar{p}$[b]
K^+-depleted (in the presence of EGTA)	4	200	30	230
K^+ (undepleted)	4	140	85	225

[a] State 4 of Chance and Williams (1956).

[b] Values are expressed in millivolts.

These figures agree with those predicted from Mitchell's calculations (Mitchell, 1966a,b).

Evidence for the existence of a mechanism of ion translocation dependent on the sign of the membrane charge is outlined in Section II,C in

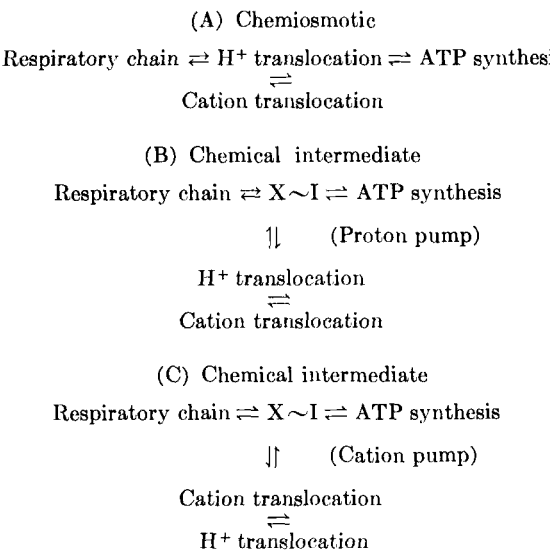

FIG. 1. Mechanisms of energy utilization. Three schemes that have been suggested for the interrelationships between respiratory chain activity, ATP synthesis, and proton translocation in mitochondria (Chance *et al.*, 1967; Slater, 1967; Mitchell, 1969b). (Taken from Greville, 1969.)

reference to Skulachev's experiments (see also Azzi, 1969; Montal *et al.*, 1970b).

In scheme B of Fig. 1 (Chappell and Crofts, 1965; Jagendorf and Uribe, 1966b), a high-energy chemical intermediate is the linkage between redox, phosphate, and electrochemical energy and drives, exclusively, a "proton pump." Cation uptake in this scheme occurs as a tertiary event in the energy tranduction process.

In scheme C of Fig. 1 (Chance, 1965; Rasmussen *et al.*, 1965; Chance and Mela, 1966b, 1967; Chance, 1967; Chance *et al.*, 1967; Pressman, 1968, 1969; Harris and Pressman, 1969; Slater, 1967; Cockrell *et al.*, 1966), the high-energy chemical intermediate drives a *cation* pump, and H^+ translocation follows the primary cation movement. Harris and Pressman (1969) attempted to measure the transmembrane potential of mitochondria on an assumption contrary to that of Mitchell and Moyle, namely, that the anions are in passive equilibrium with this potential. By measuring the accumulation of radioactively labeled substrate anions of different charge, they determined the "equilibrium transmembrane gradients" and applied the Nernst equation. Their results show that regardless of charge the various anions distribute with a potential of about +30 mV, positive inside the mitochondrion.

These experiments can also be interpreted in the following manner. The anions utilized in this work were weak acids. It has been shown by Chappell (1968) and Mitchell and Moyle (1969b) that the permeant species is the nonionized form of the acid (HA). Mitochondria in the deenergized state would have an equilibrium concentration of HA on both sides of the membrane. Energization results in the efflux of H^+ from the mitochondrion with the consequent displacement of the equilibrium $HA \rightleftharpoons H^+ + A^-$ to the right. The more $[HA]_{in}$ is ionized, the more $[HA]_{out}$ enters the mitochondrion. It superficially appears as though the membrane potential inside the mitochondrion is positive, although it could indeed be negative.

In support of this interpretation is the fact that Quagliariello and Palmieri (1968) and Palmieri and Quagliariello (1969) studied the relationship between H^+, K^+, and anion movements in the presence of nonactin, nigericin, and uncouplers and found that the distribution of succinate and malate followed the pH gradient and not the K^+ gradient across the membrane (see also Klingenberg, 1970).

Tupper and Tedeschi (1969a,b,c) made pioneering measurements of membrane potentials and resistance of *Drosophila* mitochondria (3–4 μ in diameter) using microelectrodes driven by a piezoelectric device. The first measurements of the electrical properties of an intracellular organelle were made on *Drosophila* gland cell nuclei (Lowenstein *et al.*, 1966).

The measured potential in state 4 was about 10 mV and in state 3 approximately 20 mV, both positive inside the mitochondrion. The state-3 value was insensitive to potassium cyanide or DNP. The measured resistance had the low value of about $2\ \Omega \cdot cm^2$.

There is a good correlation between the value of the membrane potential calculated by applying the Nernst equation to the distribution of weak acid anions, and Tupper and Tedeschi (1969c) conclude that "the properties of the potential suggest that it is the result of the distribution of anions imposed by a Donnan effect."

The uncertain location of the electrode seems to be one of the main criticisms of this work. The folded nature of the inner mitochondrial membrane and the relation between electrode tip size and mitochondrial size suggests that the measurement might have been made between the inner and outer membranes. Furthermore, the extremely low membrane resistance ($\sim 2\ \Omega \cdot cm^2$) is not readily compatible with the well-known impermeability of the inner membrane to K^+ or H^+, among other ions. Some other questions have been raised with regard to the origin of the measured potential concerning changes in the conductivity of the solution between the two membranes produced by mixing of contents following the impalement, changes in liquid junction potential during impalement, and plugging of the microelectrode tip. For a consideration of these questions,

the reader is referred to the original papers. The relevance of these measurements to energy conservation is, at the present time, uncertain.

Greville (1969) recently summarized Mitchell's arguments against a primary metal cation pump and in favor of a primary proton pump. These arguments include: the effect of valinomycin on H^+/O ratios in oxygen pulse experiments (Mitchell and Moyle, 1967a); the high permeability of the mitochondrial membrane to Ca^{2+} (Lehninger *et al.*, 1967; Azzi and Azzone, 1966b); the lack of uncoupling activity of valinomycin on mitochondria in low potassium chloride media; and the lack of enhancement of proton translocation by uncouplers in oxygen pulse experiments. These considerations need not be repeated here. The only point to be discussed here is one not readily compatible with a primary cation (not H^+) pump driven by a high-energy intermediate.

It is difficult to envisage a natural ion pump that selectively transports natural and unnatural ions and ions of so distinct a nature as Ca^{2+}, K^+ in the presence of valinomycin, DDA^+ (see Section II,B), or $TPAs^+$. If, however, one were to postulate a specific selective pump for each of the natural ions, how could the transport of a synthetic cation such as DDA^+ (Liberman *et al.*, 1969) be accounted for?

The mechanism seems to be more general, unspecific for the chemical nature, and dependent only on the charge of the ionized species under consideration. These are the characteristics one would predict for an electric field-driven ion translocation process associated with a primary proton pump, regardless of whether this pump is driven by a high-energy intermediate or is the primary event in the energy-coupling process.

These arguments do not rule out the occurrence of specific metal cation pumps in energy-conserving membrane systems, but if they exist they appear not to be the only device for ion uptake.

Rottenberg *et al.* (1970) and Caplan and Essig (1969) have presented a nonequilibrium thermodynamic appraisal of oxidative phosphorylation that focuses attention on the ion translocation mechanism. They stress the importance of determining both forces (free energies, potentials) and fluxes and raise the question of the constancy of permeability coefficients during different metabolic states of the mitochondria. They conclude that chemical and chemiosmotic formulations are not mutually exclusive but that under certain conditions the chemiosmotic hypothesis is nothing more than a limiting case of the chemical coupling formulation.

A word of caution is needed in regard to *nonenergy dependent passive ionic movements*. Two points deserve particular comment.

(1) Binding is an aspect of great importance considering our relative ignorance of the state of the ion within the structure. It is well known that divalent cations have relatively high chemical affinities for several

functional groups such as phosphate, carboxylate, or basic nitrogens. The consequent reduction in *ionic activity* because of binding is not as critical with monovalent cations for which the binding affinity seems to be low. This point should be taken into consideration when attempting to draw conclusions regarding free energies of transport obtained by equating activity ratios with concentration ratios.

In addition, it has been reported that binding of one cation markedly affects the transport and/or binding of other cations (Chance and Mela, 1967; Brierley, 1967; Brierley and Settlemire, 1967; Brierley and Knight, 1967; Azzi and Azzone, 1966a,b; Chappell and Crofts, 1966).

Using SMP, Scarpa and Azzi (1968) and Jacobus and Brierley (1969) observed mono- (Rb^+, K^+, Na^+) and divalent (Ca^{2+}, Mg^{2+}, Zn^{2+}) ionic competitive binding and have implicated membrane phospholipids except in the case of Zn^{2+} where a protein binding site seems to be involved.

(2) Surface ionic exchanges, "passive" ionic exchanges which occur in the absence of redox energy and in the presence of uncouplers, have been reported in mitochondria as well as in other membrane systems (cf. Harris *et al.*, 1966b). It has been suggested that mitochondria behave as cation exchangers (cf. Harris *et al.*, 1966b; Harris and Manger, 1968; Lynn and Brown, 1965), and reports exist on such nonenergy-dependent binding of Ca^{2+} (C. S. Rossi *et al.*, 1967; Reynafarje and Lehninger, 1969), Mn^{2+} (Chappell *et al.*, 1963), Zn^{2+} (Brierley and Knight, 1967), Mg^{2+} (O'Brien and Brierley, 1965), and monovalent cations such as Na^+, K^+, and Rb^+ (Gear and Lehninger, 1968). It has been suggested that these ion-exchange properties may represent an early event in the energy-linked process of ion transport (Harris *et al.*, 1966b; Rasmussen *et al.*, 1965; Lardy *et al.*, 1967; Hoeffer and Pressman, 1966; Chance and Mela, 1966b.

II. STATEMENT OF FACTS

A. Mechanism of Action of Permeability Modifiers

A large number of recent contributors to the field of ion transport and energy coupling have made use of certain antibiotics (ionophores, cf. Pressman *et al.*, 1967) and other reagents known to confer selective ionic permeability on several natural and artificial membranes. Therefore a brief introduction to the nature of these reagents seems desirable (see also, Pressman, 1969; Mueller and Rudin, 1969).

Reagents That Modify Membrane Permeability

a. Nigericin. Nigericin is a low-molecular-weight, lipid-soluble, monobasic acid antibiotic shown to induce cation-for-cation or cation-for-H^+ exchange in mitochondria (Graven *et al.*, 1966a; Lardy *et al.*, 1967; Pressman *et al.*, 1967), chloroplasts (Shavit and San Pietro, 1967; Shavit *et al.*, 1968a; Packer, 1967), bacterial chromatophores of *Rhodospirillum rubrum*, *Rhodopseudomonas spheroides*, and *Chromatium* (Jackson *et al.*, 1968; Jackson and Crofts, 1969; Thore *et al.*, 1968; Nishimura and Pressman, 1969), microsomes (Pressman *et al.*, 1967), erythrocytes (Harris and Pressman, 1967; Pressman *et al.*, 1967; Henderson, *et al.*, 1969), liposomes (cf. Bangham, 1968; Henderson *et al.*, 1969), and *Streptococcus faecalis* (Harold and Baarda, 1968). Furthermore, nigericin catalyzes alkali ion/H^+ exchange in bilayer lipid membranes without affecting the ohmic resistance of the membrane (Mueller and Rudin, 1967, quoted by Pressman *et al.*, 1967). These observations have led Pressman to suggest that "nigericin-type ionophores transport alkali ions as electrically neutral dipoles and protons in their electrically neutral, undissociated form" (Pressman, 1968).

b. Valinomycin. Valinomycin is a low-molecular-weight, lipid-soluble, cyclic dodecadepsipeptide consisting of three repeating units of D-valine, L-valine, D-hydroxyvalerate, and L-lactate and without ionizable groups (Pressman *et al.*, 1967; Pinkerton *et al.*, 1969; Ivanov *et al.*, 1969).

Reports from various laboratories have shown that valinomycin confers selective ionic permeability on a variety of natural and artificial membrane systems (Chappell and Crofts, 1966; Chappell and Haarhoff, 1967; Harold and Baarda, 1967; Henderson *et al.*, 1969; Jackson *et al.*, 1968; Karlish *et al.*, 1969; Moore and Pressman, 1964; Mueller and Rudin, 1967; Pressman, 1965, 1967, 1968; Pressman *et al.*, 1967; Silman and Karlin, 1968; Thore *et al.*, 1968).

The spectrum of selectivity of this ionophore (cf. Pressman, 1968) is K^+, Rb^+, $Cs^+ >> NH_4^+$, Na^+, $MeNH_3^+$, Li^+ but not H^+ (cf. Henderson *et al.*, 1969; Pressman *et al.*, 1967) at neutral pH. The valinomycin–cation complex (Val–K^+) is charged, and Mueller and Rudin (1967), Lev and Buzhinsky (1967), Andreoli *et al.* (1967), and Finkelstein and Cass (1968) found that valinomycin-induced K^+ permeability gives rise to membrane conductance and biionic potentials (up to 150 mV) in bilayer phospholipid membranes. The ionic selectivity between K^+ and Na^+ is of the greatest biological importance and this discrimination can be as high as 10,000 to 1 in rat liver mitochondria (Pressman, 1965, 1969), or 300 to 1 in thin lipid films (Mueller and Rudin, 1967). Recently, Johnson and Bangham (1969) reported that the permeability of single compartment liposomes

(smectic mesophases of phospholipids) to K^+ is a linear function of the valinomycin concentration and can be increased from 10^{-14} to 10^{-12} cm sec^{-1}, at 27°, using a valinomycin/phospholipid molar concentration ratio of $1:10^6$. These investigators suggest that the mechanism of action of valinomycin is compatible with the carrier hypothesis (cf. Eisenman, 1968) and indicate that the increase in K^+ permeability is the result of an entropy increase in the activated state of about 35 cal $mole^{-1}$ $degree^{-1}$.

Shemyakin *et al.* (cf. Ivanov *et al.*, 1969), using nuclear magnetic resonance, infrared, and optical rotatory dispersion spectroscopy established the structure of valinomycin and studied the changes in conformation upon complexation with K^+. This spectroscopic evidence, together with the x-ray diffraction analysis of valinomycin (Pinkerton *et al.*, 1969) and of macrotetralide ionophores (Kilbourn *et al.*, 1967), suggests that the general principles involved in the design of antibiotics having cation carrier properties are (see Wipf *et al.*, 1969; Mueller and Rudin, 1969):

(1) The molecular periphery of the carrier–cation complex is hydrophobic, thus enhancing its lipid solubility in the lipophilic core of the membrane. The presence in the external surface of the hydrophobic branched side chains of the molecule provides an effective protection of the alkali ion and the internal system of hydrogen bonds from solvent attack (Ivanov *et al.*, 1969).

(2) The ionophore contains polar groups on the inside.

(3) The alkali–cation in the clathrate is not hydrated.

(4) Alkali–cation complexes of the valinomycin type (Pressman *et al.*, 1967) (valinomycin, enniatins, gramicidin, and the nactins) bear the positive charge of the cation.

Wipf *et al.* (1969) have contributed evidence in favor of electrophoretic migration of the macrotetralide–K^+ complex. They measured the movement of K^+ across a bulk membrane containing ^{14}C-labeled macrotetralide (75% nonactin, 23% monactin, and 2% dinactin) when an electrical potential was imposed upon the system and observed that, in agreement with the x-ray data of 1:1 complex formation (Kilbourn *et al.*, 1967), one K^+ ion was accompanied by one molecule of the macrotetralide; this also provides strong evidence in favor of the carrier-mediated transport proposal.

(5) Alkali–cation complexes of the nigericin type (Pressman *et al.*, 1967) (nigericin and monensins) (Agtarap *et al.*, 1967; Estrada-O *et al.*, 1967) are electrically neutral.

c. Gramicidin. Gramicidins A, B, C, and D are linear polypeptide units with the following generic formula: *N*-formyl–pentadecapeptide–ethanol-

amine (Lardy *et al.*, 1967; Pressman, 1965). These ion-transporting antibiotics show little ionic discrimination, the spectrum of ionic selectivity being K^+, Rb^+, Cs^+, NH_4^+, Li^+, $MeNH_3^+$, as well as H^+ (Lardy *et al.*, 1967; Henderson *et al.*, 1969; Pressman, 1965; Harris, 1968; Mueller and Rudin, 1967, 1969). The ion-transporting properties of gramicidin have been studied in mitochondria (Chappell and Crofts, 1966; Harris *et al.*, 1967a; Harris, 1968), chloroplasts (Avron and Shavit, 1965; Dilley and Shavit, 1968; Shavit *et al.*, 1968a; Karlish *et al.*, 1969; Witt *et al.*, 1968), bacterial chromatophores (Baltscheffsky and Baltscheffsky, 1960; Jackson *et al.*, 1968; Von Stedingk and Baltscheffsky, 1966), red blood cells (Henderson *et al.*, 1969; Chappell and Crofts, 1966; Chappell and Haarhoff, 1967; Harris, 1968), liposomes (Chappell and Crofts, 1966; Chappell and Haarhoff, 1967; Henderson *et al.*, 1969), bilayer phospholipid membranes (Mueller and Rudin, 1967; Skulachev *et al.*, 1969), *S. faecalis* (Harold and Baarda, 1967), and the electroplax membrane (Silman and Karlin, 1968; Podleski and Changeux, 1969).

d. Conventional Uncouplers. We consider as conventional uncouplers a variety of organic compounds that are lipid-soluble weak acids and in which the electronic charge of the ionized species is delocalized in a π-electron orbital system (cf. Mitchell, 1962, 1966a,b, 1968). Among these substances are: nitrated (e.g., DNP), halogenated (e.g., PCP), or oxygenated (e.g., dicoumarol) phenols; derivatives of carbonylcyanide phenylhydrazone (e.g., FCCP, the trifluoromethoxy derivative), and halogenated and nitrated derivatives of salicylanilide (cf. Lardy and Fergusson, 1969).

Mitchell (1962, 1966a,b, 1968) proposed that uncoupling agents increased the permeability of the mitochondrial membrane to H^+ and suggested that these "proton-conductors" catalyzed H^+ equilibration by passing one way through the "coupling membrane" in the protonated form and the other way in the ionized anionic form. This prediction of the chemiosmotic coupling hypothesis has been confirmed in artificial thin phospholipid membranes by Thompson *et al.* (Bielawski *et al.*, 1966; Hopfer *et al.*, 1968), Skulachev *et al.* (1967, 1968, 1969), and Lea and Croghan (1969). It has been observed that the uncoupling capacity of these agents in mitochondria varies linearly with their proton conductor faculty in artificial membranes (Liberman *et al.*, 1969).

The groups of Thompson (Bielawski *et al.*, 1966; Hopfer *et al.*, 1968), Skulachev (1967, 1968, 1969), and Liberman (Liberman *et al.*, 1969; Liberman and Topaly, 1968a,b) provided compelling evidence of the proton conduction capacity of more than 40 chemical uncouplers of distinct structure. They showed that:

(1) The conductance of bilayer lipid membranes of the Mueller–Rudin

type (cf. Mueller *et al.*, 1964; Mueller and Rudin, 1969) increased several orders of magnitude upon addition of the lipid-soluble substance (see also Bielawski *et al.*, 1966).

(2) The conductance was a linear and in some cases a square law function of the uncoupler concentration.

(3) The conductance had a maximum value coincident with the pK of the weak acid. Increasing the concentration of uncoupler resulted in larger conductances without shift in pH for peak maximum activity. For example, trinitrophenol (picric acid) was most active at pH ~1, DNP pH ~4, and C-CCP at pH ~7. This suggests that at low pH the conductance is rate limited by the anionic form of the uncoupler and that at high pH it is limited by the ionic species transported (H^+). (see also Hopfer *et al.*, 1968).

(4) A transmembrane concentration gradient of either H^+ or the charged form of the uncoupler (carrier) generates a diffusion potential (potential difference) that exhibits a maximum at the same pH as the conductance. The measured potentials agree well with those calculated from the Nernst equation.

(5) A plot of the concentration of uncoupler required to induce twofold stimulation of succinate oxidation in rat liver mitochondria in state 4 as a function of the concentration of uncoupler that increases the proton conductance of the membrane by 5×10^{-9} mhos·cm^2 results in an almost straight line at 45°. This indicates good correlation between these two experimental parameters.

Ting (1970) and Ting *et al.* (1970) obtained a 500-fold discrepancy between the activity of uncouplers in increasing the electrical conductance of lipid bilayers 10-fold and fully releasing respiratory control in rat liver mitochondria. One of the areas of disagreement between the groups of Skulachev and Chance may be the difference in lipid solubilities (different partition coefficient of the uncoupler between the aqueous and lipid phases) and lipid specificity of the different uncouplers. It must be pointed out that DNP is more effective in lecithin-cholesterol bilayer lipid membranes (Bielawski *et al.*, 1966; Hopfer *et al.*, 1968) than in brain phospholipid tocopherol bilayer lipid membranes (Mueller and Rudin, personal communication). This may also be the explanation of why higher concentrations of uncoupler (DNP or FCCP) are required for complete inhibition of photophosphorylation in chloroplasts (Karlish and Avron, 1968a; Karlish *et al.*, 1969).

Based on these experiments, Liberman and Topaly have put forward a classification of uncouplers according to their ion-carrying property (Table III). They have classified the uncouplers in three groups (see Table III):

(1) Proton carriers*:
T-TH type: derivatives of phenol, benzimidazole, carbonylcyanide phenylhydrazone, dicoumarol, pinacol, and other lipid-soluble acids
T-TH$^+$ type: mono-, bi-, and trisubstituted amines and phosphines, and other lipid-soluble bases
Lipid-soluble esters existing in enolic and ketonic forms
(2) Cation carriers:
Neutral: valinomycin
Negatively charged: alamethicin
(3) Lipid- and water-soluble cations of the triethyltin$^+$ type

If uncoupler induces H^+ permeability and valinomycin induces K^+ conduction under specified conditions, a combination of uncoupler and valinomycin may catalyze K^+/H^+ exchange similar to that induced by nigericin, although the mechanism of the reaction is conceptually different. Only upon addition of an uncoupling agent (FCCP or DNP) does rapid K^+/H^+ exchange occur in valinomycin-treated red blood cells (Harris and Pressman, 1967), liposomes (Chappell and Haarhoff, 1967; Chappell *et al.*, 1968; Henderson *et al.*, 1969), mitochondria (Mitchell and Moyle, 1967b, 1969b; Carafoli and Rossi, 1967; Pressman *et al.*, 1967; Caswell, 1968), and chloroplasts (Karlish *et al.*, 1969).

Another possibility that follows from the above-mentioned considerations is that a combination of nigericin and valinomycin would catalyze an overall net H^+ conduction. This is discussed in detail in Section II, E. A third possible combination, uncoupler and nigericin, would mimic the ion permeability-inducing effects of valinomycin. Mitchell and Moyle (1969b) have reported that under specified conditions such a combination gave effects similar to those induced by valinomycin in mitochondria as far as light-scattering phenomena are concerned. Regardless of the net effects observed with combinations of permeability-modifying substances, the mechanism of action of each of the above-mentioned reagents is relatively clear-cut and specific.

B. The Sidedness of the Coupling Membrane

As becomes apparent later, the membrane systems involved in energy conservation possess well-defined asymmetric functional properties that are of importance in the energy-coupling process.

* T, translocator; T^-, anionic form; TH, neutral form; TH^+, cationic form.

TABLE III

EFFECT OF VARIOUS SUBSTANCES ON BILAYER LIPID MEMBRANE CONDUCTIVITY[a,b]

Substance	Conductivity of bilayer lipid membrane (mhos/cm²)[c]			Type of carrier	Penetrating ion
	5×10^{-9} *M*	10^{-7} *M*	10^{-6} *M*		
Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone	10^{-10}	2×10^{-7}	5.5×10^{-6}	$T^- - TH$	H^+
Carbonylcyanide-*m*-chlorophenylhydrazone	10^{-7}	2.3×10^{-6}	3.5×10^{-5}	$T^- - TH$	H^+
Carbonylcyanide-2,4,5-trichlorphenylhydrazone	6×10^{-11}	2.8×10^{-9}	8×10^{-8}	T^-–TH	H^+
Tetrachloro-2-trifluoromethylbenzimidazole	1.5×10^{-7}	1.7×10^{-6}	6×10^{-6}	T^-–TH	H^+
5-Nitro-2-trifluoromethylbenzimidazole	2×10^{-6}	4×10^{-5}	1.4×10^{-4}	T^-–TH	H^+
2-Methyl-4-chlorophenol	2×10^{-4}	1.2×10^{-3}	4.5×10^{-3}	T^-–TH	H^+
2-4-Dichlorophenol	7×10^{-6}	1.4×10^{-4}	10^{-3}	T^-–TH	H^+
2,4,5-Trichlorophenol	3×10^{-6}	1.7×10^{-5}	6×10^{-5}	T^-–TH	H^+
Pentachlorophenol	1.2×10^{-6}	3×10^{-5}	—	T^-–TH	H^+
m-Nitrophenol	2.4×10^{-4}	1.3×10^{-3}	3×10^{-3}	T^-–TH	H^+
2,4-Dinitrophenol	1.7×10^{-4}	1.4×10^{-3}	—	T^-–TH	H^+
2,4,6-Trinitrophenol (TNP)	3×10^{-6}	7×10^{-5}	8×10^{-4}	T^-–TH	TNP^- and H^+
Acetoacetic ester	4.5×10^{-2}	3×10^{-1}	—	T^-–TH	H^+
Perfluoropinacol	4×10^{-7}	5×10^{-6}	3×10^{-5}	T^-–TH	H^+
Decylamine (DA)	10^{-4}	7.5×10^{-4}	2×10^{-3}	T^-–TH^+	DA^+ and H^+
Tributylamine (TBA)	10^{-4}	2×10^{-3}	8×10^{-3}	T^-–TH^+	TBA^+ and H^+
Gramicidin A	2×10^{-11}	4.5×10^{-10}	3.8×10^{-9}	T–T–$cation^+$	K^+
Gramicidin A[d]	2×10^{-9}	8.5×10^{-9}	2.3×10^{-8}	—	H^+
Valinomycin	10^{-10}	3×10^{-9}	2.3×10^{-8}	T–T–$cation^+$	K^+
I_2	1.3×10^{-5}	5×10^{-5}	8×10^{-5}	T–T–$anion^-$	I^-
I_2[e]	—	—	4×10^{-6}	T–T–$anion^-$	I^-

TABLE III (*Continued*)

Substance	Conductivity of bilayer lipid membrane (mhos/cm²)[c]			Type of carrier	Penetrating ion
	5×10^{-9} *M*	10^{-7} *M*	10^{-6} *M*		
Tetraphenylboron sodium (TPB)	8×10^{-9}	3×10^{-7}	3×10^{-6}	T^-–TH	TPB^-
Dimethyldibenzyl-ammonium chloride (DDA)	4×10^{-5}	10^{-3}	—	—	DDA^+
Tributyl stannous oxide (TBO)	4×10^{-7}	6.3×10^{-6}	—	$T\text{–}TH^+$	TBO^+ and H^+
N,*N*-Diphenyl-*m*-aminodiphenyl (DAP)	6×10^{-6}	2×10^{-3}	—	—	DAP

[a] Taken from Liberman and Topaly (1968a).

[b] The table gives the concentration of substances in aqueous solution at which bilayer lipid membranes have the conductivities indicated. The bilayer lipid membranes were obtained in an incubation medium for mitochondria containing 0.25 *M* sucrose, 20 m*M* potassium chloride, 10 m*M* potassium phosphate, 5 m*M* magnesium chloride, 30 m*M* tris–HCl (pH 7.5), and 2 m*M* EDTA. The mean square deviations did not exceed 30%.

[c] This refers to the conductivity induced by a carrier and equals the total conductivity of the bilayer lipid membrane minus the conductivity in the absence of a carrier.

[d] The bilayer lipid membranes were obtained in 30 m*M* tris–HCl (pH 7.5).

[e] The incubation medium for mitochondria contained 10 m*M* potassium iodide.

Electron microscopy has shown that the inner mitochondrial membrane is a folded continuous sheath giving rise to structures known as cristae (Palade, 1956) which exhibit smooth membrane structure when examined under an electron microscope after positive staining. When the structures are negatively stained with phosphotungstic acid, however, the surface of the inner membrane appears to be covered with 85-Å spheres spaced at regular intervals and connected by a narrow "stalk" to the unstained membrane (Fernández-Morán, 1962; Fernández-Morán *et al.*, 1964; Stoeckenius, 1963; Parsons *et al.*, 1967). Detailed evidence has been presented identifying these membrane subunits with the cold-labile ATPase (Racker's F_1), the terminal enzyme of the phosphorylative pathway (Kagawa and Racker, 1966; Racker *et al.*, 1965; Racker, 1967).

It is of great importance that the location of the coupling factor 1 on one side of the membrane provides a marker for the sidedness of the inner membrane of mitochondria, chloroplasts, and bacterial chromatophores

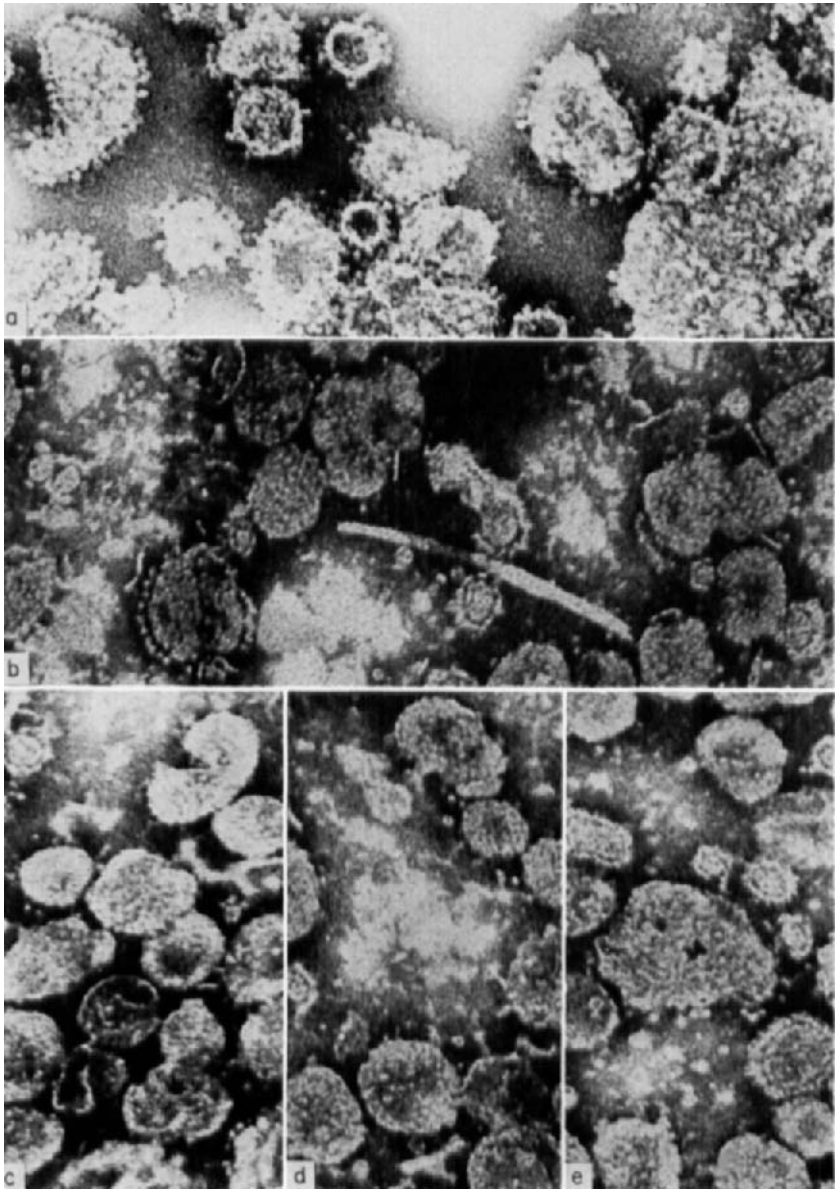

FIG. 2. Electron micrographs of negatively stained SMP, chloroplasts, and chromatophores. ×160,000. (a) Negatively stained SMP from beef heart. (Courtesy of Dr.H. Löw.) (b–e) Negatively stained chromatophore preparations from *R. rubrum*.(Courtesy of Dr. H. Löw.)

(Fig. 2). In intact mitochondria the spheres face the matrix space, whereas in SMP they are in direct contact with the solution. This constitutes the structural basis of the now generally accepted concept of the "inside-out" relationship of mitochondria and SMP (Lee and Ernster, 1966; Mitchell, 1966a,b; Racker 1969a,b).

Whereas F_1 in SMP is readily available to its specific antibody, this is not true in mitochondria (DiJeso *et al.*, 1969). During reversal of electron transfer, sonic SMP oxidize only endogenous cytochrome c, whereas mitochondria (or SMP prepared by digitonin treatment) oxidize only exogenous cytochrome c (Chance and Fugman, 1961; Lee, 1963). Endogenous cytochrome c is retained in SMP under conditions that release it from mitochondria (Jacobs and Sanadi, 1960a,b; Lenaz and MacLennan, 1966). Cytochrome c is readily washed out from swollen mitochondria at high ionic strengths (Jacobs and Sanadi, 1960a,b; MacLennan *et al.*, 1966), whereas cytochrome c in SMP is removed only after treatment with phospholipase (Ambe and Crane, 1959) or detergents (Ball and Cooper, 1957). There is stimulation of cytochrome c oxidation by the detergent Lubrol in SMP but not in mitochondria (Muscatello and Carafoli, 1969). The binding of cytochrome c to cytochrome c-deficient mitochondria in the presence of potassium chloride is unaffected by the presence or absence of valinomycin (see Section II,A) which modifies the K^+ concentration in the matrix space. This suggests that the binding site for cytochrome c is on the outer surface of the inner membrane (Nicholls *et al.*, 1969).

The asymmetric nature of the inner mitochondrial membrane is also illustrated by the reactions in which pyridine nucleotides are involved. Heart mitochondria oxidize externally added NADH at a very slow rate and exhibit extremely low P/O ratios (cf. Racker, 1965), whereas SMP oxidize exogenous NADH at high rates and at efficiencies close to theoretical P/O ratios (Hansen and Smith, 1964; Ernster and Lee, 1964). The pyridine nucleotide content of mitochondria is very high (Purvis, 1960), while there are no endogenous coenzymes in SMP (Chance *et al.*, 1966a). It has been shown that the transhydrogenase reaction can be monitored in SMP only with exogenous NADH and $NADP^+$ (Danielson and Ernster, 1963a,b). A similar situation is observed with the energy-linked reduction of NAD^+ by succinate (reversal of electron transfer), driven either by electron transfer reactions supported by ascorbate and TMPD at the third site of the respiratory chain, or by ATP hydrolysis (Löw and Vallin, 1963; Löw *et al.*, 1963) where the exogenous NAD^+ can be utilized only by SMP. Mitochondria, however, can reduce only endogenous coenzyme (Chance, 1961a,b). As early as 1961 Thompson and McLees suggested that mitochondria and sonic particles have membranes of opposite polarity.

This suggestion was based on the differences in the electrophoretic mobility of mitochondria and SMP. Thompson and McLees (1961) also suggested that the surface of the cristae membrane in SMP was in direct contact with the medium.

The chloroplasts, the chlorophyll-containing organelles of green plants, perform the energy transduction reactions that follow the absorption of light energy from the sun. Electron microscopy has shown that the chloroplast has an internal structure of lamellae that appear as double membranes, the total thickness of which is of the order of 250 Å. These "sac-like structures" or thylakoids (cf. Menke, 1966) are considered the photosynthetic units in which the two photoevents and the coupling process take place. Negatively stained preparations of chloroplast thylakoid membranes (frequently called grana discs) reveal numerous 100- to 150-Å particles located on the surface of the membrane (Vambutas and Racker, 1965; Parsons *et al.*, 1965; Howell and Moudrianakis, 1967; Moudrianakis, 1968). These particles have been identified as a Ca^{2+}-dependent ATPase involved in the dark terminal step in photophosphorylation. Therefore the one-sided location of the ATPase provides a marker for the sidedness of the inner mitochondrial and chloroplast membrane; in both SMP and chloroplasts the coupling factor is in direct contact with the solution (Fig. 2f).

Photosynthetic bacteria are capable of performing oxidative phosphorylation if grown in the dark, and photosynthetic phosphorylation if incubated in the presence of light. Electron microscope studies of the structure of *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* show intracytoplasmic membranes in continuity with the plasma membrane, with a rough lamellar arrangement reminiscent of that present in mitochondria and chloroplasts. The photosynthetic apparatus has been shown to be associated with these membranous structures. Cohen-Bazire and Kunisawa (1963) and Holt and Marr (1965a,b) have suggested that the *chromatophores* (vesicular membranous structures formed after the disruption of the membranes of photosynthetic bacteria) are formed from these membrane invaginations by "pinching off." High-resolution electron microscopy of negatively stained preparations has revealed numerous particles approximately 100 Å in diameter located on the surface of the chromatophore membrane (Löw and Afzelius, 1964). (Fig. 2b–e). It has been suggested that these particles represent the coupling factor (ATPase) involved in the last stage of photophosphorylation, in complete analogy to the mitochondrial and chloroplast F_1 (cf. Racker, 1969b). The recent isolation of this coupling factor from chromatophores (Baccarini-Melandri *et al.*, 1970) by methods similar to those used in the mitochondrial and chloroplast systems gives further support to this suggestion.

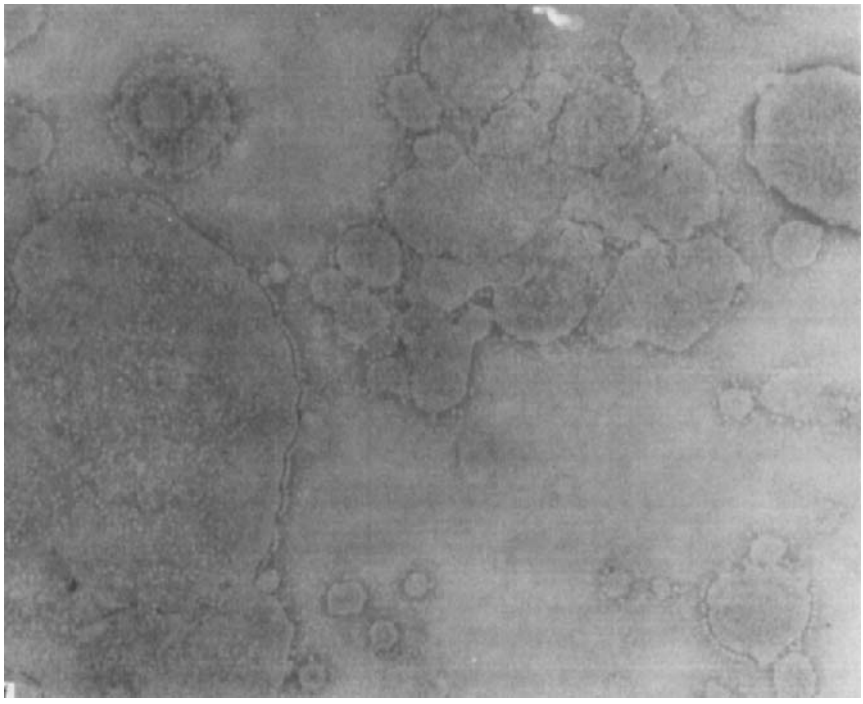

FIG. 2f. Negatively stained fragments of chloroplast lamellae. (Courtesy of Dr. E. Racker). ×160,000.

Therefore, from the morphological point of view SMP, chloroplasts and chromatophores exhibit the same membrane sidedness (see Fig. 2).

C. H^+ Translocation in Energy-Conserving Membrane Systems

Table IV summarizes our present knowledge of the characteristics of H^+ translocation in mitochondria, SMP, chloroplasts, and photosynthetic bacteria and their chromotophores.

In general, H^+ translocation can be driven by electron transfer reactions, supported either by substrate oxidation (mitochondria, SMP, and bacteria grown in the dark) or light (chloroplasts and bacteria grown in the light). This transport process exhibits a defined vectorial nature, being in the direction of intravesicular alkalinization (i.e., redox-driven H^+ efflux from the vesicular structure) in mitochondria and intact bacteria and of intravesicular acidification (i.e., redox-driven H^+ uptake) in SMP, chloroplasts, and chromatophores.

The stoichiometry of H^+ translocated per electron transferred along the chain (H^+/e^-) still remains a matter of controversy.

The process is sensitive to electron transfer inhibitors and uncouplers. Energy transfer inhibitors stimulate the extent of the redox-driven pH gradient in SMP, chloroplasts, and chromatophores, a point that is discussed in Section III.

In general terms, valinomycin ($+K^+$) stimulates and nigericin ($+K^+$) inhibits the rate, extent, or both, of the redox-driven proton translocation, whereas a combination of the two ionophores ($+K^+$) prevents or abolishes the reaction.

When H^+ translocation is driven by ATP hydrolysis, the reaction is sensitive to uncouplers and energy transfer inhibitors but not to electron transfer inhibitors.

Other cation and anion movements have been reported to be associated with the proton movements in order to maintain charge balance. The particular case of SMP is now reviewed in detail.

1. Redox Dependence

Mitchell and Moyle (1965) and Chance and Mela (1967) reported that activation of electron transport reactions in SMP resulted in the uptake of protons by the particles (intravesicular acidification). The former investigators also reported H^+/O ratios of 0.83 and 1.29 for succinate and NADH oxidation, respectively, under conditions they regard as "suboptimal." Furthermore, they observed that the H^+ translocation induced by succinate and NADH oxidation was inhibited by antimycin A and rotenone, respectively, and in both cases was stimulated (about 25%) by oligomycin.

Chance and Mela (1967) reported a H^+/O of 1.5 during succinate oxidation, and Packer and Utsumi (1969) observed a H^+/O ratio of 0.88 in the presence of choline chloride and oligomycin.

Montal *et al.* (1970b) observed an almost 4-fold stimulation of H^+ uptake by SMP induced by oligomycin during succinate oxidation, a reaction that was sensitive to antimycin A. Furthermore, they reported that oligomycin could trigger the H^+ translocation reactions, provided the preparation was supplemented with a respiratory subtrate, in a manner analogous to that observed for K^+ translocation. (see Section II,E).

2. Phosphate Bond Dependence (ATP-Driven H^+ Translocation)

Mitchell and Moyle (1965) reported that hydrolysis of ATP induced an inward H^+ translocation, giving an H^+/P ratio of 0.53, which was insensitive to electron transfer inhibitors (rotenone and antimycin A) and

TABLE IV

H^+ MOVEMENTS IN ENERGY-CONSERVING MEMBRANE SYSTEMS[a,b]

Characteristics	Mitochondria	SMP	Chloroplasts	Photosynthetic bacteria	Chromatophores
Redox dependence	Yes (*1–5*)	Yes (*10–12*)	Yes (*15–20*)	Yes (*29, 55*)	Yes (*29–38*)
Stoichiometry (H^+/e^-)	±1 (*1–5*)	0.3–0.8 (*4, 10, 11*)	1–5 (*15–21*)	(H^+/O) 0.55–4.2 (*29, 55*)	(H^+/O) 0.4–0.6 (*29, 38*); ~1.5 (in the presence of valinomycin and oligomycin (*29*)
Directionality	Intravesicular alkalinization (*1–5*)	Intravesicular acidification (*10–12*)	Intravesicular acidification (*15–20*)	Intravesicular alkalinization (*29, 55*)	Intravesicular acidification (*29–38*)
Effect of inhibitors					
Electron transfer	P and A (*1–5*)	P and A (*10–12*)	P and A (*15–20*)	Inhibited (*55*)	P and A (*29–38*)
Energy transfer	NR (*1–5*)	S (*10–12*)	S (*21*)	NR	S (*30–32*)
Uncouplers	P and A (*1–5*)	P and A (*10–12*)	P and A (*15–20*); stimulates rate but not extent at low concentrations (*23*)	P and A (high); S (low) (*29–55*)	P and A (high) (*29–38*)
Effect of ionophores					
Valinomycin	S (rate and extent) (*1*)	S (rate and extent) (*13*)	Stimulates rate (*22–23*) but not extent	S (rate and extent) (*29, 55*)	S (*29, 30, 34, 36, 38*) (rate and extent)

(*Continued*)

TABLE IV (*Continued*)

Characteristics	Mitochondria	SMP	Chloroplasts	Photosynthetic bacteria	Chromatophores
Nigericin	NR	Partial inhibition (*13*)	P and A (*24–27*)	NR	Inhibits extent (*34, 36, 38*)
Val + Nig.	P and A (*6*)	P and A (*12*)	No significant difference from nigericin alone (*23*)	NR	P and A (*36, 38*)
Gramicidin	P and A (*7–9*)	P and A (*14*)	P and A (*23, 26, 28*)	NR	P and A (*36*)
ATP hydrolysis dependence	Yes (*5, 39*)	Yes (*10, 11*)	Yes (*54*)	Yes (*29*)	Yes (*29*)
Stoichiometry (H^+/P_i)	2.0 at pH 7.0–7.1 (*39*)	~0.5 (*10*)	2.0 (*54*)	NR	~0.4 (*29*)
Directionality	Intravesicular alkalinization (*5, 39*)	Intravesicular acidification (*10, 11*)	Intravesicular acidification (*54*)	Intravesicular alkalinization (*29*)	Intravesicular acidification (*29*)
Effect of inhibitors					
Electron transfer	I (*39*)	I (*10, 11*)	NR	I (*29*)	I (*29*)
Energy transfer	P and A (*5, 39*)	P and A (*10*)	NR	P and A (inferred) (*29*)	P and A (*29*)
Uncouplers	P and A (*5, 39*)	P and A (*10, 11*)	Partial inhibition (*54*)	P and A (*29*)	P and A (*29*)

Proton movements associated with the movement of other ions					
Monovalent cations	Yes	Yes	Yes	NR	Yes
Stoichiometry (K^+/H^+)	0.1–1.0 (*40–44*)	~1 (*12, 13, 45*)	0.3–1 (*18, 19*)	—	~1 (*36*)
Divalent cations	Yes	Yes	Yes	NR	NR
Stoichiometry (H^+/C^{2+})	0.7–2.0 (*46–50*)	~1 (*51–52*)	~1 (*18*)	NR	NR
Anions	~1 (*41, 43*)	NR	~1 (*19*)	NR	NR

[a] Italicized numbers in parentheses indicate the following references:
(*1*) Mitchell and Moyle (1967b). (*2*) Snoswell (1966). (*3*) Wenner (1966). (*4*) Packer and Utsumi (1969). (*5*) Chance (1967), Chance and Mela (1966a, b, c, d, e). (*6*) Pressman *et al.* (1967). (*7*) Harris *et al.* (1967a). (*8*) Pressman (1965). (*9*) Chappell and Crofts (1966). (*10*) Mitchell and Moyle (1965). (*11*) Chance and Mela (1967). (*12*) Montal *et al.* (1970b). (*13*) Montal (1970), Papa *et al.* (1970). (*14*) Montal *et al.* (1970a). (*15*) Schwartz (1968), Dilley and Vernon (1967), Lynn and Brown (1967), Lynn (1968), Karlish and Avron (1967). (*16*) Neuman and Jagendorf (1964). (*17*) Jagendorf and Uribe (1966a,b). (*18*) Dilley and Vernon (1965), Crofts *et al.* (1967). (*19*) Deamer and Packer (1969), Packer and Crofts (1967), Izawa and Hind (1967). (*20*) Avron and Neuman (1968), Good *et al.* (1966), Avron and Chance (1966). (*21*) Dilley (1970), Karlish and Avron (1968b). (*22*) Avron and Shavit (1965). (*23*) Karlish *et al.* (1969). (*24*) Shavit and San Pietro (1967). (*25*) Packer (*1967*). (*26*) Shavit *et al.* (1968a). (*27*) Karlish and Avron (1968a), Karlish *et al.* (1969). (*28*) Dilley and Shavit (1968). (*29*) Scholes *et al.* (1969). (*30*) Von Stedingk and Baltscheffsky (1966). (*31*) Von Stedingk (1967). (*32*) Nishimura (1970). (*33*) Nishimura *et al.* (1968). (*34*) Nishimura and Pressman (1969). (*35*) Chance *et al.* (1966b). (*36*) Jackson *et al.* (1968). (*37*) Shavit *et al.* (1968b). (*38*) Thore *et al.* (1968). (*39*) Mitchell and Moyle (1968), C. S. Rossi *et al.* (1967). (*40*) Moore and Pressman (1964). (*41*) Chappell and Crofts (1965). (*42*) Harris *et al.* (1966b). (*43*) Mitchell and Moyle (1969b). (*44*) Ogata and Rasmussen (1966). (*45*) Cockrell and Racker (1969). (*46*) Rasmussen *et al.* (1965). (*47*) C. S. Rossi *et al.* (1966). (*48*) C. Rossi *et al.* (1966). (*49*) Carafoli *et al.* (1966). (*50*) Lehninger *et al.* (1967). (*51*) Loyter *et al.* (1969). (*52*) Christiansen *et al.* (1969b). (*53*) Cockrell and Racker (1969, and personal communication). (*54*) Carmeli (1970). (*55*) Edwards and Bovell (1969, 1970).

[b] P, prevented; S, stimulated; NR, not reported; I, insensitive; A, abolished.

inhibited by energy transfer inhibitors (oligomycin). Chance and Mela (1967) also reported an ATP-supported inward H^+ translocation (as judged from a decrease in the absorbance of bromthymol blue (3,3′-dibromothymolsulfonphthalein) which was inhibited by both oligomycin and uncouplers.

3. Effects of Nigericin

Preincubation of SMP with nigericin results in inhibition of the respiration-driven and oligomycin-dependent H^+ uptake; this effect is dependent on the K^+ concentration of the medium. Addition of nigericin to oligomycin-supplemented SMP oxidizing succinate after steady-state H^+ uptake has been accomplished results in further H^+ uptake at low concentrations of potassium chloride in the external medium. Raising the concentration in the medium results in effects that vary from no change induced by addition of nigericin to H^+ efflux at higher potassium chloride concentration, in agreement with the effects observed during K^+ translocation (see Section II,E). The results have been used to determine the approximate magnitude of the pH gradient in the high-energy state of SMP; the value is approximately 1 pH unit (Montal *et al.*, 1970b). Similar experiments and results have been obtained by Jackson *et al.* (1968) for *R. rubrum* chromatophores.

4. Effect of Valinomycin

Preincubation of SMP with valinomycin in the presence of K^+ results in a 2- to 3-fold stimulation of both rate and extent of oligomycin-dependent and respiration-driven H^+ translocation (Montal, 1970; Papa *et al.*, 1970).

When both valinomycin and nigericin were present, they prevented or abolished the pH gradient normally present during respiration (Montal *et al.*, 1970b), in analogy with the effect of conventional uncouplers such as FCCP or the ionophore gramicidin (Montal *et al.*, 1970a).

We have repeatedly referred to observations that support the fact that SMP have membrane sidedness opposite that of mitochondria. In this section reference is made only to the opposite behavior performed by mitochondria and SMP with regard to ion translocation and membrane charge.

Skulachev and co-workers (1969) and Liberman, *et al.* (1969), in a series of elegant experiments, utilized the bilayer lipid membrane (cf. Mueller and Rudin, 1969) in an assay of ion uptake (as an ion-specific electrode). They observed that certain lipid-soluble charged molecules increased the

electrical conductance of the bilayer lipid membrane severalfold (Liberman, and Topaly, 1968a,b). A membrane was formed, and equimolar concentrations of the lipid-soluble cation or anion were placed on both sides of the membrane. Mitochondria or SMP were added to one side of the membrane and upon energization these investigators measured a membrane potential resulting from the transmembrane diffusion of ions toward the side where mitochondria or SMP had been added. They therefore concluded that the particles accumulated ions.

The lipid-soluble cation DDA^+ was taken up by mitochondria but not by SMP, whereas the lipid-soluble anions TPB^- and PCB^- were taken up by SMP and not by mitochondria. When ions were accumulated at the expense of redox energy, this process was sensitive to antimycin A. When accumulation took place at the expense of phosphate bond energy, the process was reversed by oligomycin.

The ion movements were associated with acidification of the medium when mitochondria were involved, and with alkalinization in the case of SMP. The uncoupler TTFB reversed the pH changes and released the accumulated ions. Skulachev *et al.* (1969) concluded that "charge specific ion accumulation in mitochondria and SMP is due to ion-movement in electric field created by the energy-dependent H^+/OH^- separation."

Montal *et al.* (1970b) have reported, in agreement with Skulachev *et al.*, that TPB^- migrates (probably electrophoretically) into SMP but not into mitochondria, whereas tetraphenylarsonium, $TPAs^+$ (a cation), a ring system indentical to that of TPB^-, in which the boron metal has been replaced by arsonium, does not migrate into SMP and cannot substitute either for NO_3^- or valinomycin in the K^+ + nigericin- or NH_4^+-dependent uncoupling effect. It uncouples intact mitochondria, however (see Section II,E). Azzi (1969) studied the response of charged fluorescence probes to the energy state of mitochondria and SMP and reported that the energization of mitochondria was accompanied by increased fluorescence of the cationic probe Auramine-o (tetramethyldiaminodiphenyl ketoimine hydrochloride) and decreased fluorescence of the anionic probe ANS. In SMP, however, opposite changes have been observed, that is, enhancement of fluorescence of ANS (see also Datta and Penefsky, 1970; Azzi *et al.*, 1969) and decrease of fluorescence of Auramine-o. The results were interpreted as being attributable to changes in binding of the dye "induced by charge changes of membrane."

All these experiments strongly suggest the existence of a charge-specific mechanism of ion uptake by the inner mitochondrial membrane which operates in one direction in the intact structure and in the opposite direction when the structure is fragmented.

D. Cation and Anion Transport in Mitochondria

K^+ Transport

This topic has been covered in great detail by Lehninger *et al.* (1967), Pressman (1969), and Greville (1969), and our contribution is an attempt to systematize (classify) these ion movements in a way suitable to stimulate discussion on mechanisms of transport. In particular, Ca^{2+} transport in mitochondria is not discussed because it is the main subject of another publication (cf. Chance and Mela, 1971). Table V summarizes the main types of K^+ transport observed in intact mitochondria. Types (1) and (2) illustrate that the intrinsically low K^+ permeability of the mitochondrial membrane can be enhanced by alterations of the membrane that result from interaction with different disturbing factors such as pH, temperature, heavy metals, chelators, sulfhydryl reagents, or hormones. What the chemical or physicochemical basis of this modification of permeability is still remains an unsolved problem.

Types (3) and (4) have been extensively reviewed in recent years (cf. Pressman, 1968, 1969; Mitchell, 1968; Greville, 1969; Mueller and Rudin, 1969), and although compelling evidence has been produced showing that these ionophores can induce passive permeability or act as primary translocators in the absence of any other transport device (cf. Mueller and Rudin, 1969; Andreoli *et al.*, 1967; Lev and Buzhinsky, 1967), their possible interaction with a natural ion pump cannot at present be ruled out (cf. Pressman, 1969).

Type (5) transport raises the question whether uncouplers translocate primarily H^+ or K^+ in mitochondria and whether valinomycin induces primary K^+ or H^+ permeability. Moreover, type 5 transport raises the problem whether results obtained on model systems can be applied or extrapolated to natural membranes. The argument can be settled only by further experimentation, although current opinion favors the idea of H^+ conduction mediated by uncouplers and K^+ conduction induced by valinomycin in both biological and artificial membrane systems.

Type (6) illustrates that in the absence of redox and phosphate bond energies mitochondrial membranes can reversibly transform the electrochemical energy of one ionic species (K^+) into that of another species (Ca^{2+}) and gives strong support to the view that transport phenomena in mitochondria are just particular cases of the generalized process of "membrane transport."

The controversy relating to the nature of mitochondrial swelling associated with spontaneous or ionophore-induced cation and coupled anion uptake has not been resolved. Chappell and Crofts (1965), Rasmussen *et al.* (1964), Ogata and Rasmussen (1966), and Azzi and Azzone (1966a,

b), have proposed that the volume changes are attributable to a primary transport of solutes. This would then increase the intramitochondrial osmotic pressure and lead to a secondary water transport until a new isosmotic equilibrium is attained.

Pressman (1965) and Cockrell *et al.* (1966) have suggested, however, that the extent of swelling was greater than predicted and that this deviation is not a function of the amount of K^+ taken up. They therefore proposed the existence of simultaneous mechanochemical changes (Lehninger, 1962), or conformational changes of the type described by Hackenbrock 1966, 1968, 1969) and Blondin and Green (1967).

The recent work of Rottenberg and Solomon (1969) has provided evidence that "water movement follows solute movement in accordance with conventional physical forces, so that there is no need to invoke any other physical forces for mitochondrial volume changes which are linked to solute transport."

Anion Transport

The subject of anion transport in mitochondria has been repeatedly reviewed in recent years, and the reader is referred to the papers of Chappell (1968), Chappell and Haarhoff (1967), Chappell *et al.* (1968), Lardy and Fergusson (1969), and Klingenberg (1970). For the sake of completeness, we reproduce a table (Table VI) from Klingenberg's review (1970), partially supplemented with Chappell's data (1968).

In a recent contribution Mitchell and Moyle (1969b) have classified the anion movements that occurr in mitochondria into four classes:

(1) Fast net translocation of *anion* as compared with that of the corresponding *acid*; for example: SCN^- and HSCN.

(2) Fast net translocation of *acid* as compared with that of the corresponding *anion*, e.g., SO_4^{2-} and H_2SO_4; HPO_4^{2-} or $H_2PO_4^-$ and H_3PO_4.

(3) Very slow translocation of both acid and anionic forms, e.g., $Fe(CN)_6^{3-}$ and $H_3Fe(CN)_6$; $Fe(CN)_6^{4-}$ and $H_4Fe(CN)_6$.

(4) Very fast translocation of both acid and anionic species. Mitchell and Moyle state that this class has not yet been experimentally identified but "is probably characteristic of the classical-proton conducting uncoupling agents."

E. Cation and Anion Transport in SMP

This section deals with the ion translocation properties of SMP prepared by sonic disruption of beef heart mitochondria (Löw and Vallin,

TABLE V

K^+ TRANSPORT IN MITOCHONDRIA

Type	Inducer	Characteristic	Reference
(1) Spontaneous	—	Slow uptake of K^+ from the medium; mitochondria can double their original K^+ content in about 30 minutes	Gamble (1957, 1963); Share (1958); Ulbricht (1960); Rottenberg and Solomon (1965)
(2) Spontaneous stimulated	Temperature (38°), sucrose–EDTA; EDTA; Zn^{2+} and SH-group reagents; parathyroid hormone (PTH)	Association with H^+ countermovement or coupled anion movement (except PTH); accumulation against chemical gradient that exhibits dependence on redox energy and/or phosphate bond energy; rate = 2–6 ng K^+ ions/minute/mg protein	Christie *et al.* (1965); Judah *et al.* (1965); Gamble (1957, 1962); Rottenberg and Solomon (1965); Azzone and Azzi (1966); Brierley *et al.* (1966, 1968); Rasmussen *et al.* (1964); Rasmussen and Ogata (1966)
	Acid pH	Induced efflux of endogenous mitochondrial K^+; absent at neutral pH	Carafoli and Rossi (1967)
(3) Valinomycin induced	Valinomycin	Uptake of K^+; ejection of H^+; uptake of anions; stimulation of respiration that continues even after K^+ uptake has reached completion; pyridine nucleotide oxidation; mitochondrial swelling; accumulation against a chemical gradient which exhibits dependence on redox energy and/or phosphate bond energy; release down electrical and chemical gradients (redox coupled or phosphate bond coupled) stoichiometry: K^+:$\sim$ = 7 (ATP), K^+:$\sim$ = 4 (glutamate + malate + acetate), K^+:$\sim$ = 1.67 (glutamate +	Moore and Pressman (1964); Harris *et al.* (1966a); Pressman (1965); Cockrell *et al.* (1966, 1967); Chappell and Crofts (1966); Glynn (1967); Cockrell (1968); Rossi and Azzone (1970); Lynn and Brown (1967); Mitchell and Moyle (1969a)

		malate + Cl^-, K^+:$\sim$ = 4 (ATP) (with EGTA and mersalyl), K^+:$\sim$ = 2 (ATP) (with EDTA)	
	Valinomycin	Enthalpy of K^+ accumulation ΔH = -0.6 ± 0.75 kcal/mole K^+; rate = $\sim$300–1000 ng K^+ ions/minutes/mg	Poe (1968)
	Gramicidin	The main difference with respect to valinomycin is the lack of cationic discrimination and significant induction of H^+ permeability (at neutral pH) exhibited by gramicidin	Chappell and Crofts (1965, 1966); Chappell and Haarhoff (1967); Pressman (1965); Harris and Pressman (1967); Henderson *et al.* (1969); Harris *et al.* (1967a); Harris (1968); Skulachev *et al.* (1969); Mueller and Rudin (1967)
	N-actins	The main difference is the profile of cationic selectivity (Na^+, K^+, Rb^+, Cs^+, but not Li^+ or NH_4^+)	Graven *et al.* (1966b,c, 1967); Mueller and Rudin (1967); Henderson *et al.* (1969)
	Monazomycin	Li^+, Na^+, K^+ (unknown structure) induces voltage-dependent permeability changes in bilayer lipid membranes	Lardy *et al.* (1967); Estrada-O *et al.* (1967); Mueller and Rudin (1969); Pressman (1968, 1969)
(4) Nigericin-induced	Nigericin, dianemycin, monensins	Efflux of K^+ from mitochondria down the chemical gradient; differs from that induced by valinomycin in absence of redox coupling and/or phosphate bond energy coupling; no report of uptake against a chemical gradient; associated with a stoichiometric H^+ countermovement (K^+/H^+ exchange $\cong$ 1) stimulation of ATPase activity; inhibition of mitochondrial uptake and oxidation of glutamate, malate,	Lardy *et al.* (1967); Estrada-O *et al.* (1967); Graven *et al.* (1966a); Pressman *et al.* (1967); Palmieri and Quagliariello (1969); Henderson *et al.* (1969)

(*Continued*)

TABLE V (*Continued*)

Type	Inducer	Characteristic	Reference
		citrate, isocitrate, α-ketoglutarate, but not β-hydroxybutyrate or isocitrate in the presence of malate; inhibits P_i accumulation and ATP/$^{32}P_i$ exchange	
(5) Uncoupler induced	FCCP, DNP	Transient permeability change; reversible nature (?) apparent inability of H^+ to act as counterions; occurs only if phosphate (or valinomycin) is present in the medium	Caswell and Pressman (1968); Caswell (1968, 1969); Palmieri and Quagliarrello (1969); Judah *et al.* (1965); Kimmich and Rasmussen (1967); Harris *et al.* (1967b)
	Ca^{2+}	Analogous to uncoupler	Caswell (1969)
	Histones	Analogous to uncoupler	Johnson *et al.* (1967)
(6) K^+/Ca^{2+}	Ca^{2+} and valinomycin	A gradient of K^+ in a sucrose medium can be used to support Ca^{2+} uptake in rotenone + valinomycin-treated mitochondria; rate = 240 ng Ca^{2+} ions/minute/mg; insensitive to antimycin A, cyanide, and oligomycin; dependent on the concentration of Ca^{2+}, valinomycin, and K^+; competitive inhibition with La^{3+} ($K_i \cong 50$ nM), Mg^{2+} ($K_i \cong 4$ mM), H^+ (K_i = 0.3 μM), and DNP (K_i = 3 μM)	Azzone and Azzi (1966); C. Rossi *et al.* (1967a, b); Ogata and Rasmussen (1966); Scarpa and Azzone (1970)

TABLE VI

SURVEY OF MITOCHONDRIAL PERMEANT METABOLITES[a]

Metabolites	Permeant	Inhibitors	Carrier	Remarks	Occurrence in Mitochondria
Oxidative phosphorylation	ADP	Atractyloside	+	Exchange	Probably all mitochondria
	ATP	Bongkrekic acid	+	Exchange	
	P_i	SH reagent	+	Exchange	
Substrates	Pyruvate	—	—	Indirect transport by carnitine shuttle	—
	Fatty acids	—	—		—
Ketone bodies	Hydroxybutyrate	—	—		
	Acetoacetate	—	—		
Intermediates					
Dicarboxylates	Malate	Butylmalonate	+	Exchange with P_i	Absent in blowfly flight muscle mitochondria
	Succinate	Butylmalonate	+	Exchange with P_i	
	Ketoglutarate	Butylmalonate	+	Exchange with dicarboxylates	
Tricarboxylates	Citrate	—	+	Exchange with dicarboxylates	Absent in blowfly flight muscle
	Isocitrate	—	+		
Amino acids	Aspartate	Avenoceolide	+	Activator: glutamate	Probably present in all mitochondria
	Glutamate	Avenoceolide	+		

[a] Taken from Klingenberg, 1970.

1963; Lee and Ernster, 1967). As mentioned above, the membrane sidedness in this preparation is opposite that of intact mitochondria (cf. Malviya *et al.*, 1968). The reader is referred to the early work of Gamble (1957) on the energy-dependent retention of K^+ by SMP prepared by digitonin treatment, and to the work of Vasington (1963) on Ca^{2+} uptake. This preparation exhibits the same membrane sidedness as intact mitochondria (see Malviya *et al.*, 1968; Greville, *et al.*, 1965; Mitchell, 1966a; see also preceding discussion on the topography of cytochrome c) although a report has appeared that under defined conditions sonic as well as detergent treatments produce SMP with the same membrane sidedness (Loyter *et al.*, 1969; Christiansen *et al.*, 1969b); see discussion on Ca^{2+} transport in SMP).

The finding that the combination of the ionophores nigericin, and valinomycin in the presence of K^+ uncoupled SMP, whereas neither antibotic alone markedly affected several energy-linked functions performed by this preparation (Montal *et al.*, 1969a; Cockrell and Racker, 1969; Smith and Beyer, 1967), stimulated a systematic investigation of the ion-translocating properties of SMP.

1. K^+ Translocation

a. Spontaneous K^+ Translocation. Activation of electron transfer reactions by substrate oxidation in SMP results in the uptake of small amounts of K^+ from the medium (up to 30 ng K^+ ions per milligram of protein; Montal *et al.*, 1970b; Cockrell and Racker, 1969, and personal communication). This K^+ uptake is dependent on the presence of an energy transfer inhibitor (oligomycin, Montal *et al.*, 1970b; or DCCD and rutamycin, Cockrell and Racker, 1969). Lee and Ernster (1966, 1968) showed that oligomycin-induced inhibition of respiration is associated with a stimulation of oxidative phosphorylation and the energy-linked transhydrogenase reaction, as well as reversal of electron transfer (Lee *et al.*, 1969). It thus functions as a coupling factor. It has been found (Montal *et al.*, 1970b) that oligomycin can induce (trigger) the ion uptake process, provided the preparation has been supplemented with a respiratory substrate. The concentration of oligomycin required to induce half-maximal ion translocation is similar to that required to stimulate oxidative phosphorylation, its reversal, and ATP $-$ $^{32}P_i$ exchange in SMP (Lee and Ernster, 1968). These observations suggest that these various phenomena are closely related and add further support to the view that ion transport in SMP is an energy-linked function.

Cockrell and Racker (1969) reported that K^+ translocation was greatly enhanced by the presence of a permeant anion, NO_3^- being the most

active in this respect. The spontaneous uptake of K^+ by SMP can be stimulated about 3-fold in the presence of NO_3^- (Cockrell and Racker, 1969; Montal *et al.*, 1970b). Previous failures to accomplish this were attributable to the fact that NO_3^- does not permeate mitochondria but does permeate SMP.

b. Nigericin-Stimulated K^+ Translocation in SMP. Preincubation of SMP in the presence of nigericin resulted in a 3-fold enhancement of the respiration-dependent uptake of K^+ in the absence of permeant anion, and 6-fold enhancement in the presence of NO_3^- (Cockrell and Racker, 1969; Montal *et al.*, 1970b).

Addition of nigericin after the K^+ uptake had reached completion resulted in K^+ efflux when no potassium chloride was added to the reaction mixture, no effect when the concentration was slightly increased, and a K^+ uptake as the potassium chloride concentration was further increased. This is attributable to nigericin catalysis of K^+/H^+ exchange (see Section II, A) until a new equilibrium state is reached (Montal *et al.*, 1970b).

The spontaneous, nigericin-stimulated, oligomycin-dependent uptake of K^+ by SMP is an energy-linked function since respiratory inhibitors (rotenone, Cockrell and Racker, 1969, antimycin, Montal *et al.*, 1970b), and uncouplers (Cockrell and Racker, 1969; Montal *et al.*, 1970b) can reverse or prevent K^+ uptake (Chance, 1970).

c. Valinomycin-induced K^+ Translocation in SMP. It was shown (Cockrell and Racker, 1969; Montal *et al.*, 1970b) that valinomycin can reverse *both* spontaneous and nigericin-stimulated, oligomycin-dependent uptake of K^+ by SMP. When SMP are preincubated with both valinomycin and nigericin in the presence of K^+, the respiration-driven K^+ uptake is abolished.

Just as the direction of proton translocation in intact mitochondria is opposite that in SMP (Table IV), so antibiotic-induced or -modified K^+ translocation in SMP takes place in a direction opposite that in mitochondria. Thus valinomycin induces K^+ uptake in mitochondria (Cockrell *et al.*, 1966; Harris *et al.*, 1966a; Pressman, 1969), whereas K^+ efflux occurs when valinomycin is added to SMP. Nigericin induces K^+ efflux in mitochondria (Graven *et al.*, 1966a; Pressman *et al.*, 1967), whereas it stimulates spontaneous K^+ uptake in SMP (Cockrell and Racker, 1969, and personal communication; Montal *et al.*, 1970b).

d. Uncoupling Mechanisms in the Presence of Nigericin and Valinomycin Plus K^+. Measurements of K^+ transport in SMP revealed that nigericin stimulated a respiration-dependent and energy-linked uptake of K^+ in SMP, whereas valinomycin induced the efflux of the cation taken up, thus leading to a cyclic movement of K^+ across the membrane, as depicted in the upper-right-hand diagram of Fig. 3. Interpretation of the results sum-

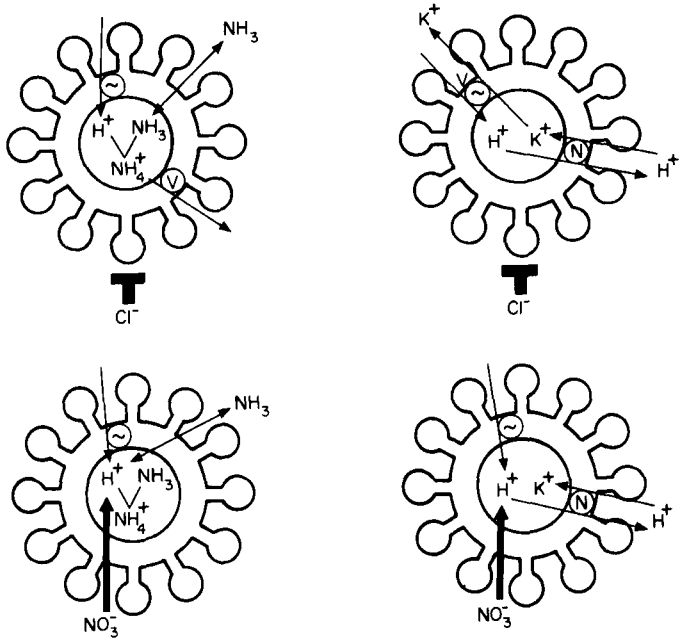

FIG. 3. Mechanisms of ion fluxes and uncoupling in SMP, chloroplasts, and chromatophores. V, Valinomycin; N, nigericin; ~, H^+-translocating respiratory chain. (Taken from Montal *et al.*, 1969b.)

marized in this section is not unique and both chemical and chemiosmotic mechanisms can account for the observed phenomena.

According to the chemiosmotic coupling proposal, protons move into SMP upon initiation of electron transfer reactions. This charge migration builds up an electrochemical activity gradient of hydrogen ions so that the transmembrane potential is attributable to two factors, a pH gradient and a membrane potential. Nigericin is thought to catalyze an electrically neutral exchange of protons accumulated in SMP for external K^+; this would lead to a decrease in the pH gradient without affecting the electrical gradient, and therefore uncoupling does not occur. When valinomycin is introduced into the system, the K^+ accumulated in the particle is allowed to diffuse out of SMP, down the electrical gradient, and up the concentration gradient. These conditions provide a situation in which the membrane is "short-circuited," H^+ and K^+ being continuously moved in and out, with the consequent dissipation of the electrochemical gradient generated during respiration. When nigericin and valinomycin are added separately, they act by displacing the main component of the electro-

chemical gradient to the membrane potential or the pH gradient, respectively. The overall effect is net H^+ conduction. In this respect the effect is analogous to that induced by conventional uncouplers (see Section II, A).

According to the chemical hypothesis, two situations are involved:

(1) A secondary proton pump is driven by the high-energy intermediate (Chappell and Crofts, 1966; Jagendorf and Uribe, 1966b). As a result, an electrochemical H^+ gradient would also be generated in the energized state, with the results interpreted as before. It must be remembered that in the secondary proton pump scheme the primary event is X~I formation, with X~I driving ATP synthesis or H^+ translocation, which are considered to be in parallel. The X~I is not in parallel but in series with H^+ translocation, however, which therefore becomes secondary to X~I formation. In the chemiosmotic formulation, however, H^+ translocation is the primary event.

(2) A secondary alkali-ion pump exists. Here SMP (or mitochondria) would transport ions by utilizing the pool of the nonphosphorylated high-energy chemical intermediate (X~I) generated during respiration. Valinomycin induces an energy-dissipating efflux of K^+ (Pressman *et al.*, 1967; Pressman, 1968, 1969), which in the absence of nigericin is limited by the low K^+ content of SMP. This would explain the partial inhibitory effect of valinomycin on energy-linked functions and oxidative phosphorylation (see also Papa *et al.*, 1969). Nigericin-induced K^+/H^+ exchange is not by itself an uncoupling process (Graven *et al.*, 1966a; Pressman *et al.*, 1967), but its loading of SMP with external K^+ provides a continuous supply of internal K^+ that is ejected by valinomycin; this in turn creates a cyclic energy-dissipating movement of K^+ across the membrane.

The fact that valinomycin increases the K^+ conductance of several natural and artificial membrane systems renders the possibility of its interaction with specific cation pump improbable. This is more evident in the discussion of the effect of anions.

2. NH_4^+ Translocation

Papa (1969), Papa *et al.* (1969), and Cockrell and Racker (1969) observed that ammonium chloride combined with valinomycin uncoupled SMP, while neither ammonium chloride nor valinomycin alone did. This finding stimulated an investigation of the capacity of SMP to translocate NH_4^+. Montal *et al.* (1970b) showed that oligomycin initiated NH_4^+ uptake by succinate-supplemented SMP, accompanied by parallel inhibition of the respiration-driven and oligomycin-dependent H^+ uptake. NH_4^+ translocation

in SMP was prevented and abolished by antimycin A or uncouplers, or by valinomycin, but not by nigericin. The results were interpreted (see also Cockrell and Racker, 1969; Crofts, 1967; McCarty, 1969) as follows: Activation of the electron transfer reaction induces an internal acidfication of SMP; this in turn leads to a displacement of the equilibrium distribution of ammonia across the membrane. The more ammonia is protonated to NH_4^+ inside SMP, the more ammonia enters to replace it; this situation leads to the dissipation of the pH gradient established during respiration. The charge-transferring antibiotic valinomycin provides a pathway for the migration of NH_4^+ down the electric gradient toward the exterior of SMP, thus collapsing the membrane potential (Mitchell, 1966a,b, 1968). (See upper-left-hand diagram of Fig. 3.)

3. Anion Translocation

The valinomycin-dependent uncoupling of SMP in the presence of either potassium chloride and nigericin or ammonium chloride suggested that valinomycin caused the discharge of the electrical gradient created by the accumulation of the charged species (K^+ or NH_4^+) in SMP (an electrophoretic cation efflux). Substitution of valinomycin by a permeant anion (an electrophotetic anion influx) led to the same uncoupling effect (Montal *et al.*, 1969b; Cockrell and Racker, personal communication). Thus it was found that NO_3^- (Cockrell and Racker, 1969), TPB^-, and picrate anions [which have been reported to induce voltage-dependent permeability changes in bilayer lipid membranes (see Mueller and Rudin, 1967; Skulachev *et al.*, 1969; LeBlanc, 1969)] replaced valinomycin, whereas Cl^-, Ac^-, P_i^-, SCN^-, I^-, did not. This series agrees well with that reported by Christiansen *et al.* (1969a), who noted that NO_3^- and SO_4^{2-} are better uncouplers of oxidative phosphorylation in SMP than Cl^- or Ac^-. The last-mentioned acid (see Section II,D) is permeant in intact mitochondria. Perhaps the inversion of membrane sidedness is also reflected in this opposite anion permeability. (See lower right- and left-hand diagrams of Fig. 3.)

4. Ca^{2+} Transport in SMP

Loyter *et al.* (1969) and Christiansen *et al.* (1969b) have reported energy-linked Ca^{2+} uptake in SMP.

Sonic disruption of beef heart mitochondria followed by either osmotic shock or digitonin treatment yielded SMP preparations with the same membrane sidedness, that is, the side on which F_1 was located was in direct contact with the suspending medium. Consequently, they were inside-out with respect to intact mitochondria (Lee and Enster, 1966).

The following observations apply:

(1) Ca^{2+} uptake requires succinate as well as ATP (or ADP).
(2) Ca^{2+} uptake is prevented or abolished by FCCP.
(3) Ca^{2+} uptake is rutamycin-insensitive.
(4) Ca^{2+} uptake in the presence of NADH and ATP is insignificant.
(5) An NADH-regenerating system can lead to a small but significant Ca^{2+} accumulation.
(6) Ascorbate–PMS oxidation supported Ca^{2+} uptake although less effectively than succinate.
(7) Ca^{2+}/OH^- ratio = 1 (obtained by Lubrol treatment of Ca^{2+} loaded SMP).
(8) Ca^{2+}/ATP ratio = 1.7.
(9) A type-specific antibody against F_1 considerably inhibited the ion uptake process in SMP (whereas this antibody is ineffective in mitochondria).
(10) Electron micrographs of thin sections of Ca^{2+}-loaded SMP in the presence of succinate and ATP revealed the existence of electron-dense deposits inside the particles. Control experiments, in the absence of energy source, did not show these deposits.

These experiments show that SMP are indeed capable of energy-linked Ca^{2+} uptake. The authors conclude that these observations are difficult to explain in terms of both chemical and chemiosmotic hypotheses of energy-coupling. In particular, according to the chemiosmotic formulation, energization in SMP (either from substrate oxidation or ATP hydrolysis) results in uptake of H^+ by SMP. This charge migration would build up a positive electrical gradient inside the particle that would prevent the further uptake of cations and moreover would allow internal cations to diffuse out of the particle down the electrical gradient. This is the reason why Loyter *et al.* (1969) and Christiansen *et al.* (1969b) regard their evidence as incompatible with the present formulation of the chemiosmotic hypothesis. One can reinterpret these observations however, on the basis of the K^+-translocation experiments on SMP summarized before. It was observed that SMP possess a spontaneous capacity to translocate K^+ toward the *inside* of the particles (Cockrell and Racker, personal communication), a capacity that is stimulated by nigericin. Since nigericin catalyzes an electrical neutral cation/H^+ exchange, it may be that both K^+ and Ca^{2+} translocation in SMP occur by exchange diffusion processes. In that case the experiments actually support the concept of exchange diffusion. In this regard it is worth remembering that anions seem to be electrophoretically transported *into* SMP (Liberman *et al.*, 1969; Montal *et al.*, 1969b).

The situation seems to be the opposite in intact mitochondria. In this case energization results in H^+ efflux and an inside negative membrane

potential. This force could drive the *electrophoretic influx* of Ca^{2+}, K^+ (plus valinomycin) or of lipid-*soluble* cations DDA^+ and $TPMP^+$ (Skulachev *et al.*, 1969), and $TPAs^+$ (Montal *et al.*, 1970b). Anions would be transported by exchange diffusion with OH^- or other anions (cf. Chappell, 1968; Chappell *et al.*, 1968).

The foregoing discussion is summarized in the accompanying table.

Process	Mechanisms	
	Mitochondria	SMP
Direction of H^+ translocation	Efflux (inside negative)	Influx (inside positive)
Ca^{2+} uptake	Electrophoretic	Exchange diffusion
K^+ uptake	Electrophoretic	Exchange diffusion
Anions	Exchange diffusion	Electrophoretic

It can be seen that mitochondria and SMP again function as opposites, whereas cations are taken up electrophoretically in mitochondria, they are accumulated by exchange diffusion in SMP. While an exchange diffusion system for accumulation of anions seems to operate in mitochondria, an electrophoretic anion influx appears to be operative in SMP.

This opposite functional polarity has the structural basis already discussed in Section II,B and provides another example of the alteration of membrane permeability induced by sonication.

F. Adenine Nucleotide Translocation in SMP

Löw (1963) reported that the atractyloside-sensitive reaction was absent in sonicated SMP, whereas it is partially conserved in digitonin particles (Vignais *et al.*, 1962; Bruni, 1966).

Klingenberg (1969) concluded that although the adenine nucleotide content of SMP was significant it was considerably lower than that of intact mitochondria and that these nucleotides did not undergo phosphorylation and exchange reactions. The endogenous nucleotides can be released from SMP by prolonged sonication, and reversible adsorption of exogenous nucleotides with apparent incorporation of ^{32}P has also been reported.

It is evident that more experimentation is needed in this area since very little is known about the existence, function, or importance of this translocator in SMP.

III. CONCLUSIONS

A. Analogies between Mammalian and Photosynthetic Membrane Systems

It seems to be generally accepted that the mechanisms of energy coupling, uncoupling, and ion transport are intimately related, and that analogous mechanisms may exist in mitochondrial as well as in chloroplast and bacterial membrane systems (cf. Mitchell, 1966a,b, 1968, 1969a,b; Chance *et al.*, 1970a; Packer and Crofts, 1967; Greville, 1969; Scholes *et al.*, 1969). Accordingly, a brief discussion of selected experiments in the field of photophosphorylation is attempted in order to provide a generalized scheme of the relationship between charge transfer and energy coupling in the mammalian, green plant, and bacterial energy-conserving membrane systems.

1. Green Plants

a. Postillumination ATP Synthesis (Two-Stage Phosphorylation). Hind and Jagendorf (1963; cf. Jagendorf and Uribe, 1966b), using broken spinach chloroplasts, observed that after an illumination period in the absence of P_i they could induce synthesis of ATP from ADP and P_i in the dark during the postillumination period. The yield of ATP was larger at pH 6.0 and in the presence of redox cofactor. The reaction was sensitive to several uncouplers of photophosphorylation (Hind and Jagendorf, 1965b); removal of the chloroplast coupling factor prevented this dark ATP synthesis (Vambutas and Racker, 1965; McCarty and Racker, 1966). The yield of ATP (100 μmoles ATP per gram of chlorophyll) ruled out the high-energy state or condition (X_E) as being a stoichiometric chemical entity involving any usual electron transfer component.

b. The Light-Induced pH Rise. A detailed study of the light-induced proton uptake in isolated chloroplasts was presented by Neuman and Jagendorf (1964). They observed that as much as 0.6 μeq H^+ ions per milligram of chlorophyll were taken up by chloroplast and released in the dark or after treatment with the detergent Triton X-100; uncouplers such as FCCP or ammonium chloride prevented the light-induced pH rise or reversed the H^+ uptake when added during the steady-state light. Energy transfer inhibitors such as Dio-9 (McCarty *et al.*, 1965), as well as the antibody against the coupling factor (McCarty and Racker, 1966), had no effect on proton translocation at concentrations known to inhibit photophosphorylation and coupled electron flow. This strongly suggested that the coupling factor is not involved in the light-induced proton uptake.

Karlish and Avron (1968b) observed a stimulation of the light-induced pH rise in Dio-9-treated chloroplasts (see Section III,B).

Light-induced efflux of K^+ and Mg^{2+} (Dilley and Vernon, 1965; Crofts *et al.*, 1967) and influx of Cl^- (Deamer and Packer, 1969) were shown to be associated with H^+ uptake. Uncouplers of photophosphorylation significantly accelerated the rate of dark decay of the pH shift (Jagendorf and Neuman, 1965; Rumberg and Siggel, 1968; Rumberg *et al.*, 1968).

Crofts (1967) reported the light-induced uptake of NH_4^+ by isolated chloroplasts, and a mechanism of uncoupling by amines was proposed in which, by analogy with erythrocytes and mitochondria (cf. Chappell and Crofts, 1966), ammonia freely diffuses across the thylakoid membrane and is protonated to NH_4^+ on the inside of the particle by association with the H^+ generated by the illumination process. This leads to a collapse of the pH gradient established by light. Cl^- would be electrophoretically taken up, thus collapsing the electrical gradient created by accumulation of the charged NH_4^+ species. Sonic treatment of chloroplasts results in smaller membrane fragments, the "subchloroplast particles," still capable of photophosphorylation, coupled electron flow, and light-induced H^+ uptake. Ammonium chloride alone does not inhibit photophosphorylation, however, even though it is taken up during illumination as in intact chloroplasts (McCarty, 1969). Addition of valinomycin in the light induces efflux of the NH_4^+ taken up and this is accompanied by virtually complete inhibition of photophosphorylation. The experiments suggest that sonic treatment of chloroplasts alters the permeability of the membrane toward anions. Cl^- therefore cannot migrate electrophoretically into the particle and cause the potential to collapse. Moreover, valinomycin is required to allow the accumulated NH_4^+ to exit by electrophoretic efflux with the consequent dissipation of the electrical gradient.

Shavit and San Pietro (1967), Packer (1967), and Shavit *et al.* (1968a) have reported uncoupling of photophosphorylation in chloroplasts by nigericin in the presence of K^+; this uncoupling effect is associated with the following alterations of the light-induced pH rise: Prior addition of the ionophore in the presence of K^+ results in corresponding inhibition of the light-induced H^+-uptake, whereas addition of nigericin in a K^+ medium, once steady-state proton uptake has been reached, results in a fast collapse of the photoinduced pH gradient. The experiments suggest that nigericin uncouples chloroplast photophosphorylation by equilibrating K^+ and H^+ activities across the thylakoid membrane (cf. Pressman *et al.*, 1967); Cl^- would migrate electrophoretically into the particle and collapse the electric gradient generated by the primary photoevent.

As previously mentioned, subchloroplast particles appear to be impermeant to Cl^- and other anions. It would therefore be expected that

nigericin would not fully uncouple photophosphorylation in this preparation. It has been reported (McCarty, 1969; Hauska *et al.*, 1970) that complete inhibition of photophosphorylation in subchloroplast particles occurred only with a combination of nigericin plus valinomycin in a K^+ medium, whereas neither alone significantly altered ATP synthesis.

Thus it seems that the energized state of chloroplasts and their fragments is associated with both a pH gradient and a membrane potential (cf. Mitchell, 1968, 1969a,b) and that uncoupling occurs only when these two constituents of the electrochemical proton gradient are eliminated.

c. Acid-Base-Induced Phosphorylation. Jagendorf and Uribe (1966, a,b; Jagendorf, 1967) observed synthesis of ATP driven by a pH gradient in chloroplasts. Incubation of chloroplasts in the dark, in the presence of an electron transfer inhibitor and a weak acid (optimum results were obtained with succinic acid at pH 4.0), and rapidly transferring the preparation to a medium containing ADP, P_i, and Mg^{2+} at pH 8.5, resulted in the synthesis of ATP (100 μmoles per mole of cytochrome f) which was sensitive to uncouplers (ammonium chloride, FCCP), detergents (Triton X-100), EDTA [which releases the coupling factor (see Avron, 1963)], the antibody against coupling factor (McCarty and Racker, 1966), and the energy transfer inhibitor Dio-9 (McCarty and Racker, 1966). Jagendorf and co-workers (cf. Jagendorf, 1967) have associated their high-energy condition (X_E) with a pH gradient and considered these experiments strong evidence in favor of the chemiosmotic hypothesis.

The terminal enzyme of photophosphorylation in chloroplasts is not ordinarily active as an ATPase (hydrolase) but a variety of procedures [trypsin, sulfhydryl reagents, and so on (see reviews of Good *et al.*, 1966; Vernon and Avron, 1965; Avron and Neuman, 1968)] can activate the enzyme to catalyze a light-triggered, Mg^{2+}-stimulated and a light-dependent, Ca^{2+}-stimulated ATPase (Vambutas and Racker, 1965).

It has been shown that in the presence of a sulfhydryl reagent (Kaplan, and Jagendorf, 1968; Kaplan *et al.*, 1965) light can be replaced by an acid–base transition in the dark [analogous to that used to obtain phosphorylation (cf. Jagendorf, 1967)]. Thus activation of the ATPase results from the installation of a pH gradient naturally (by light) or artificially (by acid–base transition). Inhibition of this enzymatic activity by the energy transfer inhibitor phlorizin (Izawa *et al.*, 1966) and by the anti-CF_1 antibody (McCarty and Racker, 1966) suggests that the system is the reverse of the ordinary photophosphorylation process.

Recently, Nishizaki and Jagendorf (1969) reported absence of cationic specificity to elicit the acid–base-triggered ATPase activity. This may mean that the primary event in photophosphorylation does not involve a specific cation pump, whereas "it is likely that a proton gradient is the

energetic basis for enzyme activation in these experiments and by inference for the phosphorylation of ADP."

Carmeli (1970) observed inward translocation of protons in chloroplasts driven by light-triggered, Mg^{2+}-stimulated and dithiothreitol-activated ATPase. He reports H^+/P_i ratios of 2, partially inhibited by gramicidin, which are consistent with a chemiosmotic ATPase II mechanism (Mitchell, 1966a,b).

2. Photosynthetic Bacteria

Photophosphorylation by chromatophores is essentially similar to that catalyzed by chloroplasts (Frenkel, 1954, 1956; Baltscheffsky and Baltscheffsky, 1960).

a. The Light-Induced pH rise. Upon illumination of chromatophores in a medium of low buffering power, the pH rises, levels off, and decays in the dark. This effect can be monitored with a glass electrode (Von Stedingk and Baltscheffsky, 1966; Von Stedingk, 1967; Jackson *et al.*, 1968; Scholes *et al.*, 1969), or spectrophotometrically using the indicator bromthymol blue (Chance *et al.*, 1966b; Cost and Frenkel, 1967; Nishimura *et al.*, 1968; Nishimura and Pressman, 1969). This light-induced proton translocation is sensitive to uncouplers and inhibitors of electron transfer reactions in very much the same way as the H^+-translocation process in chloroplasts (Von Stedingk and Baltscheffsky, 1966). Oligomycin, the energy transfer inhibitor, stimulates severalfold both rate and extent of photoinduced intravesicular acidification (Von Stedingk and Baltscheffsky, 1966; Nishimura *et al.*, 1968; Scholes *et al.*, 1969), notwithstanding its strong inhibition of photophosphorylation (Baltscheffsky and Baltscheffsky, 1960) and ATPase activity (Bose and Gest, 1965). This suggests that the coupling factor is not involved in the light-induced pH rise, as is also true in chloroplasts.

Jackson *et al.* (1968), Thore *et al.* (1968), and Nishimura and Pressman (1969) reported that valinomycin in the presence of K^+ stimulated both rate and extent of the light-induced pH rise, whereas nigericin inhibited the proton translocation phenomenon in a K^+-dependent manner. Neither antibiotic alone (plus K^+) significantly altered the synthesis of ATP. The combination of valinomycin and nigericin, however, completely inhibited photophosporylation in the presence of K^+ (Jackson *et al.*, 1968; Thore *et al.*, 1968). Jackson *et al.* (1968) interpreted their results in terms of the chemiosmotic hypothesis, stating that nigericin collapsed the pH gradient by mediating an electrically neutral K^+/H^+ exchange across the membrane and that the membrane potential was abolished by equilibration of K^+ activity across the valinomycin-treated membrane.

Von Stedingk and Baltscheffsky (1966) and Jackson *et al.* (1968) reported that gramicidin inhibits the light-induced pH rise and uncouples photophosphorylation (Baltscheffsky and Baltscheffsky, 1960). They interpreted their results in terms of the proton conductor properties of gramicidin, the effect of the ionophore being to mediate H^+ equilibration across the membrane. This prevents conservation of the energy of the photoinduced proton gradient.

Horio and Yamashita (1964) reported that ammonium chloride alone did not uncouple photophosphorylation in chromatophores, in contrast with chloroplasts, in which ammonium and the amines are the classic uncouplers (Krogman *et al.*, 1959; Good, 1960; Hind and Whittingham, 1963). The fact that in the presence of small amounts of valinomycin (which by itself does not significantly inhibit photophosphorylation) ammonium chloride completely abolished the phosphorylation reaction strongly suggests that one of the differences between chloroplast and chromatophore photophosphorylation is in the membrane selectivity for anions. This is further supported by the fact that TPB^-, which proved to be the most permeant anion in SMP, replaces valinomycin in the NH_4^+-dependent uncoupling effect, whereas TPB^- by itself does not dramatically alter the esterification reaction (Montal *et al.*, 1970c).

Uncoupling of photophosphorylation in chromatophores is not obtained in the presence of nigericin and K^+, whereas in chloroplasts nigericin is one of the most powerful uncouplers discovered so far (Shavit and San Pietro, 1967). The presence of valinomycin or TPB^- results in total inhibition of photophosphorylation of a nigericin-treated chromatophore, in complete agreement with the results obtained in SMP (Section, II,E). Picrate and sulfate anions were also active in the substitution of valinomycin for NH_4^+-or nigericin + K^+-dependent inhibition of photophosphorylation, although not to the same extent as TPB^- (Montal *et al.*, 1970c).

Moreover, both valinomycin (Jackson *et al.*, 1968; Thore *et al.*, 1968; Nishimura and Pressman, 1969) and TPB^- (Montal *et al.*, 1970c) stimulated both rate and extent of the light-induced pH rise. This suggests that the rate-limiting step is the transfer of charge associated with H^+ translocation and that the latter is neutralized by electrophoretic ion migration.

Therefore, uncoupling in chromatophores is associated with the abolition of a pH gradient, either by the K^+/H^+ exchange catalyzed by nigericin or by the internal protonation of the freely diffusible ammonia. At the same time, the membrane potential is collapsed, either by the electrophoretic cation efflux mediated via valinomycin or by the electrophoretic anion influx observed with highly lipid-soluble anions.

B. Uncoupling and Charge Transfer in Bioenergy-Conserving Membrane Systems

The morphological similarities of SMP, chloroplast thylakoids, and bacterial chromatophores, and in particular the location of the coupling factor (ATPase) on the surface of these membrane systems and in direct contact with the environmental solution provide the structural basis on which membrane polarity is based (Fig. 2).

Activation of electron transfer reactions, either by substrate oxidation in SMP (Mitchell and Moyle, 1965; Chance and Mela, 1967) or by illumination of chromatophores (Von Stedingk and Baltscheffsky, 1966; Von Stedingk, 1967; Nishimura *et al.*, 1968; Scholes *et al.*, 1969) and chloroplasts (Neuman and Jagendorf, 1964; Jagendorf and Uribe, 1966b), results in H^+ uptake by the vesicular particles. Respiration or illumina- in *R. rubrum* cells and mitochondrial respiration (Mitchell, 1967; Scholes *et al.*, 1969; Edwards and Bovell, 1969, 1970) both lead to H^+ efflux. This provides further support for the view that SMP (Lee and Ernster, 1966) and chromatophores (Mitchell, 1967; Scholes *et al.*, 1969; Edwards and Bovell, 1969, 1970) are inside out with respect to intact mitochondria and *R. rubrum* cells, respectively.

The effect of oligomycin on H^+ movements in SMP is analogous to that reported by Von Stedingk and Baltscheffsky (1966), Nishimura *et al.* (1968), and Scholes *et al.* (1969) in *R. rubrum* chromatophores. It is also similar to the effects of Dio-9 and synthalin (also energy transfer inhibitors) on chloroplasts as reported by Dilley (1970) and Karlish and Avron (1968b). The latter investigators observed that the energy transfer inhibitor stimulated the extent of light-induced H^+ uptake. Oligomycin markedly stimulated respiration-driven H^+ translocation in SMP and initiated respiration-driven proton translocation (Montal *et al.*, 1970b). These results in SMP, chromatophores, and chloroplasts are consistent with the inhibitory effect of oligomycin (or synthalin or Dio-9) on energy transfer in oxidative phosphorylation (Lardy *et al.*, 1958) and photophosphorylation (Baltscheffsky, and Baltscheffsky, 1960; Dilley, 1970; Karlish and Avron, 1968b). They can also be interpreted as being attributable to a decrease in the electrolytic conductance of the membrane, however, thus allowing the maintenance of ionic gradients and their associated energy (Mitchell, 1966a,b, 1968).

The effect of ionophores on H^+ translocation in chloroplasts, chromatophores (Jackson *et al.*, 1968), and SMP are analogous (see also Cockrell and Racker, 1969). Preincubation of SMP or chromatophores with valinomycin and K^+ results in a 2- to 3-fold enhancement of the extent of respiration-(SMP) or light- (chromatophores) induced pH rise. Prior

addition of valinomycin to chloroplasts considerably enhances the rate of the light-induced pH rise, although the extent of the pH gradient is unaffected (Karlish *et al.*, 1969). As expected, a similar effect is obtained by preincubating SMP with NO_3^- and chromatophores with TPB (Montal *et al.*, 1970c). Preincubation of SMP (Montal, 1970), chromatophores (Jackson *et al.*, 1968), or chloroplasts (Shavit *et al.*, 1968a; Karlish *et al.*, 1969) with nigericin plus K^+ results in corresponding inhibition of the respiration- (SMP) or light- (chromatophores and chloroplasts) induced pH rise. Prior addition of both nigericin and valinomycin plus K^+ to SMP and chromatophores prevents respiration- or light-induced proton translocation, respectively (Montal *et al.*, 1970b; Jackson *et al.*, 1968). No further inhibition of the light-induced pH rise in nigericin-treated chloroplasts is obtained by the addition of valinomycin (Karlish *et al.*, 1969).

The four mechanisms of ion translocation-dependent uncoupling of SMP illustrated in Fig. 3 would be expected to occur in SMP, chromatophores, and chloroplasts. Indeed, Jackson *et al.* (1968) reported uncoupling of photophosphorylation in *R. rubrum* chromatophores by a combination of nigericin and valinomycin in the presence of K^+ (upper right diagram of Fig. 3). Consistent with the proposed mechanism are the inhibition of the light-induced pH gradient by nigericin and its enhancement by valinomycin. This enhancement, by allowing the electrophoretic efflux of K^+, prevents the buildup of an electric field generated by the transfer of charge associated with the proton translocation. Moreover, it has been observed that either ammonium chloride (Horio and Yamashita, 1964) or valinomycin (Von Stedingk and Baltscheffsky, 1966) alone partially inhibit (20–30%) phosphorylation in *R. rubrum* chromatophores. Fleishmann and Clayton (1968) reported that ammonium chloride plus valinomycin inhibited the light-induced change in the carotenoid absorption band of chromatophores [which in turn have been associated with the energized state of the system (see Jackson and Crofts, 1969)]. As shown by Montal *et al.* (1970c), total inhibition of photophosphorylalation is obtained with ammonium chloride and valinomycin (upper left diagram of Fig. 3). Furthermore, uncoupling of *R. rubrum* chromatophores is obtained with a combination of potassium chloride plus nigericin plus TPB^- or potassium sulfate (lower right diagram of Fig. 3), and by ammonium chloride plus TPB^- or ammonium sulfate (lower left diagram of Fig. 3), in complete analogy with the observations reported for SMP (Table VII, Section II,E).

It has been inferred that the chloroplast membrane is permeable to Cl^- (Crofts, 1967; Hind *et al.*, 1969); direct demonstration of this suggestion has been obtained by Deamer and Packer (1969), who showed that illumination of chloroplasts suspended in a solution of $Na^{36}Cl$ results in

TABLE VII

UNCOUPLING AND CHARGE TRANSFER IN BIOENERGY-CONSERVING MEMBRANE SYSTEMS[a]

Membrane system	Abolition of pH gradient	Abolition of membrane potential	Net effect	Reference[b]
SMP	$NH_4^+ \xrightarrow{NH_3} H^+$; $H^+ \rightarrow NH_4^+$	$NO_3^-(TPB^-) \rightarrow In$; $Val(NH_4^+) \leftarrow Out$	U U	(*1*) (*1–3*)
	$K^+ \xrightarrow{N} H^+$; $H^+ \rightarrow K^+$	$NO_3^-(TPB^-) \rightarrow In$; $Val(K^+) \leftarrow Out$	U U	(*1*) (*1, 2*)
	$H^+ \xrightarrow{G} H^+$	$H^+ \xrightarrow{G} H^+$	U	(*1*)
Chromatophores	$NH_4^+ \xrightarrow{NH_3} H^+$; $H^+ \rightarrow NH_4^+$	$SO_4^{2-}(TPB^-) \rightarrow In$; $Val(NH_4^+) \leftarrow Out$	U U	(*16*) (*16, 12*)
	$K^+ \xrightarrow{N} H^+$; $H^+ \rightarrow K^+$	$SO_4^{2-}(TPB^-) \rightarrow In$; $Val(K^+) \leftarrow Out$	U U	(*16*) (*13–15*)
	$H^+ \xrightarrow{G} H^+$	$H^+ \xrightarrow{G} H^+$	U	(*13*)
Chloroplasts	$NH_4^+ \xrightarrow{NH_3} H^+$; $H^+ \rightarrow NH_4^+$	$Cl^- \rightarrow In$	U	Cf. (*4*)
	$K^+ \xrightarrow{N} H^+$; $H^+ \rightarrow K^+$	$Cl^- \rightarrow In$	U	(*5–7*)
	$K^+ \xrightarrow{Val} K^+$; $H^+ \xrightarrow{F} H^+$	$Cl^- \rightarrow In$	U	(*8, 9*)
	$H^+ \xrightarrow{G} H^+$	$H^+ \xrightarrow{G} H^+$	U	(*7, 9–11*)

[a] Val, valinomycin; N, nigericin; G, gramicidin; U, uncoupling; F, FCCP.

[b] Key to references:

(*1*) Montal *et al.* (1970a,b). (*2*) Cockrell and Racker (1969). (*3*) Papa *et al.* (1969). (*4*) Crofts (1967). (*5*) Shavit and San Pietro (1967). (*6*) Packer (1967). (*7*) Shavit *et al.* (1968a). (*8*) Karlish and Avron (1968a). (*9*) Karlish *et al.* (1969). (*10*) Whatley *et al.* (1959). (*11*) Dilley and Shavit (1968). (*12*) Fleishmann and Clayton (1968). (*13*) Jackson *et al.* (1968). (*14*) Thore *et al.* (1968). (*15*) Nishimura and Pressman (1969). (*16*) Montal *et al.* (1970c).

the stoichiometric uptake of H^+ and Cl^-. Thus it would be expected that the dominant component of the electrochemical proton gradient would be the pH gradient. In this case valinomycin would not, and does not (Avron and Shavit, 1965; Karlish and Avron, 1968a; Karlish *et al.*, 1969), uncouple photophosphorylation in chloroplasts; however, it is worth noting that the rate of the light-induced pH rise is markedly enhanced

by prior addition of valinomycin to the incubation medium. This suggests that the rate-limiting step is not the translocation of H^+ itself but the associated charge transfer which is dissipated by allowing the electrophoretic efflux of K^+ by valinomycin (Karlish *et al.*, 1969). Uncoupling is obtained with potassium chloride and nigericin (Shavit and San Pietro, 1967; Shavit *et al.*, 1968a; Packer, 1967) in chloroplasts, with potassium nitrate (or potassium chloride plus TPB^-) and nigericin in SMP, and with potassium sulfate (or potassium chloride + TPB^-) and nigericin in chromatophores. Uncoupling is obtained with ammonium chloride in chloroplasts (Krogman *et al.*, 1959), with ammonium nitrate (or ammonium chloride plus TPB^-) in SMP, and with ammonium sulfate (or ammonium chloride plus TPB^-) in chromatophores. These results strongly suggest that the uncoupling mechanism is the same for SMP, chromatophores, and chloroplasts, but some differences are attributable to membrane anion selectivity, the permeant anion being NO_3^- (Cockrell and Racker, 1969), TPB^-, or picrate (Montal *et al.*, 1970b) in SMP; SO_4^{2-}, TPB^-, or picrate (Montal *et al.*, 1970c) in chromatophores; and Cl^- (Deamer and Packer, 1969) in chloroplasts. As mentioned above, subchloroplast particles obtained by sonic disruption of chloroplasts behave similarly to SMP and chromatophores. In other words, they are uncoupled by a combination of ammonium chloride and valinomycin, *or* potassium chloride plus nigericin and valinomycin, and it is not clear by what mechanism the selectivity of the membrane for anions can be altered by sonication. (McCarty, 1969; Hauska *et al.*, 1970).

It was mentioned in Section II,A that an effect equivalent to that induced by nigericin (K^+/H^+ exchange) can be obtained by a combination of valinomycin and uncoupler, although the mechanism of action is different in the two cases. Thus uncoupling of photophosphorylation in chloroplasts is obtained with nigericin and potassium chloride (Shavit and San Pietro, 1967), or with a combination of low concentrations of valinomycin (plus K^+) and uncoupler, whereas either alone partially inhibits phosphorylation (Karlish *et al.*, 1969; Karlish and Avron, 1968a).

Finally, the effect of gramicidin in SMP (Montal *et al.*, 1970a), chromatophores (Jackson *et al.*, 1968; Baltscheffsky and Baltscheffsky, 1960; Von Stedingk and Baltscheffsky, 1966), and chloroplasts (Whatley *et al.*, 1959; Shavit *et al.*, 1968a; Dilley and Shavit, 1968) is to uncouple oxidoreduction from phosphorylation. Gramicidin inhibits respiration-driven and oligomycin-dependent H^+ and K^+ translocation in SMP (Montal *et al.*, 1970a) and light-induced proton uptake in chromatophores (Von Stedingk and Baltscheffsky, 1966; Jackson *et al.*, 1968) and chloroplasts (Dilley and Shavit, 1968; Shavit *et al.*, 1968a; Karlish *et al.*, 1969). Gramicidin inhibits the energy-dependent bromthymol blue response in

SMP (Montal *et al.*, 1970a), light-induced absorption changes of the carotenoids in chromatophores (Fleishmann and Clayton, 1968), and light-induced absorption changes at 518 nm in chloroplasts (Witt, 1967; Witt *et al.*, 1968). Both signals have been associated with the membrane potential of these organelles (Witt *et al.*, 1968; Jackson and Crofts, 1969). The mechanism of uncoupling induced by gramicidin is most probably attributable to proton conduction (Mueller and Rudin, 1967; Henderson *et al.*, 1969; Skulachev *et al.*, 1969). By mediating H^+ equilibration across the coupling membrane and thus collapsing the electrical and chemical components of the proton gradient, gramicidin uncouples oxidoreduction from phosphorylation in SMP, chromatophores, chloroplasts, and mitochondria (Harris *et al.*, 1967a; Chappell and Crofts, 1966).

Table VII presents a summary of the conditions of uncoupling and charge transfer in SMP, chromatophores, and chloroplasts as described in this chapter. It specifically points out the mechanisms by which the components of the energized state are affected and the overall effects obtained.

It seems remarkable that energy-conserving membrane systems as distinct from those present in mammals, green plants, and bacteria possess a common device for energy transduction. Even if the suggestion of reversible transformation of electrical energy into chemical energy proves to be equivocal, it has already been extremely fruitful in drawing the attention of workers in the field to new and unexpected areas. If the membrane potential is not the direct link between oxidoreduction and hydrodehydration, it certainly is responsible for the direction and catalysis of one of the main functions of these membrane systems, that of ion transport. The importance of the membrane and the phenomena associated with it emerges clearly from the interconversion of electrochemical into phosphate bond or redox energy. This suggestion has led to a fruitful search for indicators of membrane function, for example, electric field or conformational changes. Witt (Witt *et al.*, 1968; Witt, 1967) interpreted findings on the light-induced absorption changes at 515 nm as compatible with an electric field across the thylakoid membrane of chloroplasts. This has resulted in the measurement of one of the fastest biological reactions as they report that the half-time of rise of the 515 nm pigment upon illumination with a giant laser is less than 2×10^{-8} seconds.

After the flash there occurred a biphasic relaxation in the dark, with a fast component ($T_{1/2} < 10^{-2}$ second) and a slow component ($T_{1/2} > 10^{-2}$ second). The rate of decay was greatly accelerated by gramicidin and uncouplers. Witt *et al.* also observed a linear relationship between phosphorylation activity and extent of the slow component of the absorption change at 515 nm. Another interesting finding is that when one photo-

system was eliminated the absorption change was halved (50%). Witt *et al.* conclude that the rise at the 515 nm band indicates the installation of an electric field across the membrane, which in the presence of permeability- modifying agents such as uncouplers or gramicidin would be dissipated. The slow phase is related to H^+ efflux. The fact that the change in H^+ efflux was linearly related to the phosphorylation activity of the preparation suggested that H^+ efflux is used to synthesize ATP, as suggested by Mitchell. Witt *et al.* estimate a value of 50–200 mV for the electrical potential. In separate experiments Rumberg and Siggel (1968) and Rumberg *et al.* (1968) estimated a pH difference across the thylakoid membrane at an external pH of 8, equal to 3 pH units in the absence of uncoupler and phosphorylation. The pH gradient was decreased to 2.6 units during phosphorylation, equivalent to about 160 mV. Added to the calculated electrical potential, this gives a total proton motive force of 210–360 mV, sufficient to drive ATP synthesis (Slater, 1967).

Jackson and Crofts (1969) generated diffusion potentials in the dark across the chromatophore membranes of *R. spheroides*. They generated K^+ gradients in the presence of valinomycin, and H^+ gradients in the presence of uncouplers, and observed a linear relationship with a shift in the spectrum of the carotenoids, entirely analogous to that obtained in the light. They concluded that the carotenoids are sensitive indicators of the membrane potential of chromatophores.

At present, two major alternatives can be envisaged as explanation for the primary event: (1) an anisotropic distribution of electron and H-atom carriers in the coupling membrane so as to provide a source of electrical potential to energy-conserving membrane systems (Mitchell, 1961, 1966a,b, 1968, 1969a,b) or (2) primary electron transfer-linked conformational changes that secondarily induce charge separation in or across the membrane or alter the proton-binding affinity of membrane groups (the "membrane Bohr effect," Wyman, 1948; Chance *et al.*, 1970b). It remains for future investigations to choose between these possibilities.

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ABBREVIATIONS USED IN THIS CHAPTER

ADP, Adenosine diphosphate; ANS, 8-anilino-1-naphthalene sulfonate; ATP, adenosine triphosphate; C-CCP, carbonyl cyanide, *m*-chlorophenyl-

hydrazone; DCCD, *N*-*N*′-dicyclohexyl-carbodiimide; DDA^+, *N*-*N*-dimethyl, *N*-*N*-dibenzylammonium; DNP, 2-4-dinitrophenol; EDTA, ethylenediaminetetracetate; EGTA, ethylenebis(oxyethylenenitrilo)tetracetate; FCCP, carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone; PCB^-, phenyldicarbaundecaboran; PCP, pentachlorophenol; P_i, inorganic phosphate; PMF($\Delta\bar{p}$), proton motive force; PMS, phenazine methosulfate; SMP, submitochondrial particles; TMPD, tetramethyl-*p*-phenylenediamine; $TPAs^+$, tetraphenylarsonium, chloride salt; TPB^-, tetraphenylboron, sodium salt; $TPMP^+$, tetraphenyl-methylphosphonium; TTFB, 4,5,6,7-tetrachloro-2-(trifluoromethyl)benzimidazole; ΔpH, pH difference; $\Delta\psi$, membrane potential.

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Structure and Biosynthesis of the Membrane Adenosine Triphosphatase of Mitochondria

ALEXANDER TZAGOLOFF

The Public Health Research Institute of the City of New York, Inc.
New York, New York

I. INTRODUCTION

It has been known for some time that the ATPase activity of mitochondria is markedly enhanced by reagents that cause uncoupling of oxidative phosphorylation or conditions that lead to a loss in the efficiency of phosphorylation. These observations were the earliest suggestions that part of the coupling system of mitochondria is an enzyme that functions as an ATPase when its normal association with the electron transfer chain is disturbed.

A crucial development in the study of mitochondrial ATPase and its relationship to oxidative phosphorylation was the isolation by Pullman *et al.* (1960) of a soluble ATPase (F_1) that increased the efficiency of phosphorylation in certain types of submitochondrial particles (Penefsky *et al.*, 1960). These studies provided the first direct evidence for the participation of the ATPase in oxidative phosphorylation and pointed out the usefulness of the tactic of resolution and reconstitution as an experimental approach to the study of the coupling mechanism.

Although the soluble ATPase had the hallmarks of being the same enzyme that functions in uncoupled mitochondria, it differed from the latter in several significant respects. In particular, it was noted that in contrast to the membrane-bound ATPase, which was inhibited by oligomycin and was stable at low temperatures, purified F_1 was completely insensitive to oligomycin and was rapidly inactivated in the cold. An explanation for this discrepancy was given by Racker (1964), who showed that the inner membrane of mitochondria contains a factor that can combine with F_1 with concomitant restoration of oligomycin sensitivity. This finding suggested that in the membrane, F_1 is a component of a larger complex. Confirmatory evidence for this was later obtained when a protein fraction was isolated which in conjunction with phospholipid was capable of conferring oligomycin sensitivity and cold stability upon F_1. More recently, the availability of purified preparations of the oligomycin-sensitive ATPase complex has allowed characterization of its subunit proteins and in some instances a definition of their function (Tzagoloff *et al.*, 1968a; MacLennan and Tzagoloff, 1968).

Studies on the mitochondrial ATPase complex have helped to clarify some aspects of coupling factors of oxidative phosphorylation, particularly their relationship to the subunit components of the complex and their function in energy transduction. For a detailed discussion of these topics, the recent reviews by MacLennan (1970b) and Sanadi *et al.* (1968) are recommended.

In addition to its specific role in the terminal reactions of oxidative phosphorylation, the ATPase system has been of interest from the more general standpoint of the biochemistry of membrane enzymes. In this context the areas of special interest are (1) the internal structure of the complex and the function of the subunit proteins in the catalysis of phosphate transfer, (2) the contribution of the complex to the ultrastructure of the membrane and, (3) the *in vivo* synthesis of the subunit proteins and the mechanism of their assembly into a functional enzyme. Each of these areas is discussed in this chapter, and where major gaps in our knowledge prevent definitive statements, plausible alternatives are presented.

II. PURIFICATION AND PROPERTIES OF THE OLIGOMYCIN-SENSITIVE ATPase COMPLEX

The oligomycin-sensitive ATPase of mitochondria is a high-molecular-weight complex containing six to seven different subunit proteins and phospholipid. The ATPase is firmly associated with the inner membrane of the mitochondrion and, similar to most membrane lipoprotein complexes, can be solubilized with surface-active reagents. These reagents cause dissociation of lipid–protein complexes (Green and Fleischer, 1964), and therefore purified preparations of the enzyme have differed with respect to their content of phospholipid. The significant point, however, is that the ATPase activity of the enzyme is absolutely dependent on phospholipid.

It has also been noted that the specific activity of different preparations of the complex has varied from very low to very high values. These differences are probably unrelated to the purity of the ATPase but are probably attributable to the presence of inhibitors. One such inhibitor is a low-molecular-weight protein tightly bound to the ATPase.

A. ATPase Complex of Beef Heart Mitochondria

1. Purification

During the past several years, a number of procedures have been developed for purifying the ATPase complex of beef heart mitochondria (Kagawa and Racker, 1966a; Kopaczyk *et al.*, 1968; Tzagoloff *et al.*, 1968a). These procedures differ in minor details, but in each case cholate and/or deoxycholate was used to solubilize the ATPase. The key steps in one such procedure (Tzagoloff *et al.*, 1968a) are summarized in Fig. 1. Submitochondrial particles are extracted with low levels of deoxycholate. This solubilizes the ATPase as well as some components of the electron transfer chain. Dialysis of the extract causes cytochromes b, c_1, and most of the flavoproteins to precipitate. The ATPase remains soluble under these conditions and is precipitated by passage through Sephadex. The precipitated enzyme is further purified by fractionation with cholate and ammonium sulfate.

This procedure achieves a separation of the ATPase from most of the electron transfer components of the mitochondrion. A comparison of the concentrations of cytochromes and flavin in submitochondrial particles and in the purified ATPase (see Table I) shows that with the exception

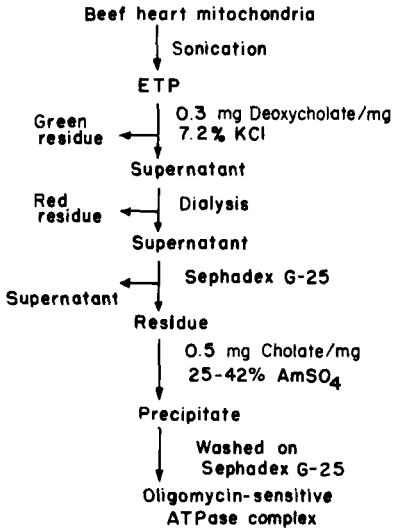

FIG. 1. Purification of oligomycin-sensitive ATPase from beef heart mitochondria.

of flavin the extent of contamination is negligible. This is also true of other preparations reported in the literature (Kagawa and Racker, 1966a; Kopaczyk *et al.*, 1968).

Additional criteria for purity include specific activity, which is approximately 5 times higher than that of submitochondrial particles, and electrophoretic analysis of the depolymerized complex. Under dissociating conditions (Takayama *et al.*, 1966), six major protein bands are resolved

TABLE I

CONCENTRATION OF ELECTRON TRANSFER COMPONENTS IN THE ATPASE COMPLEX AND ETP[a]

Components	Concentration (nmoles/mg)	
	ATPase complex	ETP
Cytochromes a + a_3	0	1.62
Cytochrome b	0.06–0.08	0.85
Cytochromes c + c_1	0.02–0.03	0.63
Total flavin	0.52	0.66

[a] Data taken from Tzagoloff *et al.* (1968a). ETP refers to beef heart submitochondrial particles.

by polyacrylamide gel electrophoresis (see Fig. 2). These proteins have been shown to be intrinsic components of the oligomycin-sensitive complex (Tzagoloff *et al.*, 1968a; MacLennan and Tzagoloff, 1968).

2. Physical Properties

Both phospholipids and bile salts have a profound effect on the physical state of the ATPase complex. Thus removal of bile salts leads to a rapid polymerization of the complex. The nature of the polymers depends on the endogenous content of phospholipid; if the phospholipid content is

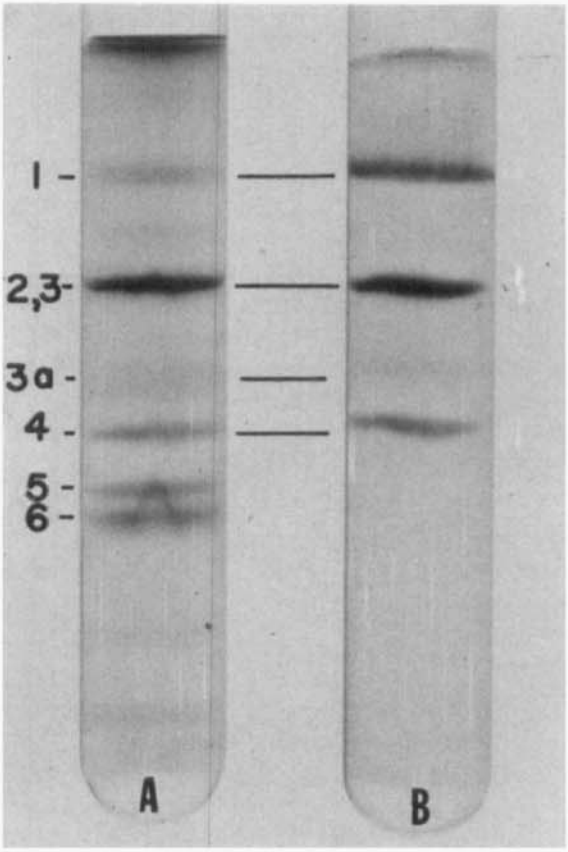

Fig. 2. Polyacrylamide gel electrophoresis of beef F_1 and beef oligomycin-sensitive ATPase complex. The two enzymes were depolymerized in a mixture of phenol–acetic acid–urea and separated by electrophoresis on polyacrylamide gels according to the method of Takayama *et al.* (1966). Gel A, oligomycin-sensitive complex; gel B, F_1. Reproduced from Tzagoloff *et al.* (1968a).

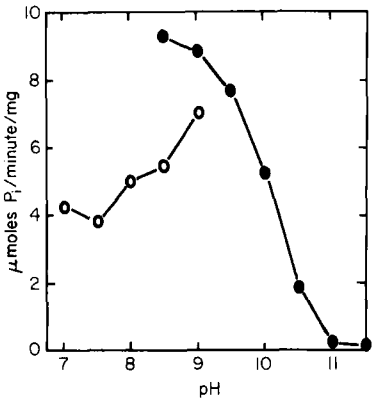

FIG. 3. Effect of pH on the ATPase activity of the oligomycin-sensitive complex. Tris–HCl buffer (O——O), glycine buffer (●——●). For experimental details see Tzagoloff *et al.* (1968a).

low (<10%), the polymerization results in bulk phase aggregates which lack a definable structure. At higher phospholipid-to-protein ratios, however, the complex reassociates into membranes. A similar requirement of phospholipid has also been found for membrane formation from electron transfer complexes of mitochondria (McConnell *et al.*, 1966).

Kagawa and Racker (1966b) have reported that their preparation of the ATPase consists of large amorphous aggregates. These aggregates are converted to vesicular membranes when supplemented with phospholipids. A similar phenomenon was found by Kopaczyk *et al.* (1968). The preparation described here contains approximately 30% phospholipid and consists of vesicular membranes after the last step of the purification. This step is a Sephadex treatment which removes residual bile salts from the complex.

3. Enzymic Properties

The purified complex exhibits the same enzymic properties as the ATPase of submitochondrial particles (Tzagoloff *et al.*, 1968a). This is true of the pH optimum, nucleotide specificity, metal activation, and sensitivity to various inhibitors. In Fig. 3 it can be seen that the enzyme functions best at pH 8–9. At more alkaline values the activity declines rapidly. A divalent cation is required for optimal activity. Of the metals tested Mg^{2+} and Mn^{2+} were found to be the most stimulatory. Ca^{2+} did not stimulate the enzyme to any appreciable extent (see Table II). The results shown in Table III. show the rates of hydrolysis of several nucleotides with reference to ATP. Although ATP is the best substrate, other triphosphonucleotides are

TABLE II

STIMULATION OF MITOCHONDRIAL ATPASE BY Ca^{2+} AND Mg^{2+}[a]

Preparation tested	ATPase activity (μmoles/minute/mg) With Mg^{2+}	With Ca^{2+}
ETP	2.35	0.28
Oligomycin-sensitive complex	10.65	0.31
F_1	44.60	32.50

[a] Data taken from Tzagoloff *et al.* (1968a).

TABLE III

HYDROLYSIS OF TRIPHOSPHONUCLEOTIDES BY MITOCHONDRIAL ATPASE

Preparation tested	Activity (% of ATP) GTP	ITP	UTP	CTP
ETP	34	43	4	9
Oligomycin-sensitive complex	26	17	5	5
F_1	110	100	48	18

[a] Data taken from Tzagoloff *et al.* (1968a).

TABLE IV

INHIBITORS OF THE OLIGOMYCIN-SENSITIVE ATPASE COMPLEX[a]

Inhibitor	Concentration required for 50% inhibition
Oligomycin	0.5 μg/mg protein
Rutamycin	0.5 μg/mg protein
Aurovertin	4.0 μg/mg protein
Mitochondrial inhibitor[b]	0.3 mg/mg protein
Tri-*n*-butyltin chloride	10^{-7} *M*
p-Chloromercuribenzoate	10^{-4} *M*
ADP	10^{-2} *M*

[a] Data taken from Tzagoloff *et al.* (1968a).

[b] The mitochondrial inhibitor was prepared by the procedure of Pullman and Monroy (1963).

hydrolyzed but at a slower rate. The order of nucleotide specificity of the isolated complex and of submitochondrial particles is ATP > GTP, ITP > UTP, CTP.

Inhibitors of the ATPase are listed in Table IV. These reagents fall into two groups: those that inhibit both the lipoprotein complex and the soluble ATPase F_1, and those that inhibit only the complex. In the latter group belong oligomycin, rutamycin (oligomycin and rutamycin are chemically related compounds; because of the present scarcity of oligomycin, most investigators now use rutamycin), tri-*n*-butyltin chloride, and mercurials. The concentrations at which these reagents inhibit the ATPase activity of the complex are also effective in inhibiting oxidative phosphorylation in mitochondria and in submitochondrial particles.

Although these data suggest that the basic properties of the ATPase remain unmodified after purification, two partial reactions of oxidative phosphorylation, probably carried out by the ATPase complex in mitochondria are not catalyzed by the isolated complex. The inability of the complex to catalyze the exchange of the terminal phosphate of ATP with inorganic phosphate and with ADP indicates that in mitochondria the ATPase is part of a more elaborate coupling system.

B. ATPase Complex of Yeast Mitochondria

1. Purification

The ATPase complex of yeast mitochondria has been purified by a procedure involving differential extraction of submitochondrial particles with deoxycholate and salt fractionation (Tzagoloff, 1969a). This preparation has a low content of phospholipid and is particulate.

A much simpler procedure allows the ATPase complex to be isolated in a dispersed form. A suspension of submitochondrial particles is treated with 0.25% Triton X-100 to solubilize the ATPase complex. The Triton extract is layered on top of a linear gradient of glycerol (5–15%) containing either 0.1% Triton X-100 or 0.2% Tween 80. After centrifugation at 25,000 rpm for 17 hours in a Spinco SW-27 rotor, the ATPase sediments approximately midway into the gradient. This centrifugation separates the ATPase from other solubilized membrane components by virtue of differences in molecular size. The distribution of ATPase activity on the glycerol gradient (see Fig. 4) indicates that the ATPase is homogeneous with respect to size. Data on the purification of the ATPase by this procedure are summarized in Table V. The purified complex has a specific activity that is 10-fold higher than that of submitochondrial particles.

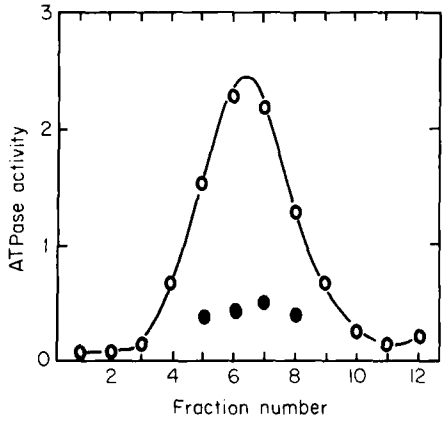

FIG. 4. Distribution of ATPase activity on glycerol gradient. Yeast submitochondrial particles were extracted with 0.25% Triton X-100. The extract (4 ml) was applied to 32 ml of 5–15% glycerol (w/v) gradient containing 5 m*M* tris–acetate (pH 7.5) and 0.1% Triton X-100. The gradient was centrifuged at 23,000 rpm for 17 hours. Thirteen fractions were collected and assayed for ATPase activity in the absence (○——○) and presence (●——●) of rutamycin. Ordinate refers to total ATPase units (μmoles/min/mg).

The protein profile of the dissociated complex is shown in Fig. 5. The electrophoretic separation was carried out under the same conditions used for the beef enzyme. It is evident that both enzymes have the same major protein bands with the exception of band 5a (Fig. 5) which is absent or unresolved in the beef complex. Since these preparations are obtained by entirely different procedures, the similarity of the two profiles confirms the notion that the proteins observed on the gels are intrinsic components of the ATPase complex.

TABLE V

RECOVERY OF PROTEIN AND ENZYME ACTIVITY[a]

Fraction	Total protein (mg)	Specific activity (μmoles P_i/minute/mg)
Submitochondrial particles	125	2.3
Triton extract	25	10.8
Active peak from gradient	2.3	28.0

[a] Submitochondrial particles were extracted with 0.25% Triton X-100 as described in the text. The extract was centrifuged through a 5–15% glycerol gradient containing 0.1% Triton X-100 in an SW-27 rotor for 17 hours at 23,000 rpm. Twelve fractions were collected from each tube and assayed for ATPase.

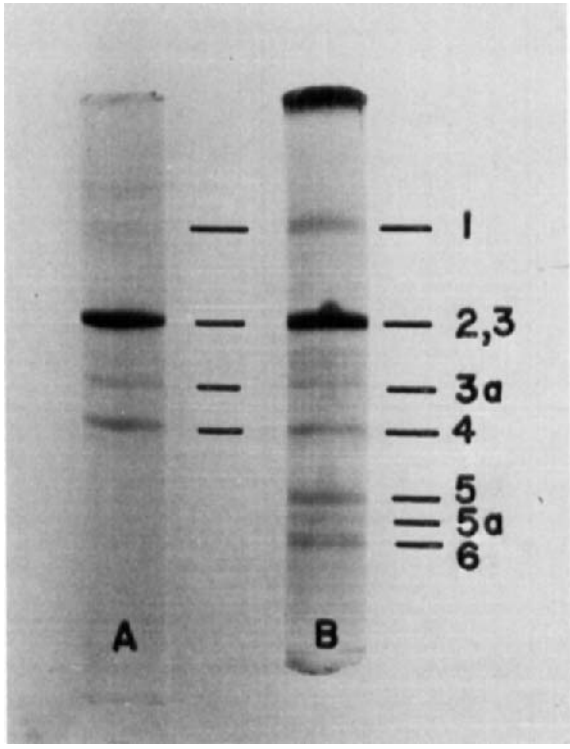

FIG. 5. Polyacrylamide gel electrophoresis of yeast F_1 and yeast oligomycin-sensitive ATPase complex. The conditions for electrophoresis were the same as described in the legend to Fig. 2. Gel A, F_1; gel B, oligomycin-sensitive complex.

The phospholipid content of the Triton enzyme depends on the type and concentration of detergent used in the gradient. Values of 31, 18, and 10% were found when the complex was isolated from gradients containing, respectively, 0.2% Tween, 0.1% Triton, and 0.2% Triton.

2. PHYSICAL PROPERTIES

The fact that low concentrations of Triton maintain the yeast complex in a dispersed form has made it possible to make some estimate of its molecular size. The sedimentation of the complex and of F_1 in a 5–20% linear gradient of sucrose containing 0.2% Triton X-100 is shown in Fig. 6. β-Galactosidase from *Escherichia coli* was used as a molecular weight marker. By using the procedure of Martin and Ames (1961), the $s_{20,w}$ of the complex was calculated to be 15.3 and of F_1, 12.1. If it is assumed that the partial specific volume of the protein is close to 0.74 (the partial

specific volume of F_1) and of phospholipid 0.99 (Huang, 1969), the ATPase complex containing 10% phospholipid would have a partial specific volume of 0.77. A correction for the effect of the increased partial specific volume would raise the $s_{20,w}$ of the complex to 15.9 (Martin and Ames, 1961). These values correspond to molecular weights of 520,000 for the complex and 240,000 for F_1. The former value corrected for 10% phospholipid would be 468,000 or 128,000 in excess of F_1. These molecular weights, however, are only approximations since no attempts have been made to correct for any detergent that may be bound to the enzyme. Since the presence of bound detergent would tend to increase the apparent molecular weight, these values must be considered upper limits. Despite this limita-

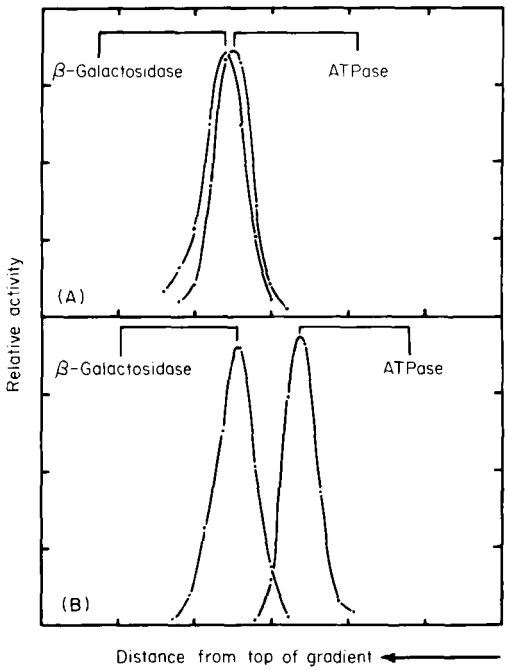

FIG. 6. Sedimentation of yeast F_1 and of the oligomycin-sensitive ATPase complex. (A) Yeast submitochondrial particles were extracted with 0.25% Triton X-100 as described in the text. The extract (0.2 ml) was mixed with 0.05 ml of β-galactosidase (0.1 mg) and applied to a 5–20% linear sucrose gradient containing 5 m*M* tris–acetate (pH 7.5) and 0.2% Triton. The gradient was centrifuged at top speed in a Spinco SW–65 rotor for 3 hours at 0°. Thirty fractions were collected and assayed for ATPase and β-galactosidase. (B) Yeast F_1 (0.4 mg) and β-galactosidase (0.1 mg) in a volume of 0.25 ml were layered on top of a 5–20% linear gradient of sucrose containing 5 m*M* tris–acetate (pH 7.5), 2 m*M* ATP, and 0.2% Triton X-100. The gradient was centrifuged in a Spinco SW–65 rotor for 135 minutes at top speed with the temperature set at 24°. Twenty-eight fractions were collected and assayed for ATPase and β-galactosidase.

tion the results indicate that the oligomycin-sensitive complex is not very much larger than F_1.

The particulate or membranous nature of previous preparations of the ATPase complex has hindered an ultrastructural description of the unit enzyme. Electron micrographs of the complex isolated by the Triton procedure are shown in Figs. 7B and C. These electron micrographs show the complex to be an oval-shaped particle with dimensions of 100×150 Å. For comparison, and electron micrograph of F_1 is shown in Fig. 7A. The dimensions of the lipoprotein complex are consistent with a structure composed of F_1 attached to a smaller unit 50–70 Å long. Viewed from the top, such a composite structure appears as a spherical particle with the same dimensions as F_1. Such particles can be seen in the field shown in Fig. 7B (see arrows). The smaller unit in the complex probably comprises the protein components that determine oligomycin or rutamycin sensitivity.

3. Enzymic Properties

The enzymic properties of the yeast and beef complexes are very similar. It is interesting, however, that much higher concentrations of oligomycin or rutamycin are required to inhibit the ATPase activity of yeast mitochondria (Ohnishi *et al.*, 1966) and also of the isolated complex (Tzagoloff, 1969a). It has been reported that when yeast F_1 is bound to beef submitochondrial particles the reconstituted hybrid ATPase is inhibited by oligomycin at the same concentrations that inhibit the beef ATPase (Schatz *et al.*, 1967). This finding indicates that the lower sensitivity of the yeast complex is not attributable to differences that may exist in F_1 but rather to the factor concerned with conferral of oligomycin sensitivity. It has also been shown that the extent of inhibition of the yeast complex by rutamycin decreases in the presence of increasing concentrations of phospholipid (Tzagoloff, 1969a). The ability of phospholipids to compete for rutamycin (probably by virtue of their lipophilic character), together with a poorer affinity of the yeast complex for oligomycin or rutamycin, may explain why higher concentrations of these reagents are needed for inhibition in the case of yeast.

III. COMPOSITION OF THE ATPase COMPLEX

A. Protein Components

1. Oligomycin-Insensitive ATPase (F_1)

Pullman *et al.* (1960) purified from beef heart mitochondria a water-soluble ATPase which has since been shown to be a component of a larger

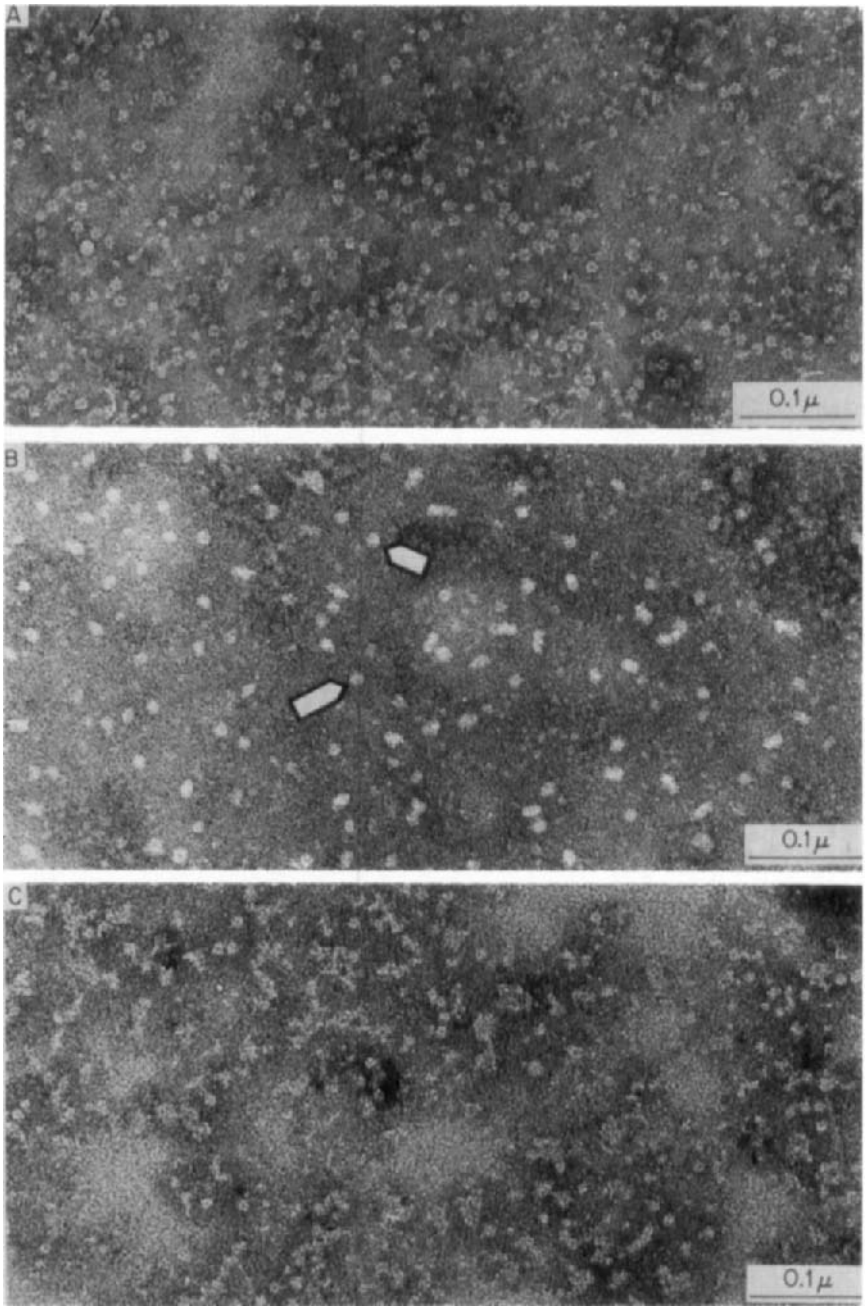

FIG. 7. Electron micrographs of yeast F_1 and of the yeast oligomycin-sensitive ATPase complex. (A) Yeast F_1. (B) Yeast oligomycin-sensitive complex. (C) Yeast oligomycin-sensitive complex sampled from a reaction assay containing 0.1% Tween 80.

TABLE VI

PROPERTIES OF F_1 AND OF THE OLIGOMYCIN-SENSITIVE ATPASE COMPLEX

Property	F_1	Oligomycin-sensitive complex
Enzymic		
Ca^{2+} activation	+	−
Inhibition by oligomycin, rutamycin, tributyltin, and mercurials	−	+
Phospholipid requirement	−	+
Physical		
Phospholipid content	−	+
Water solubility	+	−
Cold lability	+	−
Number of protein subunits	3–4	6–7
Molecular weight (yeast complex)	340,000	468,000
Capacity for membrane formation	−	+

lipoprotein complex. That F_1 is the rudimentary ATPase of the oligomycin-sensitive complex has been established from the following evidence. (1) F_1 becomes sensitive to oligomycin when it is combined with another lipoprotein factor which in itself has no demonstrable ATPase activity (Kagawa and Racker, 1966a). (2) A water-soluble ATPase with the same properties as F_1 has been isolated from the oligomycin-sensitive complex (Tzagoloff, 1968a). (3) the subunit proteins of F_1 are present in the oligomycin-sensitive complex (Tzagoloff *et al.*, 1968a). (4) Antibody against F_1 inactivates the oligomycin-sensitive ATPase activity of submitochondrial particles (Fessenden and Racker, 1966; Schatz *et al.*, 1967).

F_1 is tightly bound to the inner membrane of the mitochondrion. It has been solubilized by fragmentation of mitochondria in a Nossal shaker (Pullman *et al.*, 1960; Schatz *et al.*, 1967) and by sonic irradiation of submitochondrial particles (MacLennan *et al.*, 1968; Datta and Penefsky, 1970; Tzagoloff, 1969a). Homogeneous preparations of F_1 have been obtained both from beef heart and from yeast mitochondria.

Some of the salient differences between F_1 and the oligomycin-sensitive ATPase complex are listed in Table VI.

a. Physical Properties. Beef F_1 is a globular protein with an s_{20w} of 11.9 and an estimated molecular weight of 285,000 (Penefsky and Warner, 1965). Almost identical sedimentation constants have been found for the yeast enzyme (Schatz *et al.*, 1967; see also Section II,B).

An interesting property of F_1 is its marked lability to cold. This phenomenon was investigated by Penefsky and Warner (1965). These investi-

gators found that cold inactivation of F_1 is accompanied by a dissociation of the active 11.9 S polymer into lower-molecular-weight species. When the enzyme was exposed to 0°, the ATPase activity declined over a period of several hours. During this period there appeared two new species having $s_{20,w}$ of 9.1 and 3.5. The depolymerized subunits could be induced to reassociate into an active enzyme provided the incubation was limited to 2 hours. Over longer times of exposure to cold, the 3.5 S subunits aggregated irreversibly into large inactive polymers. Penefsky and Warner (1965) also found that the rate of dissociation of F_1 was strongly influenced by the ionic composition of the medium. Anions of the Hofmeister series such as I^-, NO_3^-, and Br^- were particularly effective in promoting dissociation in the cold. The recent findings of Hatefi and Hanstein (1969) that these reagents weaken the hydrophobic interactions of proteins suggest the involvement of such interactions in the quaternary structure of F_1.

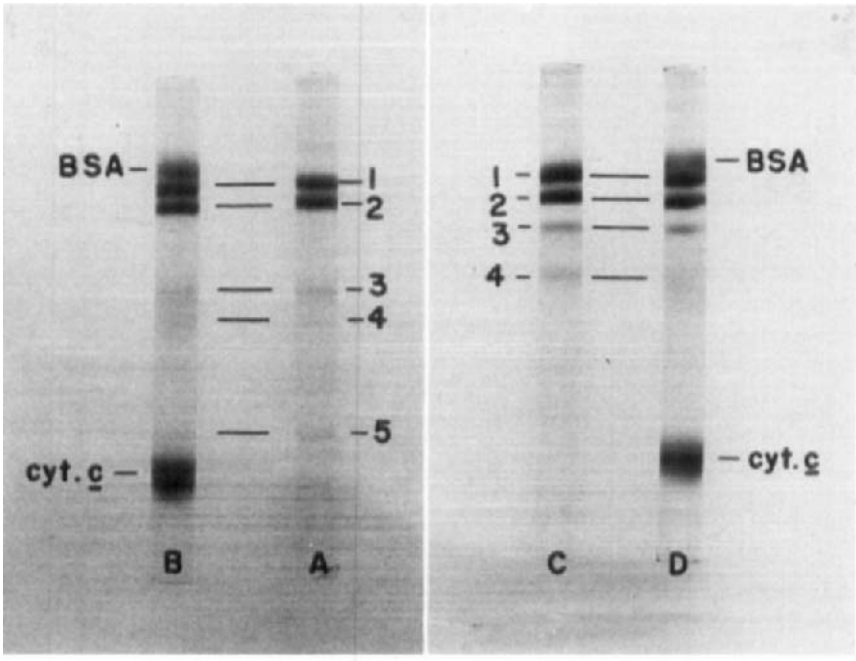

FIG. 8. Polyacrylamide gel electrophoresis of yeast F_1 and of beef F_1. The two enzymes were depolymerized in a solvent containing 1% sodium dodecyl sulfate, 1% mercaptoethanol, and 0.01 *M* sodium phosphate (pH 7.0). The samples were separated on 10% acrylamide gels in the presence of 0.1% sodium dodecyl sulfate according to the procedure of Weber and Osborn (1969). (A) Beef F_1. (B) Beef F_1 plus bovine serum albumin and cytochrome c. (C) Yeast F_1. (D) Yeast F_1 plus bovine serum albumin and cytochrome c.

Although the oligomycin-sensitive complex is stable in the cold under conditions of low ionic strength, it is rapidly inactivated in the presence of sodium bromide (MacLennan *et al.*, 1968; Tzagoloff *et al.*, 1968b). The mechanism of this inactivation is probably similar to the enhancement of cold inactivation of soluble F_1 since the salt extracts subunits of F_1 from the complex.

Two solvents systems have been used to analyze the subunit protein components of F_1 and of the oligomycin-sensitive complex. In phenol–acetic acid–urea (Takayama *et al.*, 1966), beef F_1 separates into four distinct protein bands (see Fig. 2). These protein bands are also present in the oligomycin-sensitive complex; the latter, however, contains two additional faster-migrating bands (bands 5 and 6). Almost identical patterns are obtained with the yeast enzymes, the sole difference being that the two major bands of F_1 (bands 2 and 3) are not resolved in this system (see Fig. 5). The relative amount of band 1 is variable and may represent an incompletely depolymerized enzyme or some secondary aggregation product.

A similar correspondence of bands is observed when the two enzymes are depolymerized in 1% sodium dodecyl sulfate and separated on polyacrylamide gels containing 0.1% sodium dodecyl sulfate (Weber and Osborn, 1969). In this system, however, the two major subunits of both yeast and beef F_1 are separated (see Fig. 8). Another advantage of the sodium dodecyl sulfate system is that it allows the molecular weights of the subunit proteins to be estimated. In Table VII are summarized the molecular weights of the two major (bands 1 and 2) and the two minor (bands 3 and 4) proteins of yeast and beef F_1. These values were calculated

TABLE VII

MOLECULAR WEIGHTS OF F_1 SUBUNITS[a]

Protein subunit	Molecular weight	
	Beef F_1	Yeast F_1
1	59,000	57,000
2	53,000	54,000
3	33,000	39,000
4	27,000	32,000

[a] F_1 was depolymerized in a solvent containing 1.0% sodium dodecyl sulfate, 0.01 *M* sodium phosphate (pH 7.0), and 1.0 mercaptoethanol. The proteins were separated on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The protein subunits correspond to the protein bands shown in Fig. 8.

from a standard curve in which the log of the molecular weights of bovine serum albumin, yeast alcohol dehydrogenase, trypsin, and cytochrome c were plotted against the distance of migration from the top of the gel (Weber and Osborn, 1969). To account for the known molecular weight of F_1, a 2:1 ratio of the major to minor bands is postulated. This seems reasonable from the relative staining intensity of the four proteins.

F_1 was shown to be a spherical particle with a diameter of 80–100 Å (Racker *et al.*, 1965; Schatz *et al.*, 1967). At high resolution it appears to be composed of six subunits arranged in a hexagonal array (see Fig. 7A) Since the gel patterns obtained in sodium dodecyl sulfate indicate at least six subunit proteins, it is probable that each morphological subunit represents a single subunit protein of the enzyme.

b. Enzymic Properties. F_1 is isolated free of phospholipids and does not require phospholipid for enzymic activity. In addition to its lack of sensitivity to certain inhibitors of oxidative phosphorylation (see Table VI), F_1 differs from the oligomycin-sensitive complex in its nucleotide specificity and response to metal activators. Thus Ca^{2+}, which does not stimulate the oligomycin-sensitive complex, is almost as effective as Mg^{2+} in stimulating the ATPase activity of F_1 (see Table II). F_1 also shows a lesser specificity toward triphosphonucleotides. The rates of hydrolysis of GTP and ITP are equal to that of ATP (see Table III).

2. Oligomycin Sensitivity-Conferring Protein (OSCP)

This protein component has been isolated from the beef complex and directly from submitochondrial particles. OSCP is extracted from the ATPase complex with ammonium hydroxide and purified to near homogeneity by salt fractionation and adsorption on carboxymethyl cellulose (MacLennan and Tzagoloff, 1968). Bulos and Racker (1968a) have reported the isolation from beef heart mitochondria of a factor (F_c) with the same properties as OSCP. OSCP has also been purified from the yeast complex (Tzagoloff, 1970).

OSCP is a positively charged protein with an isoelectric point between 9 and 10. On Sephadex G-100 the beef protein cochromatographs with metmyoglobin, indicating a molecular weight around 18,000 (MacLennan and Tzagoloff, 1968). This value is in agreement with electron microscope data. MacLennan and Asai (1968) have reported that beef OSCP is a cylindrical particle 50 ± 5 Å in length and 30 ± 5 Å in diameter. A particle with these dimensions was calculated to have a minimum molecular weight of 17,000 and a maximum of 40,000 (MacLennan and Asai, 1968).

Based on its electrophoretic behavior in the phenol–acetic acid–urea

system, OSCP has been identified with band 6 of the complex (MacLennan and Tzagoloff, 1968; see Fig. 2).

Purified OSCP has no ATPase activity nor has it been shown to carry out any definable enzymic function. Although the role of this component is not clearly understood at present, it has been shown to be required for the restoration of oligomycin or rutamycin sensitivity and for the binding of F_1 to submitochondrial particles (MacLennan and Tzagoloff, 1968; Bulos and Racker, 1968b). This evidence is discussed in Section III,C.

3. Juncture Protein

The term juncture protein was used by MacLennan (1970b) in order to indicate that this component probably links the ATPase system to the electron transfer chain. Juncture protein has not been purified, but its existence is implicit from the following observations. (1) Reconstitution of oligomycin-sensitive ATPase requires in addition to F_1 and OSCP another yet uncharacterized component which is heat and trypsin labile (Bulos and Racker, 1968a; Tzagoloff, 1970). (2) The gel profiles of the depolymerized complex show that in addition to the protein bands of F_1 and OSCP the complex contains one other protein subunit (band 5, see Fig. 2).

Despite the fact that juncture protein has not been purified, some of its properties are known. Juncture protein is the least soluble protein component of the complex. Crude preparations of juncture protein are obtained by sequential extraction of the beef complex with sodium bromide and ammonium hydroxide (MacLennan and Tzagoloff, 1968). This procedure extracts F_1 and OSCP. The particulate residue contains largely band 5 and some denatured OSCP. Attempts to solubilize band 5 from this material have failed so far.

The crude juncture protein residue contains the bulk of the phospholipid of the original ATPase complex (Tzagoloff *et al.*, 1968b). This suggests that juncture protein is the phospholipid-binding component of the complex. Evidence that juncture protein may contain the oligomycin binding site is discussed in Section III,D.

4. ATPase Inhibitor

The presence in mitochondria of a naturally occurring ATPase inhibitor was demonstrated by Pullman and Monroy (1963), who purified this component from beef heart mitochondria. Most of the ATPase activity of beef heart submitochondrial particles is masked by the inhibitor. Thus Racker and Horstman (1967) achieved a 10-fold activation of the ATPase activity of submitochondrial particles by passage through Sephadex G-

50. This treatment presumably removes bound inhibitor from the particles. Yeast mitochondria, however, seem to contain relatively little inhibitor. Sephadex treatment of yeast particles achieves only nominal activation of their ATPase activity (Tzagoloff, 1969a). Attempts to purify this component from yeast mitochondria have not succeeded.

The ATPase inhibitor is a low-molecular-weight protein (15,000) which inhibits the activity of isolated F_1 and of the oligomycin-sensitive complex (Pullman and Monroy, 1963; Tzagoloff *et al.*, 1968a). The beef protein also inhibits yeast F_1 (Schatz *et al.*, 1967) but fails to inhibit nonmitochondrial ATPases (namely, microsomes, myosin) (Pullman and Monroy, 1963). F_1 and the inhibitor react to form a stoichiometric complex. When complexed to the inhibitor, F_1 is no longer cold labile; this indicates a stabilization of its quaternary structure (Pullman and Monroy, 1963). The F_1–inhibitor complex can be dissociated by low salt concentrations, aging, and heat (Pullman and Monroy, 1963). Some of these conditions are also used in the preparation of F_1. It is therefore likely that the low and variable amounts of endogenous inhibitor found in purified preparations of F_1 are the result of a dissociation of the complex during the purification of the enzyme (Horstman and Racker, 1970). One exception to this, however, is the factor-A preparation of Andreoli *et al.* (1965). The ATPase activity of factor A is very low but can be stimulated 3- to 4-fold by heating at 65°. This preparation of F_1 probably contains sufficient inhibitor to mask most of its ATPase activity. Recently, Warshaw *et al.* (1968) reported that heating of factor A at 70° causes the release of a protein with an inhibitory activity toward F_1.

The physiological role of the inhibitor is not known. There have been some speculations that it plays a role in the respiratory control of mitochondria (Pullman and Monroy, 1963; Horstman and Racker, 1970), but at present there is no experimental evidence in support of this. Undoubtedly the inhibitor is involved in the regulation of the ATPase.

B. Phospholipid Components

There are numerous examples of enzymes both from mitochondria and other membrane systems which have been shown to be dependent on phospholipids for enzymic activity, suggesting that this may be a general property of all membrane enzymes. It is therefore not surprising that phospholipid is also an essential component of the oligomycin-sensitive ATPase complex. Various values have been reported for the phospholipid content of the complex. The preparations obtained from beef mitochondria by the procedures of Kagawa and Racker (1966a) and Kopaczyk *et al.*

(1968) contain about 10% phospholipid. Both enzyme preparations require exogeneously added phospholipid for maximal ATPase activity. The ATPase complex obtained by the procedure of Tzagoloff *et al.* (1968a) has a higher phospholipid content (30%) and its activity is not significantly enhanced by added phospholipids. A requirement of phopholipid for enzymic activity has also been shown for the yeast complex (Tzagoloff, 1969a).

The activation of oligomycin-sensitive ATPase by phospholipids was studied extensively by Kagawa and Racker (1966a,c). Several interesting points have emerged from these studies. Both phospholipids and free fatty acids were found to stimulate the ATPase activity of the complex. Of these two classes of lipids, however, only phospholipids activated the enzyme in an oligomycin-sensitive fashion. Linoleic acid, the major fatty acid constituent of mitochondrial phospholipids, gave maximal stimulation, but the ATPase activity was virtually insensitive to rutamycin (Kagawa and Racker, 1966a). Even though fatty acids do not cause a physical separation of F_1 from the oligomycin sensitivity factor, they appear to modify the internal structure of the complex, thereby causing a conversion of the catalytic properties to an F_1 type of activity. A similar effect has been observed with tributyltin chloride. This compound inhibits the complex at low concentrations ($< 10^{-6}$ *M*) but fails to inhibit at high concentrations ($> 10^{-5}$ *M*). At high concentrations of tributyltin chloride, F_1 remains associated with the oligomycin sensitivity-conferring factor but is not inhibited by rutamycin (Tzagoloff *et al.*, 1968a).

Crude mixtures of phospholipids from beef heart mitochondria and from soybean as well as purified lecithin were found to be equally effective in restoring the ATPase activity of the beef complex (Kagawa and Racker, 1966a). The yeast ATPase was also found to be equally well activated by both mixed phospholipids isolated from yeast and soybean (Tzagoloff, 1969a).

Correlative studies on the effect of phospholipid on the ultrastructure and catalytic activity of mitochondrial ATPase (Kagawa and Racker, 1966b; Kopaczyk *et al.*, 1968), the Ca^{2+} transport ATPase of sarcoplasmic reticulum (Martonosi, 1968; MacLennan, 1970a), and cytochrome oxidase (McConnell *et al.*, 1966) have suggested a common mechanism for the observed phospholipid activation of these membrane enzymes. In each example cited the requirement could be satisfied by a large variety of phospholipids. This lack of specificity argues against the involvement of a specific phospholipid component in the catalytic function of these complexes. More significantly, however, the phospholipid activation is accompanied by a change in the state of aggregation of the complex. Thus phospholipid has been shown to convert aggregates of the mitochondrial

and sarcoplasmic reticulum ATPases (Kagawa and Racker, 1966b; MacLennan, 1970a), as well as cytochrome oxidase (McConnell *et al.*, 1966), into membranes, and in each case it is the latter form that is enzymically active. These observations have suggested a molecular explanation for the phospholipid effect (Green and Tzagoloff, 1966b), namely, that the bulk phase aggregates, which is the state membrane enzymes assume in the absence of phospholipid, are enzymatically inactive because of poor accessibility of the substrate to the enzyme. Phospholipid, by converting the aggregates into membranes, provides the means for maximal exposure of the enzyme complex to the surrounding medium, and this constitutes the basis for the requirement of phospholipid for enzymic activity.

In addition to allowing enzymes to exist in a membrane form, phospholipids may also stimulate the activity of membrane enzymes by other means. For example, in the case of cytochrome oxidase, the substrate, cytochrome c, was shown to form complexes with phospholipids (Das *et al.*, 1965). If the enzyme poses a hydrophobic barrier which must be penetrated by the substrate in order to reach the catalytic site, or if the site itself is highly hydrophobic in character, a complex with phospholipid may have to be formed for the substrate to be active. Studies on cytochrome oxidase (Tzagoloff and MacLennan 1965) have in fact indicated that phospholipids function as substrate activators. A similar mechanism may also function in the activation of the ATPase by phospholipids.

C. Resolution and Reconstitution of the ATPase Complex

Various methods have been described for the selective removal of the ATPase components from submitochondrial particles and also from the isolated complex. At present, there are methods available for extracting F_1, subunits of F_1, OSCP, and the ATPase inhibitor.

1. Extraction of F_1 and Subunits of F_1

Enzymically active F_1 has been extracted from beef and yeast submitochondrial particles by sonic irradiation (MacLennan *et al.*, 1968; Datta and Penefsky, 1970; Tzagoloff, 1969a), and from beef particles and the ATPase complex by incubation at 65° in the presence of low concentrations of ATP and EDTA (Tzagoloff *et al.*, 1968a). Neither procedure, however, achieves a quantitative extraction of F_1. The data summarized in Table VIII show that while heat treatment of the ATPase complex causes virtually complete loss of oligomycin sensitivity, only 50% of the total activity is recovered in the soluble fraction as F_1.

TABLE VIII

RECOVERY OF PROTEIN AND ATPASE ACTIVITY IN FRACTIONS OBTAINED AFTER EXPOSURE OF THE ATPASE COMPLEX TO 65°[a]

Fraction	Total protein (mg)	Specific activity (μmoles/minute/mg)	
		Minus oligomycin	Plus oligomycin
Unheated complex	8	6.9	0.6
Heated complex	8	6.2	6.0
Protein extracted by heating	0.7	34	33
Residue after heating	4.8	5.4	5.1

[a] For experimental details see Tzagoloff *et al.* (1968a).

A complete extraction of F_1 from submitochondrial particles or from the isolated complex is achieved with urea (Kagawa and Racker, 1966a; Racker and Horstman, 1967), sodium bromide (Tzagoloff *et al.*, 1968b), and silicotungstate (Racker *et al.*, 1969). These reagents are potent depolymerizers of F_1 and extract it in the form of inactive subunits. Particles depleted of F_1, however, still contain the protein components necessary for binding and to confer oligomycin sensitivity.

The protein components of the lipoprotein residue obtained from the ATPase complex after extraction with 3.5 *M* sodium bromide have been analyzed by gel electrophoresis in the phenol–acetic acid–urea system. The sodium bromide has been shown to extract the bulk of the subunits of F_1 from the complex, causing an enrichment of OSCP and juncture protein in the lipoprotein residue (Tzagoloff *et al.*, 1968b). When soluble F_1 is mixed with the lipoprotein residue, a complex between the two is formed. The resultant particle-bound ATPase is sensitive to rutamycin (see Table IX). A factor capable of conferring oligomycin sensitivity upon F_1 has also been obtained from submitochondrial particles (Kagawa and Racker, 1966a). Submitochondrial particles exposed to short digestion with trypsin were extracted with urea in order to remove F_1. The oligomycin sensitivity-conferring factor (CF_o) was isolated from the F_1-deficient membranes by fractionation with cholate and ammonium sulfate (Kagawa and Racker, 1966a).

An interesting resolution of the ATPase occurs when submitochondrial particles are extracted with 2 *M* sodium chloride. These conditions cause a 5- to 6-fold diminution of the ATPase activity of the membranes, while removing only a very small percentage of F_1 subunits (MacLennan *et al.*, 1968). Oligomycin-sensitive ATPase can be restored in the mem-

branes with native F_1 as well as with subunits of F_1 obtained by cold depolymerization. This type of resolution was also demonstrated with the isolated ATPase complex (Tzagoloff *et al.*, 1968b). In Fig. 9 are shown data on the relative efficiency of native F_1 and F_1 subunits in reconstituting rutamycin-sensitive ATPase of the complex extracted either with 2 *M* sodium chloride or with 2 *M* sodium chloride plus 2 *M* urea. The extraction with 2 *M* sodium chloride plus 2 *M* urea is comparable to 3.5 *M* sodium bromide since the lipoprotein residue contains very little of the F_1 subunits (Tzagoloff *et al.*, 1968b). The results of these experiments disclose that subunits of F_1 are active in reconstituting ATPase only when the basic F_1 structure is still present in the complex.

MacLennan *et al.* (1968) have concluded that sodium chloride causes a partial depolymerization of F_1. The partially depolymerized F_1 that remains bound to the membrane may be lacking in one or only a few subunits and can be reactivated when the missing subunit is supplied. Although polymeric F_1 was also effective in reconstituting the ATPase activity of the sodium chloride-treated membranes, it was not established whether only the missing subunits were transferred from F_1 or whether an exchange of a complete F_1 polymer occurred.

2. Extraction of OSCP

OSCP is an essential component for the reconstitution of oligomycin- or rutamycin-sensitive ATPase (MacLennan and Tzagoloff, 1968; Bulos

TABLE IX

Reconstitution of Rutamycin-Sensitive ATPase from F_1 and the Lipoprotein Factor Obtained After Extraction of the Complex with Sodium Bromide[a]

F_1 added per milligram of lipoprotein factor (μg)	Specific activity (μmoles/minute/mg)	
	Minus rutamycin	Plus rutamycin
0	0	0
200	2.00	0.16
400	2.95	0.42
600	3.35	0.52

[a] The oligomycin-sensitive ATPase complex of beef heart mitochondria was extracted with 3.5 *M* sodium bromide. The lipoprotein residue obtained after this extraction was mixed with the indicated amount of F_1. The particle-bound ATPase was assayed. For further experimental details see Tzagoloff *et al.* (1968b).

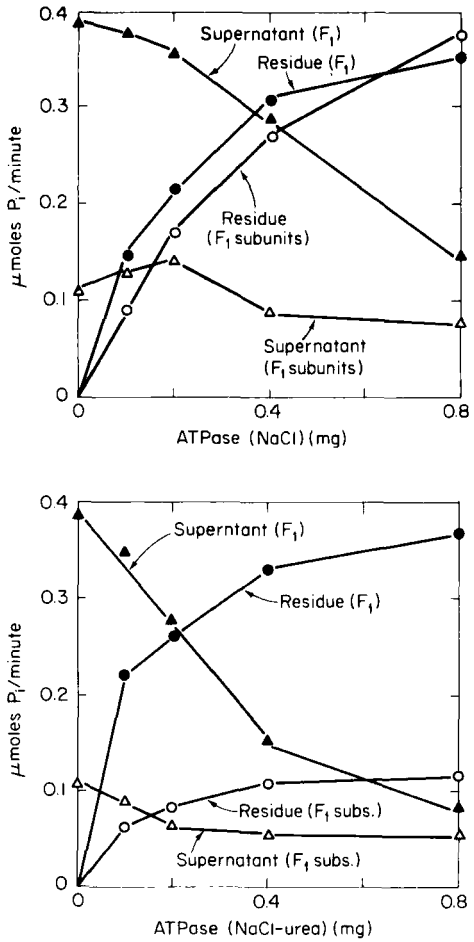

FIG. 9. Reconstitution of ATPase with F_1 and subunits of F_1. (A) Beef oligomycin-sensitive ATPase complex was extracted with 2 *M* sodium chloride. The lipoprotein obtained by this extraction [ATPase(NaCl)] was mixed either with 100 μg of F_1 or 100 μg of cold-inactivated F_1 (subunits). The mixture was centrifuged and both the residue and supernatant were assayed for ATPase activity. (B) Beef oligomycin-sensitive ATPase complex was extracted with 2 *M* sodium chloride plus 2 *M* urea. The lipoprotein obtained by this extraction [ATPase(NaCl–urea)] was reconstituted with F_1 and depolymerized F_1. For further experimental details see Tzagoloff *et al.* (1968b).

and Racker, 1968b; Tzagoloff, 1970). Thus crude juncture protein obtained by the sequential extraction of the beef ATPase complex with sodium bromide and ammonium hydroxide binds F_1 but fails to confer sensitivity to rutamycin (MacLennan and Tzagoloff, 1968). Rutamycin sensitivity

TABLE X

RECONSTITUTION OF RUTAMYCIN-SENSITIVE ATPASE FROM F_1, OSCP, AND CRUDE JUNCTURE PROTEIN[a]

Additions to 1 mg of crude juncture protein	Specific activity (μmoles/minute/mg)	
	Minus rutamycin	Plus rutamycin
None	0.00	0.00
200 μg F_1	1.39	1.30
200 μg F_1 + 3 μg OSCP	1.30	0.92
200 μg F_1 + 6 μg OSCP	1.34	0.70
200 μg F_1 + 12 μg OSCP	1.40	0.70

[a] Crude juncture protein was obtained by extraction of the oligomycin-sensitive ATPase complex of beef heart mitochondria with 3.5 *M* sodium bromide followed with 0.33 *M* ammonium hydroxide. For further experimental details see MacLennan and Tzagoloff (1968).

is reconstituted, however, provided OSCP is also added (see Table X). These results were originally interpreted to indicate that OSCP is the oligomycin sensitivity-conferring element of the complex, while the juncture protein is involved in the binding of F_1 (Tzagoloff *et al.*, 1968b; MacLennan and Tzagoloff, 1968). Several observations have necessitated a reappraisal of this earlier interpretation. In studies on the resolution of the beef complex it was pointed out that while ammonium hydroxide extracts active OSCP a significant amount of this protein remains associated with the juncture protein fraction (MacLennan and Tzagoloff, 1968). Furthermore, it was observed that the binding of F_1 to beef submitochondrial particles extracted with amonmium hydroxide was occasionally stimulated as much as 2-fold by OSCP (MacLennan, unpublished observations). In fact, recent experiments with yeast submitochondrial particles showed that the binding of F_1 is almost completely dependent on OSCP (Tzagoloff, 1970). The results shown in Fig. 10 indicate that submitochondrial particles of yeast extracted with sodium bromide and ammonium hydroxide [YETP (NaBr, NH_4OH)] do not bind F_1 unless supplemented with OSCP. These results suggest that OSCP is in some way involved in the binding of F_1 to juncture protein.

The previously mentioned observation that crude juncture protein obtained from the beef complex is capable of binding F_1 without added OSCP can be explained by the incomplete extraction of the protein with ammonium hydroxide. The presence in the juncture protein fraction of

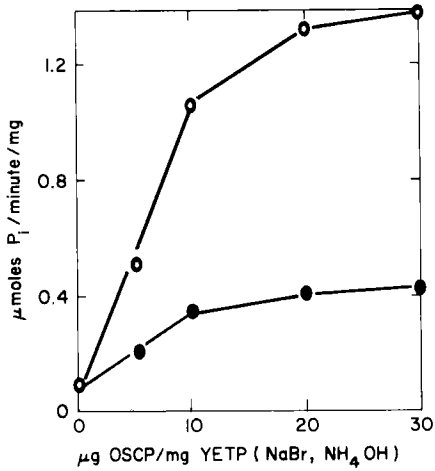

FIG. 10. Reconstitution of rutamycin-sensitive ATPase in yeast submitochondrial particles. Yeast submitochondrial particles were extracted with 3 *M* sodium bromide followed by 0.33 *M* ammonium hydroxide. The extracted particles (1 mg) were incubated in the presence of 100 μg of F_1 and the indicated amounts of OSCP. The particles were reisolated by centrifugation and assayed for ATPase. For further experimental details see Tzagoloff (1970).

OSCP in a denatured form or dislocated from its normal site could thus account for the observed binding but lack of rutamycin sensitivity.

If OSCP serves as a link between F_1 and juncture protein, the implication is that it has a binding site for each of these components. Only partial experimental evidence for this has been obtained (Tzagoloff, 1970). The experiment summarized in Table XI shows that yeast membranes extracted with ammonium hydroxide acquire competence to bind F_1 after initial incubation with OSCP. The fact that there is also a measurable loss of OSCP from the supernatant indicates that OSCP is capable of interacting with the membrane component (juncture protein) in the absence of F_1. Mixtures of F_1 and OSCP, however, not only fail to reconstitute rutamycin-sensitive ATPase but also do not form a physical complex (Tzagoloff, 1970). A possible clue to this negative result stems from the observation of MacLennan and Asai (1968) that purified OSCP tends to aggregate into tetramers. If the F_1 binding site is masked in the tetrameric form, an intitial interaction of OSCP with juncture protein may provide the necessary conformation for subsequent binding of F_1. Alternatively, juncture protein may itself in conjunction with OSCP provide the binding site for F_1.

Figure 11 summarizes the types of resolution and reconstitution that have been achieved with the ATPase complex.

TABLE XI

BINDING OF YEAST OSCP TO YETP(NaBr, NH_4OH)[a]

Addition		Specific activity (μmoles/minute/mg)	
First incubation	Second incubation	Minus rutamycin	Plus rutamycin
None	None	0.050	0.046
F_1	None	0.105	0.059
F_1	OSCP	0.105	0.076
OSCP	F_1	1.039	0.327
OSCP + F_1	None	0.968	0.236

[a] Yeast submitochondrial particles were extracted with sodium bromide followed by ammonium hydroxide. The particles were incubated in the presence of the components shown under the first incubation. They were re-isolated by centrifugation and incubated in the presence of the components shown under the second incubation. They were then assayed for ATPase activity. For further experimental details see Tzagoloff (1970).

D. Nature of Oligomycin Sensitivity

The mechanism of inhibition of the ATPase by oligomycin and related compounds is still not understood. Presumably, these inhibitors react with an active site of the enzyme, thereby preventing catalysis of phosphate transfer. Although the location and chemical nature of the oligomycin site are not known, some speculations may be offered. There is some circumstantial evidence, for example, that a sulfhydryl function is involved in the oligomycin binding site of the complex. The fact that oligomycin, tributyltin chloride, and mercurials inhibit only the lipoprotein complex and not F_1 suggests that each operates at the same site. It is well known that both mercurials and organic tin form complexes with sulfhydryl groups. There is some evidence that oligomycin is also capable of reacting with certain sulfhydryl groups. Thus it has been found that oligomycin forms a complex with cycteine and glutathione. The complex has no inhibitory activity toward the ATPase (Byington and Tzagoloff, unpublished observations).

The lack of sensitivity of F_1 to oligomycin or rutamycin suggests that the binding site for these inhibitors resides in the protein subunits associated with the oligomycin sensitivity-conferring factor. Kagawa and Racker (1966a) found that radioactive rutamycin interacts with CF_o but not with F_1. There is also some evidence that the site is in the juncture protein. When the ATPase complex is labeled with radioactive tributyltin chloride, which probably reacts with the same site as oligomycin (Aldrige,

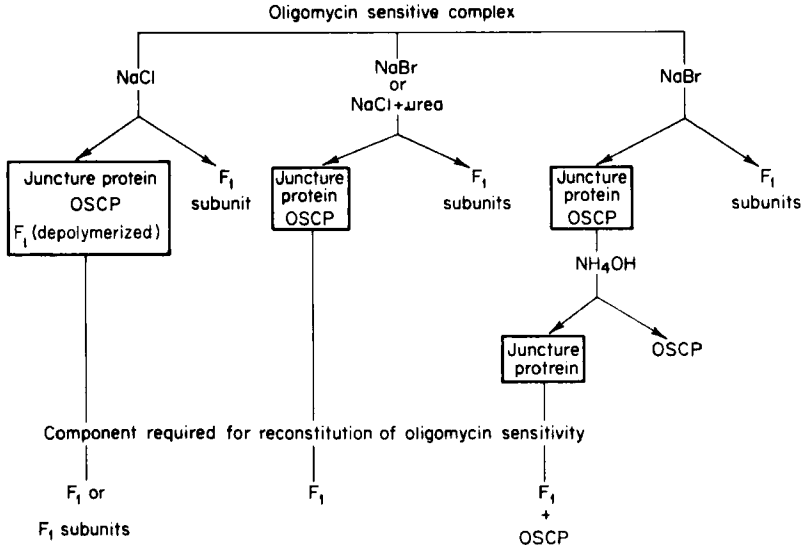

FIG. 11. Resolution and reconstitution of the ATPase complex with respect to F_1 and OSCP.

1958), the radioactivity is recovered exclusively in the crude juncture protein fraction (Tzagoloff, unpublished experiments). These reagents, however, are not covalently bound to the complex, and internal transfer of either inhibitor to some other site cannot be excluded at present.

The difference in oligomycin sensitivity of the lipoprotein complex and of soluble F_1 may be rationalized in two ways. For example, hydrolysis of ATP by the complex may involve the transfer of phosphate to two separate intermediates. One of these transfers would be catalyzed by F_1 and the other by the oligomycin sensitivity-conferring factor. In this scheme oligomycin inhibits only the latter reaction. Alternatively, the difference between the two enzymes can be explained by conformational differences in F_1 when complexed to OSCP and juncture protein. The term allotopy has been proposed by Racker (1967) to account for the altered properties of enzymes in soluble and membrane-bound form. Although the differences in the catalytic properties of F_1 and the lipoprotein complex could be attributable to conformational changes induced by the oligomycin sensitivity conferring factor, it is unlikely that the mere existence of the enzyme in a membrane form per se determines oligomycin or rutamycin sensitivity. The yeast complex isolated by the Triton procedure is a dispersed preparation and does not contain any membranes. When assayed in the presence of 0.1% Tween, the enzyme remains dis-

persed (see Fig. 7C) and is fully sensitive to rutamycin. Rutamycin sensitivity therefore appears to be a function of the association of F_1 with OSCP and juncture protein and does not depend on the presence of the complex in membrane form.

IV. ULTRASTRUCTURE OF THE ATPase COMPLEX

The ATPase complex is a major constituent of the mintochondrial inner membrane. In beef heart mitochondria F_1 alone has been estimated to contribute as much as 10% to the total mass of the inner membrane (Racker and Horstman, 1967). The architecture of this enzyme and its localization vis-à-vis the electron transfer chain is crucial for understanding the ultrastructure of the membrane and the role of this structure in the coupling mechanism. Although there are still numerous gaps in our knowledge of the macromolecular organization of the mitochondrial inner membrane, the important advances made in recent years permit some models to be constructed. The model presented here is based on studies dealing with the ultrastructure of the inner membrane, of the isolated electron transfer and ATPase complexes, and of the reconstituted membranes derived from these complexes.

A. Localization of F_1, OSCP, and Juncture Protein

Fernández-Morán (1962) reported that the inner membrane of the mitochondrion is lined with spherical particles 80–100 Å in diameter. These particles were visualized by negative staining and were shown to be linked to another globular element in the membrane itself through a stalk (Fernández-Morán *et al.*, 1964). To account for these ultrastructural features, Green and Perdue (1966) postulated the membrane to be composed of a repeating unit whose elements are a spherical headpiece, a connecting cylindrical stalk, and a basepiece. In this model the membrane continuum arises from planar stacking of the basepieces of the tripartite units.

The identity of F_1 with the headpiece has been conclusively established by the studies of Racker and his associates (Racker *et al.*, 1965; Kagawa and Racker, 1966b). Their evidence in support of this may be summarized as follows. (1) The size and shape of the inner membrane headpiece is identical to that of purified F_1 (see Section III,A). (2) Procedures that are fairly selective in extracting F_1 from submitochondrial particles also remove the headpieces. (3) Binding of F_1 to the extracted membranes and reconstitution of membrane-bound ATPase are accompanied by a morphological reconstitution of headpieces.

The localization of F_1 in the headpiece sector of the tripartite unit is also supported by ultrastructural studies on the isolated ATPase complex (Kagawa and Racker, 1966b; Kopaczyk *et al.*, 1968) and the oligomycin sensitivity-conferring factor (CF_o) (Kagawa and Racker, 1966b). It has already been pointed out that phospholipid-containing preparations of the ATPase complex consist of vesicular membranes lined with headpieces similar to those observed on the mitochondrial inner membrane. The CF_o preparation, however, which has virtually no ATPase activity because of its low content of F_1, appears as vesicles with smooth membranes. Such membranes can be reconstituted with respect to headpieces when supplemented with F_1 (Kagawa and Racker, 1966b).

Since it has been postulated that OSCP is concerned with the binding of F_1 to juncture protein, OSCP must be localized in part or *in toto* in the stalk part of the tripartite unit. This is supported by the ultrastructure of the inner membrane and of the isolated ATPase complex. Thus negatively stained specimens of the inner membrane and of the ATPase complex show that F_1 is attached to the membrane through the stalk (Fernández-Morán *et al.*, 1964; Kopaczyk *et al.*, 1968). Additional evidence for this view comes from the work of MacLennan and Asai (1968), who have shown that OSCP is an essential component for the morphological reconstitution of the tripartite unit. In their experiments the membranes formed from F_1 and crude juncture protein in the absence of OSCP failed to show a spatial separation between F_1 and the membrane continuum. F_1 appeared to be closely affixed to the membrane. An attachment of F_1 to the membrane through the stalk was observed, however, when ATPase membranes were reconstituted from a mixture of juncture protein, OSCP, and F_1. These experiments, as well as the molecular size and ultrastructural features of OSCP (see Section III,A), constitute the best evidence to date for the identification of OSCP with the stalk.

The precise localization of the third component of the ATPase, namely, juncture protein, is still not known. Kopaczyk *et al.* (1968) have proposed that the ATPase complex is localized exclusively in the headpiece and stalk sectors of the inner membrane. This would mean that the stalk is composed of both OSCP and juncture protein. Several observations argue against this interpretation. Crude juncture protein derived from the ATPase complex by sodium bromide and ammonium hydroxide extraction contains phospholipid and when examined in the electron microscope appears as smooth vesicular membranes (MacLennan and Asai, 1968). The membrane-forming capacity of juncture protein implies that it is localized in the basal part of the tripartite unit. The molecular weight of juncture protein has not been determined, but estimates based on its migration on polyacrylamide gels indicate a size in the range 20,000–

30,000. The combined molecular weight of OSCP and juncture protein would therefore be 38,000–48,000. Although still imprecise, the best available measurements of the stalk indicate that it is 30 Å in diameter and 50 Å in length (Fernández-Morán *et al.*, 1964). These dimensions are too small to accommodate both OSCP and juncture protein but are more in line with the notion that the stalk consists of OSCP only.

B. Ultrastructure of the Inner Membrane

The complexes of the electron transfer chain and the ATPase complex comprise the major constitutive enzymes of the mitochondrial inner membrane. The electron transfer complexes have been purified from beef heart mitochondria (Hatefi *et al.*, 1962a,b; Fowler *et al.*, 1962; Ziegler and Doeg, 1962), and each complex has been shown to have the capacity to form vesicular membranes in the presence of phospholipid (McConnell *et al.*, 1966; Green *et al.*, 1967; Tzagoloff *et al.*, 1967). In contrast to the ATPase membranes, the membranes formed from the electron transfer complexes do not exhibit the stalks and headpieces characteristic of the inner membrane. The reconstituted electron transfer membranes are composed of nesting globular particles which approximate the unit complex in their dimensions (Green and Tzagoloff, 1966a). Based on these findings, it has been proposed that a single complex of the electron transfer chain corresponds to a single basepiece of the tripartite unit (Green and Tzagoloff, 1966a) and that consequently the electron transfer complex is the building block of the membrane. How can this model be reconciled with observations of Kagawa and Racker (1966b) that CF_o (OSCP and juncture protein) also forms vesicular membranes?

The model diagramatically shown in Fig. 12 attempts to reconcile these conflicting observations. The basic features of this model include: (1) localization of the electron transfer complexes in the basepieces of the membrane, (2) localization of F_1 in the headpiece and OSCP in the stalk, (3) localization of juncture protein in the basepiece, separated from the electron transfer part of the basepiece by phospholipid, (4) the electron transfer complexes and juncture protein are the only components of the membrane that interact directly with phospholipid, and (5) protein–phospholipid interactions are considered hydrophobic (although the phospholipid is pictured as a continuous inverted bilayer, an arrangement in which phospholipids occur in discrete pockets could equally well be accommodated by the model).

Some of the evidence for this model (namely, localization of F_1 and OSCP) has already been discussed above. Several features, however, deserve

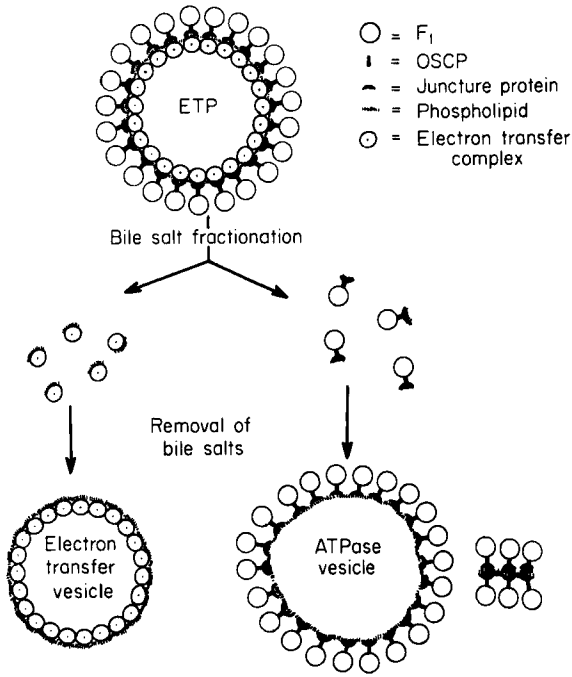

FIG. 12. Model of the structure of the ATPase complex.

further comment. The molecular weight of juncture protein is too low to make it a candidate for a complete basepiece. All the evidence at present points to the identification of the basepiece with the electron transfer components (Green and Tzagoloff, 1966a). It cannot be excluded, however, that juncture protein is an intrinsic component of the basepiece and is directly linked to the electron transfer complexes through protein–protein interactions. This possibility has been tentatively rejected because of the ready separation of the oligomycin-sensitive ATPase from the electron transfer complexes and the total absence of juncture protein activity in two of the electron transfer complexes that have been examined. Neither cytochrome oxidase nor reduced coenzyme Q–cytochrome c reductase binds F_1 or reconstitutes oligomycin-sensitive ATPase in the presence of OSCP (Tzagoloff, unpublished observations).

The interactions between phospholipid and the electron transfer complexes are predominantly hydrophobic (Green and Fleischer, 1964; Green and Tzagoloff, 1966b). This also appears to be the case with the ATPase complex, since salt fails to dissociate the phospholipids from the complex.

For this reason, a hydrophobic association of the juncture protein and phospholipids has been indicated.

In addition to explaining the membrane-forming capacity of both the electron transfer complexes and of the ATPase complex, the model also accounts for the appearance in preparations of the ATPase of short segments of membranes with projecting headpieces equally distributed on both sides of the membrane (Kopaczyk *et al.*, 1968) (see Fig. 12). This symmetrical arrangement is not seen in enclosed vesicles because of the negative staining technique which permits only the visualization of the structure on the external surface of the membrane.

V. BIOSYNTHESIS OF THE ATPase COMPLEX

Very little is known at present about the synthesis of mitochondrial enzymes. The questions that are of particular interest from the standpoint of the broader problem of mitochondrial biogenesis concern the site of biosynthesis of the subunit proteins of the mitochondrion, their assembly into functional enzymes and, finally, the mechanism of assembly of the membrane itself. It is apparent from the foregoing discussion of the structure of the ATPase complex that this mitochondrial enzyme offers a number of advantages for probing these questions. The ATPase is a quantitatively important component of the mitochondrial membrane. The protein components of the complex have been fairly well characterized and techniques are available for their quantitation either by enzymic procedures or by polyacrylamide gel electrophoresis. Furthermore, the membrane-forming capacity of the ATPase suggests that an understanding of the biogenesis of this enzyme will have direct relevance to the mechanism of assembly of the membrane itself.

A. Experimental System

The yeast cell has been a favorite experimental tool for studying the biogenesis of the mitochondrion. The reason for this stems from the fact that relatively simple manipulations of the growth conditions of the cell have a profound effect on the morphology and enzymic activities of the mitochondrial particles. Ephrussi (1950) and Slonimski (1953) showed that yeast cells grown under low oxygen tension are deficient in both the oxidative and phosphorylative activities normally associated with mitochondria. Recently, it was reported that the anaerobically grown cells contain mitochondrial particles (promitochondria) which lack the enzymes

of the electron transfer chain (Criddle and Schatz, 1969; Plattner and Schatz, 1969). These enzymes are rapidly synthesized and incorporated into the promitochondria when the cells are provided with oxygen (Schatz, 1970).

The synthesis of mitochondrial respiratory enzymes can also be repressed or induced by controlling the amount of fermentable substrate such as glucose in the medium (Utter *et al.*, 1967; Jayaraman *et al.*, 1966; Polakis *et al.*, 1965). In analogy to the anaerobic yeast, mitochondria-like organelles deficient in respiratory components are also present in glucose-repressed yeast (Jayaraman *et al.*, 1966). The synthesis of mitochondrial enzymes in glucose-repressed yeast is induced when the level of glucose in the medium is less than 0.05% (Utter *et al.*, 1967). In *Saccharomyces cerevisiae* derepression occurs within a period of 6–7 hours (Utter *et al.*, 1967).

In the studies reported here, glucose rather than anaerobiosis was used to repress yeast because of certain experimental advantages. In most experiments cells were grown for 16–17 hours in a yeast extract medium containing 5.4% glucose. At the time of harvest, the cells were completely repressed as evidenced by the low concentrations of cytochromes and respiratory activities of the mitochondrial particles. The repressed cells were washed and reinoculated into fresh medium for derepression. The derepression medium contained 0.8% glucose. The high initial concentration of cells inoculated into the derepression medium insured a rapid exhaustion of the glucose (15 minutes after inoculation the concentration of glucose was less than 0.1%), thus allowing the cells to derepress almost from the time of the inoculation. Usually, the cells were fully derepressed after 6 hours of incubation in the second medium. During this time the density of the culture increased only by 20–30%, indicating an almost

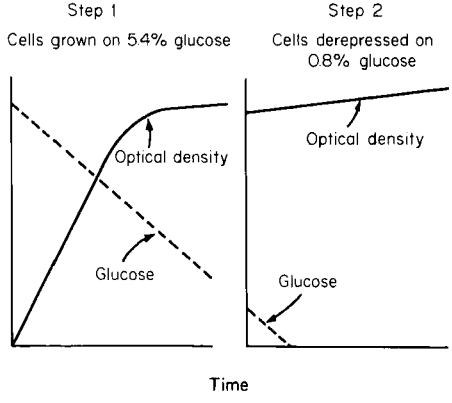

FIG. 13. Experimental conditions for repression and derepression of yeast.

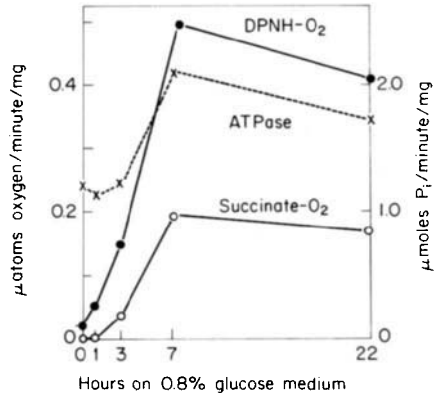

FIG. 14. Emergence of electron transfer and ATPase activity in yeast mitochondria during derepression. For experimental details see Tzagoloff (1969b).

static population of cells (Tzagoloff, 1969b). The basic experimental system is illustrated in Fig. 13.

It was essential to establish first whether or not high concentrations of glucose repress the synthesis of the mitochondrial ATPase. In Fig. 14 are shown the results of an experiment in which electron transfer activities and the ATPase activity of the mitochondrial particles were monitored during derepression. Both electron transfer and ATPase activities of the isolated particles can be seen to increase during derepression, reaching maximal values after 7 hours.

That the increase in the mitochondrial ATPase was attributable to the synthesis of new enzyme rather than an activation of preexisting enzyme was shown in several ways. First, submitochondrial particles prepared from the repressed cells revealed very few headpieces. Membranes from derepressed cells had a normal complement of headpieces (see Fig. 15). Second, electrophoresis of the membranes on polyacrylamide gel under dissociating conditions indicated that the membranes from the repressed cells were deficient in the major protein components of F_1 (see Fig. 16). The intensity of this band increased over the course of derepression (Tzagoloff, 1969b).

B. Site of Synthesis of the Protein Components

Most proteins of the cell are synthesized by the cytoplasmic–ribosomal protein-synthesizing system. In recent years it has been found that, in addition to the cytoplasmic system, certain cellular organelles such as chloroplasts and mitochondria possess their own apparatus for protein

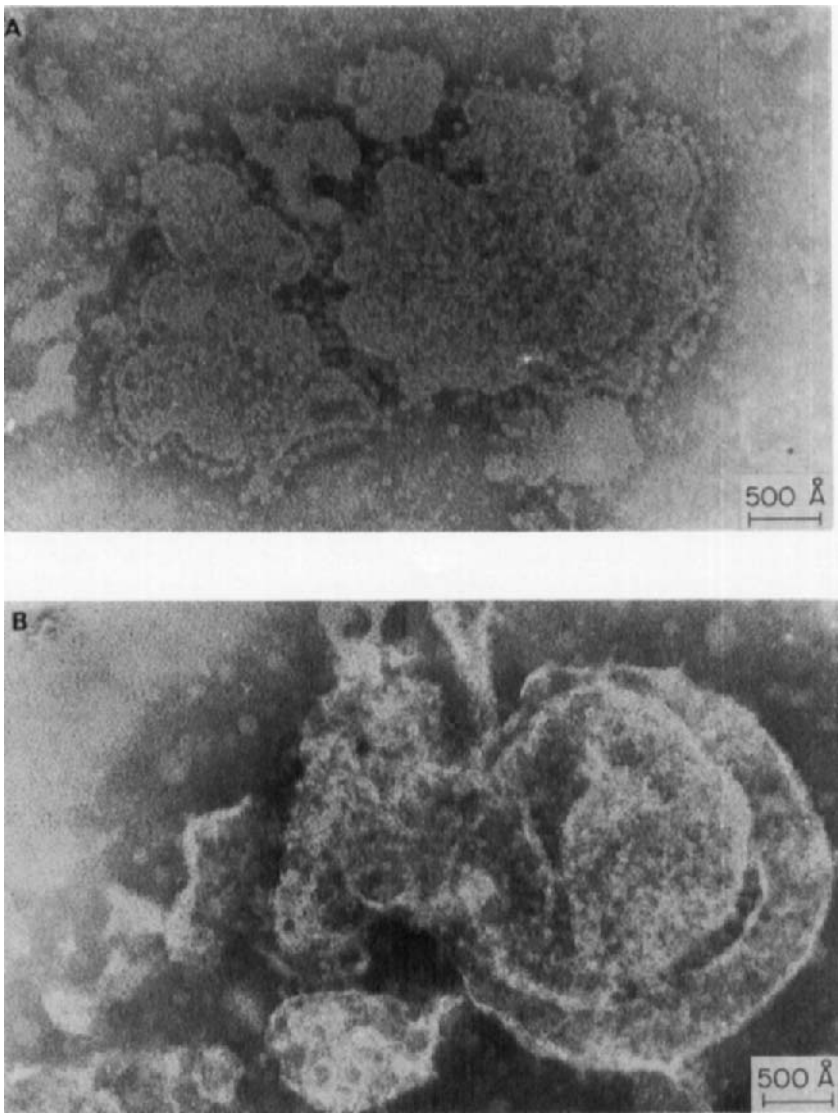

FIG. 15. Electron micrographs of submitochondrial particles from repressed and derepressed yeast. (A) Membranes from derepressed yeast. (B) Membranes from repressed yeast. The samples were negatively stained with phosphotungstate.

synthesis. *In vivo* and *in vitro* studies have shown mitochondria to be capable of incorporating amino acids into proteins by a system distinct from the classic cytoplasmic system (McLean *et al.*, 1958; Roodyn *et al.*,

1961; Beattie *et al.*, 1966). Inhibitors of protein synthesis have provided a simple tool for distinguishing cytoplasmic and mitochondrial protein synthesis. In yeast as well as other eukaryotic cells, cycloheximide has been shown to inhibit only cytoplasmic protein synthesis, while chloramphenicol inhibits only mitochondrial protein synthesis (Wintersberger, 1965; So and Davie, 1963; Lamb *et al.*, 1968).

It is now generally recognized that the elaboration of functional mitochondria involves the participation of both cytoplasmic and mitochondrial protein synthesis. This view is supported by evidence from *in vivo* studies on the kinetics of amino acid incorporation into mitochondrial proteins (Beattie *et al.*, 1966; Work, 1968), the effect of inhibitors of protein synthesis on the enzymic composition of mitochondria (Clark-Walker and

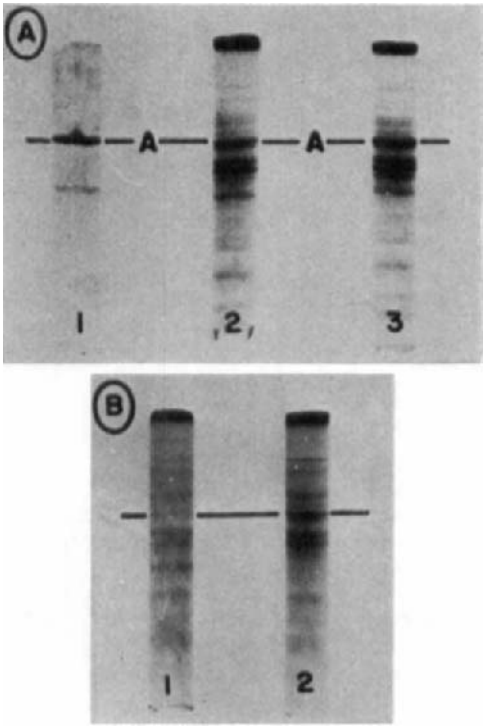

FIG. 16. Polyacrylamide gels of yeast F_1 and yeast submitochondrial particles. The conditions of Takayama *et al.* (1966) were used for the depolymerization and electrophoretic separation. (A) Gel 1, F_1; gel 2, derepressed mitochondria; gel 3, F_1 plus derepressed mitochondria. (B) Gel 1, repressed mitochondria; gel 2, derepressed mitochondria. The line marks the position of the major protein subunits of F_1. For further experimental details see Tzagoloff (1969b).

Linnane, 1967), and work performed on respiratory mutants of yeast (Schatz, 1968; Kováč and Weissová, 1968). At present, our knowledge of the site of synthesis of the various mitochondrial proteins is still fragmentary. While it is certain that some soluble components such as malic dehydrogenase (Clark-Walker and Linnane, 1967) and cytochrome c (Kadenbach, 1967; Sherman *et al.*, 1966) are products of the cytoplasmic system, virtually nothing is known about the identity of the proteins synthesized by the mitochondrion. From *in vitro* studies with isolated mitochondria it has been established that amino acids are incorporated predominantly into the least soluble proteins of the inner membrane (Beattie *et al.*, 1970; Work, 1968). These observation have led to the speculation that the mitochondrion synthesizes the constitutive enzymes of the inner membrane.

1. Synthesis of F_1

The biosynthesis of the ATPase complex has been studied in glucose-repressed yeast during adaptation on low glucose (Tzagoloff, 1969b). The aim of these studies was to determine whether the ATPase is a product

TABLE XII

Effect of Chloramphenicol and Cycloheximide on Mitochondrial ATPase Activity During Derepression[a]

Hours of deprepression	Inhibitor	Concentration	Specific activity (μmoles/minute/mg)	
			Minus rutamycin	Plus rutamycin
Experiment 1				
0	None	—	0.97	0.19
6	None	—	2.10	0.36
6	Chloramphenicol	0.1 mg/ml	2.20	0.40
6	Chloramphenicol	0.5 mg/ml	1.85	0.38
6	Chloramphenicol	2.0 mg/ml	1.30	0.50
Experiment 2				
0	None	—	1.25	0.25
6	None	—	2.20	0.44
6	Cycloheximide	10^{-7} *M*	1.80	0.50
6	Cycloheximide	10^{-6} *M*	1.35	0.48
6	Cycloheximide	10^{-5} *M*	1.30	0.55

[a] Mitochondrial particles were isolated from yeast incubated under the conditions indicated above and assayed for ATPase activity. For further experimental details see Tzagoloff (1969b).

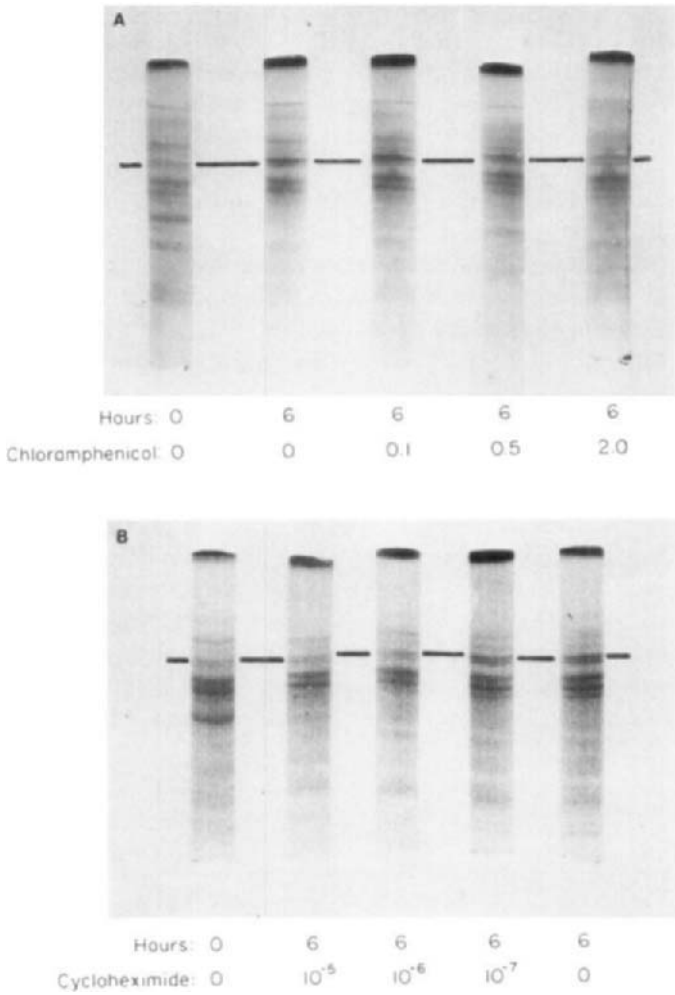

FIG. 17. Polyacrylamide gels of mitochondria isolated from yeast derepressed in the presence of chloramphenicol and cycloheximide. The mitochondrial samples were obtained from the experiments described in Table XII. The numbers under the gels refer to the concentrations of inhibitors and hours of incubation in the derepression medium. Each gel represents 160 μg of mitochondrial protein. For further experimental details see Tzagoloff (1969b).

of the cytoplasmic or mitochondrial protein-synthesizing system. In Table XII are shown data on the ATPase activity of mitochondrial particles isolated from yeast incubated under derepressive conditions without inhibitors and with different concentrations of chloramphenicol or cyclo-

heximide. These results indicate that the increase of ATPase activity in mitochondrial particles during derepression (see Fig. 14) is arrested by both inhibitors. A lower content of ATPase in the isolated membranes was also seen by electrophoretic analysis of the membranes on polyacrylamide gels under dissociating conditions. The gel profiles shown in Fig. 17 disclose that the major protein subunits of F_1 are diminished in intensity in the membranes of cells exposed to the highest concentrations of either inhibitor.

Further experimentation with this system showed that although appearance of membrane-bound ATPase was inhibited by chloramphenicol, F_1 was still synthesized in the presence of this inhibitor (Tzagoloff, 1969b). In Table XIII are shown data on the distribution of mitochondrial ATPase activity in the mitochondrial and the postribosomal fractions of the cell. To distinguish mitochondrial ATPase from other cellular ATPases, only that part of the supernatant activity inhibited by Dio-9, a specific inhibitor of F_1 (Guilliory, 1964), is shown. It is evident form the results of this experiment that most of the ATPase activity of cells incubated in a derepression medium containing chloramphenicol is recovered in the postribosomal supernatant. The identity of the supernatant ATPase with F_1 was established from its cold lability (see Fig. 18) and its inhibition by mitochondrial ATPase inhibitor Dio-9 (Pullman and Monroy, 1963), and by antibody prepared against purified yeast F_1 (Tzagoloff, 1969b). The supernatant ATPase was not inhibited by rutamycin.

TABLE XIII

DISTRIBUTION OF ATPASE ACTIVITY IN YEAST DEREPRESSED IN THE PRESENCE OF CHLORAMPHENICOL[a]

		ATPase activity			
		Mitochondria		Supernatant	
Hours of derepression	Concentration of chloramphenicol	Specific activity	Total units	Specific activity	Total units
0	0	0.80	11.8	0.025	4.3
6	0	1.75	31.0	0.052	11.7
6	0.1	1.75	29.0	0.032	7.4
6	0.5	1.45	21.5	0.08	18.5
6	2.0	0.86	9.7	0.140	31.5

[a] Yeast incubated under the conditions indicated above were fractionated to obtain mitochondria and postribosomal supernatant. The ATPase activity of the two cell fractions are reported as specific activity and total units recovered in the fraction. For further details see Tzagoloff (1969b).

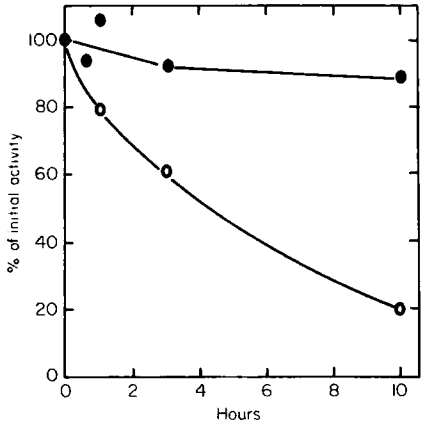

FIG. 18. Cold lability of the ATPase in the postribosomal supernatant. Repressed yeast were incubated for 6 hours in a derepression medium supplemented with 2 mg chloramphenicol per milliliter. The postribosomal supernatant was assayed for ATPase activity after incubation at 23° (●——●) and and 0° (O——O) for the indicated lengths of time.

The accumulation of F_1 in the postribosomal supernatant was not observed when derepression was carried out in the presence of cycloheximide instead of chloramphenicol. In this case both the ATPase units of the supernatant and of the supernatant plus mitochondria decreased as a function of the cycloheximide concentration in the medium (see Table XIV).

On the basis of the known site of action of the two inhibitors, these results strongly imply that the F_1 component of the ATPase complex is synthesized by the cytoplasmic system. This conclusion is corroborated by previous reports that mitochondrial particles of *petite* mutants of yeast, which are defective in mitochondrial protein synthesis, contain high levels of F_1 (Schatz, 1968; Kováč and Weissová, 1968).

2. Site of Synthesis of OSCP

Essentially the same experimental tactic was used to probe the locus of synthesis of this component. Since OSCP is a relatively minor component of the mitochondrial inner membrane, it has not been feasible to detect it by gel electrophoresis of whole dissociated membranes. It was pointed out earlier that binding of F_1 to membranes extracted with sodium bromide and ammonium hydroxide is absolutely dependent on exogenously added OSCP (see Fig. 10). The stimulation of F_1 binding to the extracted membranes of normal cells [YETP(NaBr,NH_4OH)] was used for the assay of OSCP (Tzagoloff, 1970).

TABLE XIV

DISTRIBUTION OF ATPASE ACTIVITY IN YEAST DEREPRESSED IN THE PRESENCE OF CYCLOHEXIMIDE[a]

Hours of derepression	Concentration of cycloheximide	ATPase activity			
		Mitochondria		Supernatant	
		Specific activity	Total units	Specific activity	Total units
0	0	0.97	9.2	0.015	3.1
6	0	1.60	28.0	0.019	5.9
6	10^{-7} *M*	1.65	22.0	0.000	0.0
6	10^{-6} *M*	1.20	12.0	0.011	2.0
6	10^{-5} *M*	1.1	10.0	0.006	1.1

[a] The details of this experiment are the same as those described in the footnote to Table XIII.

The presence of OSCP in the postribosomal supernatant of cells incubated in a derepression medium containing chloramphenicol was established from the following observations. First, the F_1 activity of the supernatant became bound to YETP(NaBr,NH_4OH) without added OSCP (see Fig. 19). The particle-bound ATPase activity was rutamycin-sensitive, indicating a true reconstitution of the ATPase. Second when the postribosomal supernatant was fractionated by a procedure used to purify OSCP from yeast mitochondria, a protein was obtained that was active in stimulating binding of F_1 to the extracted particles (Tzagoloff, 1970).

In Table XV are summarized the results of an experiment in which the OSCP activities of postribosomal supernatants of cells exposed either to cycloheximide or chloramphenicol were compared. The inhibition of OSCP synthesis by cycloheximide is seen from the inability of that supernatant to stimulate F_1 binding to the extracted membranes. These data constitute evidence that OSCP, similar to F_1, is a product of the cytoplasmic-ribosomal protein-synthesizing system of yeast.

In view of these findings, it was of interest to determine whether F_1 and OSCP were present in the chloramphenicol supernatant as a complex or as separate entities. Sedimentation of the supernatant through a glycerol gradient caused the two components to separate, indicating that they are not part of a complex (Tzagoloff, 1970). Nevertheless, F_1 and OSCP appeared to be present in equivalent amounts. Thus all the F_1 activity of the supernatant could be bound to YETP(NaBr,NH_4OH) with-

out an external supply of OSCP. Conversely, the OSCP concentration was sufficient to bind only that amount of F_1 already present in the postribosomal supernatant.

3. Synthesis of Juncture Protein

The lack of a good assay for juncture protein has been a major obstacle in studying the synthesis of this component. The fact that F_1 and OSCP synthesized in the presence of chloramphenicol are not assembled into a functional ATPase complex suggests that juncture protein is not synthesized under these conditions. Although this would in turn imply juncture protein to be a product of the mitochondrial protein-synthesizing system, the possibility that the postribosomal supernatant of chloramphenicol cells contains a soluble form of this protein cannot be excluded. Thus chloramphenicol could inhibit the synthesis of some other mitochondrial component essential for the incorporation of the ATPase complex into the membrane. Still another possibility is that chloramphenicol, in some fashion unrelated to its protein synthesis inhibitory activity, prevents the assembly of the ATPase components into a rutamycin-sensitive complex. The fact that it has not been possible to reconstitute a rutamycin-sensitive complex from the protein components present in the chloramphenicol supernatant alone is also inconclusive. Juncture protein has

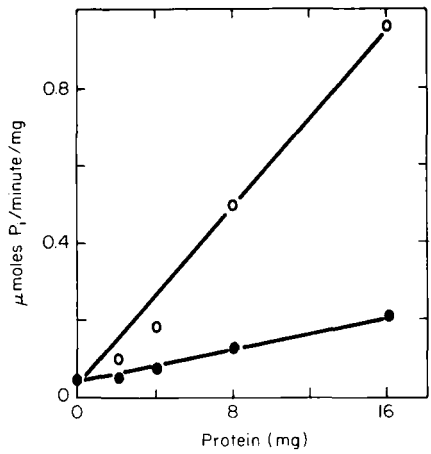

Fig. 19. Reconstitution of ATPase from YETP(NaBr,NH_4OH) and postribosomal supernatant. YETP(NaBr,NH_4OH) (1 mg) was incubated with the indicated amounts of postribosomal supernatant of yeast derepressed in the presence of chloramphenicol. The particles were reisolated by centrifugation and tested for ATPase in the absence (O——O) and presence (●——●) of rutamycin.

TABLE XV

EFFECT OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON SYNTHESIS OF OSCP DURING DEREPRESSION OF YEAST[a]

Hours of derepression	Inhibitor	Concentration	ATPase activity of reconstituted particles (μmoles/minute/mg)	
			Minus F_1	Plus F_1
0	None	—	0.066	0.135
6	None	—	0.175	0.310
6	Chloramphenicol	2 mg/ml	0.610	0.620
6	Cycloheximide	10^{-5} *M*	0.055	0.088

[a] The postribosomal supernatants of yeast derepressed under the conditions indicated above were tested for their ability to reconstitute ATPase in YETP(NaBr,NH_4OH). To one set of reconstitution mixtures, exogenous F_1 was also added. For further experimental details see Tzagoloff (1970).

not been isolated in soluble form, and the optimal conditions for reconstitution of rutamycin-sensitive ATPase from soluble components are consequently not known.

If these difficulties are taken into account, some information may still be gained from studies on the appearance of juncture protein in a membrane-bound form. The level of juncture protein in mitochondrial membranes may be estimated from reconstitution of rutamycin-sensitive ATPase in a system consisting of submitchondrial particles extracted with sodium bromide and ammonium hydroxide, OSCP, and F_1. If such a reconstitution is carried out in the presence of excess F_1 and OSCP, the specific activity of the ATPase in the reconstituted membranes should serve as a measure of the juncture protein content in the membranes. The results shown in Fig. 20 disclose that the increase of ATPase activity in mitochondrial particles during derepression is paralleled by an increase in the level of juncture protein as assayed by the above procedure. Mitochondrial membranes obtained from cells derepressed for 7 hours were 2.5 times more active in reconstituting rutamycin-sensitive ATPase than membranes obtained from fully repressed cells. The specific activity of the ATPase in unextracted membranes increased by approximately the same factor after 7 hours of derepression.

In order to determine whether or not inhibitors of protein synthesis affect the levels of membrane-bound juncture protein, glucose-repressed yeast were incubated in a derepression medium containing either chloram-

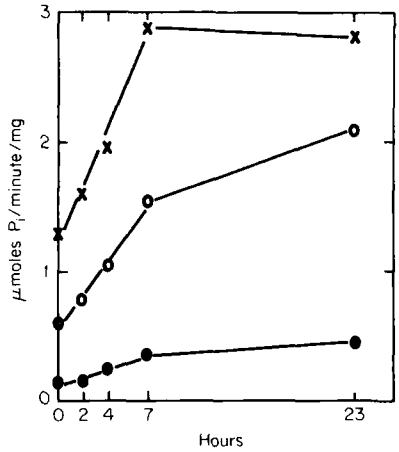

FIG. 20. Reconstitution of rutamycin-sensitive ATPase in submitochondrial particles of yeast derepressed on 0.8% glucose. Yeast were grown on 5.4% glucose medium. The repressed cells were transferred to 0.8% glucose medium and samples were taken at 0, 2, 4, 7, and 23 hours. Mitochondrial particles were isolated and assayed for ATPase (X——X). For reconstitution of ATPase, the mitochondrial particles were extracted with 3 *M* sodium bromide followed by 0.33 *M* ammonium hydroxide. The extracted particles were mixed with 100 μg of F_1 and 50 μg of OSCP. The particles were re-isolated from the reconstitution mixture by centrifugation and tested for ATPase activity in the absence (O——O) and presence (●——●) of rutamycin.

phenicol or cycloheximide. Mitochondrial particles were isolated and assayed for juncture protein. The results shown in Table XVI indicate that both reagents when used singly inhibit the increase in the level of juncture protein. The same experiment also shows a 2- to 3-fold enhancement of juncture protein activity in membranes isolated from cells that had been incubated first in the presence of chloramphenicol and then transferred to a medium containing cycloheximide. Although in this experiment the incubation in cycloheximide was 17 hours, similar results were obtained when the duration of the second incubation was only 6 hours. Exposure of yeast to the two inhibitors in reverse order also caused some increase in membrane-bound juncture protein, but the effect was not nearly as dramatic. Since the percent of protein extracted from the membranes with sodium bromide and ammonium hydroxide was approximately the same regardless of the prior history of the yeast from which they were isolated, it is unlikely that these differences are attributable to artifacts of the extraction procedure.

The increased levels of juncture protein in mitochondrial particles of cells incubated in cycloheximide suggest that in contrast to OSCP and

TABLE XVI

JUNCTURE PROTEIN ACTIVITY OF MITOCHONDRIAL PARTICLES OF YEAST EXPOSED TO CHLORAMPHENICOL AND CYCLOHEXIMIDE[a]

First incubation	Second incubation	Reconstituted ATPase (μmoles/minute/mg)	
		Minus rutamycin	Plus rutamycin
None	None	0.67	0.22
6 hours on CAP	None	0.43	0.20
6 hours on CH	None	0.75	0.30
6 hours on CAP	17 hours on CH	1.75	0.35
6 hours on CH	17 hours on CAP	1.05	0.20

[a] Mitochondrial particles were prepared from repressed yeast (line 1) and repressed yeast further incubated in 0.8% glucose medium containing either 2 mg/ml of chloramphenicol or 10^{-5} *M* cycloheximide as indicated above. The mitochondrial particles were extracted with sodium bromide and ammonium hydroxide and tested for reconstitution of ATPase with F_1 and OSCP as described in the legend to Fig. 20.

F_1, juncture protein is synthesized by the mitochondrial system. The fact that this synthesis occurs only after incubation in chloramphenicol may indicate that products of the cytoplasmic system exert a control on mitochondrial protein synthesis.

There is mounting evidence that a number of mitochondrial enzymes are formed through the intervention of two separate protein-synthesizing systems. Rouslin and Schatz (1969) have concluded on the basis of experiments in which yeast were sequentially incubated in cycloheximide and chloramphenicol that both the mitochondrial and cytoplasmic systems are required for the assembly of respiratory enzymes during adaptation to aerobic metabolism. There is evidence that the two systems are involved in the synthesis of cytochrome oxidase (Chen and Charalampous, 1969).

Our knowledge of the *in vivo* assembly of membrane enzymes is still very superficial. The scheme shown in Fig. 21 for the assembly of the mitochondrial ATPase tries to take into account some of the experimental findings discussed in this chapter. Both OSCP and F_1 are synthesized extramitochondrially and exert a positive control on the synthesis of juncture protein visualized to occur in the mitochondrion. Juncture protein, which may be synthesized directly on the membrane, might then serve as the nucleus for the further assembly of the complex. The specificity of the *in vitro* reconstitution of oligomycin-sensitive ATPase complex

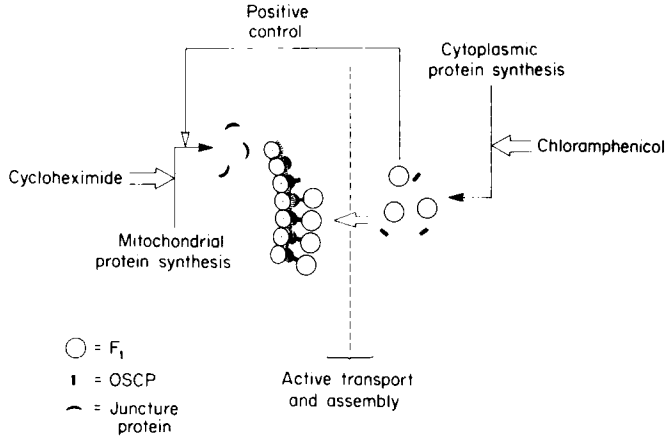

FIG. 21. Assembly of the mitochondrial ATPase complex.

suggests that this could be a self-assembling step. Cytoplasmic F_1 and OSCP are postulated to be transported into the mitochondrion by an energy-dependent process similar to that reported for the transfer of cytochrome c from microsomes to mitochondria (Kadenbach, 1968). Whatever the merits of this model, it is evident that juncture protein is the key to understanding the biosynthesis of the ATPase complex. Further progress in this area will undoubtedly depend on better understanding the chemistry and role of this component.

ACKNOWLEDGMENTS

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Mitochondrial Compartments: A Comparison of Two Models

HENRY TEDESCHI

Department of Biological Sciences,
State University of New York at Albany,
Albany, New York

I. MITOCHONDRIAL SPACES AVAILABLE TO SOLUTES: A STATEMENT OF THE PROBLEM

Observations on the permeability of isolated mitochondria to solutes of relatively low molecular weight such as sucrose have led in recent years to the formulation of two models. Both models can account for at least some of the data. In the two-space model, each mitochondrion is assumed to contain a space permeable to sucrose (or other low-molecular-weight components such as mannitol) and another space relatively impermeable to sucrose. In the one-space model, each mitochondrion is assumed to correspond to a single solute-permeable space. The mitochondria differ in penetrability because of differences in the surface areas exposed to penetration, a consequence of the size distribution of the mitochondrial population.

The two-space hypothesis is most widely accepted at this time. It should be noted, however, that neither osmotic behavior nor the permeability of the mitochondrial spaces are well understood and neither model is capable of explaining all data without further possibly questionable assumptions. This failure suggests the need for considerable revision of our conceptual framework or a more rigorous evaluation of experimental procedures.

The adoption of either model has far-reaching implications for any subsequent analysis of data related to mitochondrial properties and behavior. For example, if we were concerned with the estimation of the concentration of internal ionic components of mitochondria, we would have to decide whether it would be more appropriate to use the total internal mitochondrial volume (as required by the one-space model) or only the sucrose-impermeable portion (as required by the two-space model). In most studies the two volumes differ by a factor of 2 to 5. Estimates of internal concentrations are fundamental to calculations of the energy requirements of transport. For transport against an electrochemical gradient, the steeper the gradient the higher the necessary expenditure. In addition, knowledge of whether or not mitochondria are in osmotic equilibrium with their environment depends on knowing the internal solute concentration. Since the permeability of mitochondria to water is high (Tedeschi and Harris, 1955, 1958; Bentzel *et al.*, 1966), the absence of an osmotic equilibrium would mean the existence of special mechanisms that maintain mitochondrial volume against an osmotic pressure gradient.

Werkheiser and Bartley (1957) were the first to propose the concept of two mitochondrial spaces, one very permeable to sucrose (and other low-molecular-weight solutes), the other very impermeable to sucrose. This concept is supported by the observations that (1) part of the mitochondrial space is rapidly penetrated by sucrose, whereas the rest of the space is penetrated slowly (e.g., Jackson and Pace, 1956; Werkheiser and Bartley, 1957; Tedeschi, 1965), and (2) part of the mitochondrial volume, as measured from a centrifugal pellet, does not seem to respond osmotically and, in some experiments, corresponds to the sucrose-^{14}C-permeable space, at least in part (e.g., Malamed and Recknagel, 1959; Tarr and Gamble, 1966; Bentzel and Solomon, 1967; Harris and Van Dam, 1968). The two-space model is attractive since the sucrose-permeable space could correspond morphologically to the space between inner and outer membranes observed in some electron micrographs of isolated mitochondria (e.g., Hackenbrock, 1966).

Superficially, these observations could have a trivial interpretation. Damage in mitochondria could account for a more rapidly permeable space (see also Werkheiser and Bartley, 1957). With the aid of sucrose density gradient techniques, however, it is possible to differentiate between

intact and damaged mitochondria in the same preparation, since the two fractions by responding differently to changes in osmotic pressure should have different densities. The distribution of mitochondria does not appear bimodal, however; this argues against the presence of damaged mitochondria (Beaufay and Berthet, 1963). It should be noted, however, that it has been possible to obtain a bimodal distribution by zonal centrifugation in a Ficoll gradient (Wong *et al.*, 1970). Similar findings stem from experiments involving differential centrifugation (Amoore and Bartley, 1958). The possibility of two significantly different populations being present in some preparations cannot be disregarded at this time.

There are several indications that the observations on mitochondrial spaces are not amenable to simplistic explanations. Although data from different laboratories appear to be in agreement and to support the two-space model, the size of the sucrose-permeable space proves to be extremely variable when measured on the same material, with similar procedures, and sometimes in the same laboratory. This is shown in Table I. In addition, although measurements of the sucrose-permeable space have generally led to an interpretation based on a two-space model, experimental details and specific results differ fundamentally. Consequently, the model can be

TABLE I

MEASUREMENTS OF THE SUCROSE-PERMEABLE SPACE AS REPORTED BY SEVERAL INVESTIGATORS[a]

Reference	Concentration of major component of medium	Water volume (%)
Werkheiser and Bartley (1957)	0.25 *M* Sucrose	60
Amoore and Bartley (1958)	0.25 *M* Sucrose	34–75
Harris and Van Dam (1968)	0.28 Osmolal mixture	80
Tarr and Gamble (1966)	0.30 *M* Sucrose	80–82
Gamble and Garlid (1970)	0.25 *M* Sucrose	64
Malamed and Recknagel (1959)	0.30 *M* Sucrose	43–74
*Blondin and Green (1969)	0.272 Molal sucrose	69
*O'Brien and Brierley (1965)	0.33 *M* Sucrose	34
Share (1960)	0.3 *M* Sucrose	44
Klingenberg and Pfaff (1966)	0.3 *M* Sucrose	70
Pfaff (1967)	0.25 *M* Sucrose	33

[a] Results were selected from experiments thought to be comparable with respect to medium and source of mitochondria. The results are presented without corrections. The experiments were carried out on rat liver mitochondria except for those marked with an asterisk.

TABLE II

CHANGES IN THE INTERNAL SPACES OF MITOCHONDRIA WITH DECREASING EXTERNAL OSMOTIC PRESSURE[a]

Reference	Sucrose-permeable space	Sucrose-impermeable space	Total volume
Malamed and Recknagel (1959)	↑	↑	↑
Bartley (1961)	↑ or 0	↑ or 0	↑
Bentzel and Solomon (1967)	↓	↑	↑
Klingenberg and Pfaff (1966)	↑	↑	↑
Pfaff (1967)	↑	↑	↑
Harris and Van Dam (1968)	↓	↑	↓
Tarr and Gamble (1966)	0 (Slight)	↑	↑
Hunter and Brierley (1969)	0 (Slight)	↑	↑

[a] Upward arrows indicate increases, downward arrows indicate decreases, 0 indicates no significant change.

tailored to fit the data only after extensive additions or provisos. The discrepancies between the findings of the different studies are summarized in Table II. For these and other reasons, discussed in Section II, the question naturally arises as to whether or not either model can be supported by other data and whether or not it is possible to explain fundamental aspects of mitochondrial behavior on the basis of the two-space or the one-space model.

The one-space model, the only alternative to the two-space model presented so far, can also explain the rapid penetration of part of the mitochondrial space and its apparent nonosmotic behavior. The model assumes a partially penetrated single compartment for each mitochondrion, but a distribution of sizes in the population of mitochondria (hence different surface areas are exposed to the solute and the amount of sucrose that enters varies). That mitochondria differ in size and constitute a varied population has been shown by many independent studies (see Section II,A). The kinetics of the penetration of sucrose-^{14}C (Tedeschi, 1965) support the one-space model. In addition to these data, the evidence in the literature can be subjected to further analysis.

As discussed in Section II,D, the one-space model generally leads to much the same predictions as the two-space model. In many experimental situations, for example, there is an apparent sucrose-penetrated space which behaves as if it were not osmotically responsive. The predictions of the two models, however, differ under certain experimental conditions.

For this reason, it may prove fruitful to examine the presently available evidence on the basis of the two models. A summary of the evaluation that follows is shown in Table III. It should be noted that neither model explains all results satisfactorily, but that the two-space hypothesis has some serious difficulties.

An evaluation of the literature is particularly necessary since most articles published in this area have made little or no attempt to analyze and correlate in detail the results from other studies. For example, the classic work of Jackson and Pace (1956) has remained largely ignored. Unfortunately, the evaluation is complicated by the fact that the experiments are not always comparable. Frequently, only a few parameters have been followed and the experimental details are not fully reported. Surprisingly, in many studies neither the number of experiments nor the number of estimates for each experiment have been reported. This together with the failure to report statistical parameters of variability, such as standard deviation or standard error, make the evaluation and weighing of conflicting data a very difficult task. In addition, experimental conditions differ significantly among studies. Moreover, procedural differences are often sufficiently large to account for real differences in the proportion of damaged mitochondria, their size distribution, or their permeability properties. Consequently, it may not be possible to evaluate all reports consistently.

TABLE III

A Comparison of the Two Models in Relation to Their Predictive Value[a]

Type of observation or criterion	Two-space model	One-space model with varying mitochondrial sizes
Osmotic equilibrium	−	+[b]
Kinetics of sucrose penetration	0 or −	+
Osmotic behavior	0 or −	0 or +
Energy cost of transport	−	+
Variations in penetrable space	− or 0	+
Shifts in sucrose when mitochondria are transferred to hypotonic solutions	+	− or 0

[a] +, Indicates that the observations are compatible with the model; −, indicates that the observations are not compatible with the model; 0, indicates that the results are inconclusive or incomplete.

[b] With small discrepancies.

II. OSMOTIC BEHAVIOR AND SOLUTE SPACE

The discussion that follows reviews the kinetics of sucrose penetration and is followed by a discussion of the distribution of solutes and water in mitochondria.

A. Kinetics of Sucrose Penetration

The initial penetration of sucrose into suspended mitochondria is very rapid. Thereafter, the rate of entry is reduced considerably (e.g., Jackson and Pace, 1956; Amoore and Bartley, 1958; Tedeschi, 1965; Bentzel and Solomon, 1967). Similar experiments have been reported for mannitol in the case of beef heart mitochondria (Hunter and Brierley, 1969; Hunter *et al.*, 1969). These results have been interpreted by a number of investigators as evidence for the presence of a sucrose-permeable compartment (which equilibrates rapidly) and a sucrose-impermeable compartment (which equilibrates slowly). In at least some experiments, however, both the rapid and slow phases are quantitatively predictable on the basis of the one-space model (Tedeschi, 1965). The larger mitochondria in the suspension take a longer time to equilibrate by virtue of their lower area/volume ratios (Tedeschi, 1965). It should also be pointed out that in most studies the space available to sucrose or other low-molecular-weight solutes at equilibrium has not been determined. Water inaccessible to solute penetration has been found in other systems such as erythrocyte suspensions or hemoglobin solutions (Bobo, 1967). In the experiments of Bentzel and Solomon (1967), the sucrose-space was found to be about 70% of the total (in mitochondria suspended in 272 milliosmolal sucrose). Half of the remaining 30% is thought to correspond to water unavailable for osmotic volume changes. A similar figure for the portion of the mitochondrial space not penetrated by sucrose after long incubations comes from the reports of Amoore and Bartley (1958, see Fig. 1) and Jackson and Pace (1956). Therefore it might be argued that some preparations exhibit little penetration of sucrose with time because they are close to equilibrium and not because there is a truly separate sucrose-impermeable space. This argument is strengthened by the possibility that part of the water may be water of hydration, which would not be available to penetration. As a result, the system may be closer to equilibrium than suspected. Because a system such as this approaches equilibrium asymptotically, the rate of penetration would be expected to be very low. The rate of penetration would be slowed down further by the fact that the space remaining still unpenetrated corresponds to the larger mitochondria. In experiments in which the size distri-

bution of mitochondria was determined and the space available to the solute estimated, at least approximately (from the glycerol-^{14}C penetration), the kinetics are those predicted from a single space per mitochondrion. A number of studies, utilizing different techniques, have demonstrated a wide distribution of mitochondrial sizes that seem to determine these kinetics (deDuve *et al.*, 1955; Kuff *et al.*, 1956; Pauly *et al.*, 1960; Tedeschi, 1965; Baudhuin and Berthet, 1967). A similar explanation has been invoked by Jackson and Pace, who regard the apparent differences in the permeability of the mitochondrial preparation to be attributable to the heterogeneity of the mitochondrial population. The kinetics of penetration in the experiments of Jackson and Pace (e.g., Jackson and Pace, 1956, Figs. 8 and 9) are not consistent with the presence of two compartments but exhibit a pattern characteristic of a multicompartment system. The results are compatible with the explanation that the heterogeneity is the result of the distribution of sizes of the mitochondrial population.

This may explain, at least in part, the wide variety of kinetic characteristics of sucrose penetration curves exhibited by different preparations; the closer the system is to equilibrium, the slower should be its rate of sucrose penetration. A comparison of experiments from various laboratories shows the large variety of kinetic behavior that has been observed. For example, Gamble and Garlid observed a pronounced penetration of the mitochondrial sucrose-impermeable space with time by sucrose at 30° (Gamble and Garlid, 1970, Fig. 2A), whereas this was not seen by Bentzel and Solomon at 20°–25° (Bentzel and Solomon, 1967, Fig. 5). Amoore and Bartley (1958, Fig. 1) and Tedeschi (1965, Fig. 3) observed significant penetrations at 0°, whereas Gamble and Garlid observed a much smaller penetration (Gamble and Garlid, 1970, Fig. 2B). It is perhaps significant to note that the space in red blood cells not accessible to solute is temperature dependent and is much larger at lower temperatures (Bobo, 1967). The penetration of the sucrose-impermeable space observed as a function of time does not correspond to deterioration of the preparations since the rates of either short or long incubation periods can be explained with a single permeability constant (Tedeschi, 1965). In addition, when the preparations are exposed to sucrose-^{14}C for identical short periods of time, the sucrose penetration is the same, regardless of the total duration of incubation (Gamble and Garlid, 1970, Table I).

It should also be remembered that where osmotic swelling accompanies sucrose penetration, equilibrium can in theory be attained only after the mitochondria are swollen sufficiently to no longer respond osmotically. In effect, the percent penetration of the total space does not change as rapidly as expected. In a number of experiments, the penetration of sucrose does result in swelling (e.g., Jackson and Pace, 1956; Amoore and Bartley,

1958, Fig. 1). In many other experiments swelling is not significant, since the penetration is matched by the exit of other solutes.

The lack of penetration of mannitol in a sucrose medium of low osmotic pressure reported by Hunter and Brierley (1969, Fig. 6) may be at least in part the result of a decrease in penetrability caused by the increase in area/volume ratio that results from osmotic swelling. With the possible exception of this last result, the kinetics of penetration do not support the two-space model without significant modification.

B. Partial Penetration of the Sucrose-Permeable Space by Other Solutes

The concept of two spaces requires the sucrose-permeable space to be accessible to essentially all low-molecular-weight solutes. This point has been particularly stressed by Pfaff, who found little variation in the spaces penetrated by sucrose, AMP, ADP, ATP, and NAD^+. The solute space is reported to be between 20 and 30% (Pfaff, 1967), but this value is only rarely correct. Pfaff's values are in conflict with earlier ones obtained in the same laboratory (Klingenberg and Pfaff, 1966, Tables III, IV, Fig. 2), or with findings of other investigators. Thus O'Brien and Brierley report a sucrose space of 34% for beef heart mitochondria, with other spaces below or above this value. For example, the malate space was found to be 22% and the K^+ space 56% (O'Brien and Brierley, 1965). Birt and Bartley found the penetration of NAD^+ and NADH frequently to range from 0 to 25% of the mitochondrial volume, below the magnitude of the sucrose-permeable space. In some of their experiments, the space penetrated by NAD^+ or NADH corresponded to the space external to the mitochondria as measured with polyglucose-^{14}C (e.g., Birt and Bartley, 1960, Tables 13–15).

A similar group of experiments (Garfinkel, 1963) showed that the space penetrated by amino acids is high when the external amino acid concentration is low. When the concentration rises, however, the space approaches 27% of the mitochondrial volume, a value well below the 50–80% figure reported for the sucrose space by the same investigator (Garfinkel, 1963). The results are consistent with the explanation that there is a single mitochondrial space and that amino acids penetrate it by a saturable process.

The results just discussed are not compatible with the two-space model but can be readily explained by the one-space model.

C. Internal Solutes and the Osmotic Balance of Mitochondria

As noted above, the estimate of the internal concentration of solutes calculated from one model should differ significantly from that predicted by

the other model. This is indeed the case, since the internal volume calculated from the one-space model is generally 2 to 5 times larger than that assumed by the two-space model (see Table I). Consequently, the two models should lead to widely differing predictions for the osmotic behavior of mitochondria.

For most purposes, mitochondria can be considered in osmotic equilibrium. Since the amount of light scattered by mitochondrial suspensions is a function of mitochondrial volume (e.g., Tedeschi and Harris, 1955, 1958), the latter can be monitored photometrically. These measurements indicate that a sudden change in the osmotic pressure of the suspending medium leads to a new steady state in much less than 1 second (Tedeschi and Harris, 1955; Bentzel *et al.*, 1966), whether the new medium is hyper- or hypoosmotic with respect to the original medium (Tedeschi and Harris, 1955). Consequently, the activity of the internal solutes must equal that of the medium even after very short exposures. In the case of dilute solutions, activity and concentration are approximately the same.

Conceivably, some mechanism could exist that permits the osmotic pressure of the internal medium to exceed that of the external medium. For example, the organelles might be enclosed in rigid capsules. The extreme osmotic volume changes of which mitochondria are capable (Tedeschi and Harris, 1955) and their osmotic behavior, which approaches the ideal (Tedeschi and Harris, 1955; Tedeschi, 1961), preclude this alternative, however. Furthermore, the high permeability to water (Tedeschi and Harris, 1955; Bentzel *et al.*, 1966) makes it energetically prohibitive to maintain an osmotic gradient by active transport of water. In fact, it is well known that inhibitors of the electron transport chain do not induce mitochondrial swelling (e.g., Hunter *et al.*, 1959). Alternatively, it could be assumed that as a result of binding the activity of the ions taken up is low. Unfortunately, this does not account for the reciprocal relationship between the internal K^+ and the sucrose present (for discussion, see this section), nor for the situation in which, with the two-space model, it becomes necessary to assume that the concentration in the internal space is lower than that of the medium. Finally, the amount of possible binding as reflected in the amount of cations present in disrupted preparations [e.g., in digitonin fragments (Gamble, 1957) or repeatedly washed mitochondria (Ulrich, 1959; Gamble, 1962)] is too low to explain these massive discrepancies.

To assume that the ions inside the mitochondrion are in the sucrose-impermeable space, as does the two-space model, leads to severe discrepancies between inside and outside concentrations. Smaller discrepancies arise with the one-space model, which can be accounted for either by the difference between concentration and activity at high ion concentrations

or, in the case of low internal ion concentrations, by assuming that the list of inside solutes is incomplete.

The data shown in Table IV correspond to data taken or calculated from the report of Harris and Van Dam (1968). The concentrations of K^+ have been calculated either on the assumption that K^+ is present only in the sucrose-impermeable space as predicted by the two-space hypothesis (column 5), or that it is distributed in the total mitochondrial volume as predicted by the one-space hypothesis (column 6). The sucrose concentration calculated to be in the total mitochondrial volume is shown in column 7. The internal concentration of K^+ alone, calculated from the two-space model, is 2 to 4 times higher than that of all the osmotically active components in the suspending medium. Many other experiments show a similar osmotic imbalance if the total internal concentration of the measured cations is taken into consideration (e.g., Rottenberg and Solomon, 1969, Figs. 3–5).

The discrepancies between inside and outside concentrations derived on the basis of a two-space model are clearly inconsistent with an osmotic equilibrium. It is difficult to see from the published figures whether or not the inside solute concentrations in the one-space model agree exactly with those of the medium. Any discrepancy, however, is likely to be small (for example, if in Table IV it is assumed that concentration equals activity and that the penetrating anionic species is monovalent, the maximum deviation would be 30%).

Experiments have been published in which several solutes present in mitochondria have been determined. In many of these, if it were to be assumed that no sucrose had entered the osmotically active compartment, the internal osmotic pressure would be much below that of the medium. This is shown in Table V (Amoore and Bartley, 1958, Table 10). Column 3 presents the concentration in the external medium and column 4 the concentration on the assumption that the solute is distributed in the total mitochondrial volume according to the one-space hypothesis. Column 6 presents the concentrations calculated on the assumption that the salts but not sucrose are present in the sucrose-impermeable space (as required by the two-space hypothesis).

The deviations from the predictions of the two-space model are too large for a simple explanation (column 7). The one-space model, however, has good predictive value (the deviations are shown in column 5). A similar analysis can be carried out with data from other published experiments (e.g., see Ulrich, 1960, Tables I–III and V–VII; Carafoli *et al.*, 1964, Figs. 4 and 6; Harris *et al.*, 1966b).

It is possible on the basis of cationic estimates to arrive at an approximate maximum estimate of anionic concentrations. In several instances

TABLE IV

K^+ CONCENTRATION IN THE MITOCHONDRIAL COMPARTMENTS[a,b]

Conditions	Water volume (ml/gm protein)	Sucrose impermeable (ml/gm protein)	Amount of K^+ (mmoles/gm protein)	K^+ Concentration in sucrose-impermeable space (mM)	K^+ Concentration in total space (mM)	Sucrose concentration in total space (mM)
Control	1.80	0.24	270	1120	150	66
$+P_i$	2.25	0.33	263	770	117	66
+ATP	1.80	0.43	257	600	143	59
+Magnesium sulfate	1.78	0.42	240	570	135	59

[a] Harris and Van Dam (1968, Table 2).

[b] The medium contained 83 mM potassium chloride, 77 mM sucrose, 15 mM tris, 3 mM tris-glutamate, and 3 mM tris-malate.

TABLE V

INTERNAL SOLUTE CONCENTRATION IN MITOCHONDRIA WITH A LOW INTERNAL IONIC CONTENT[a]

Experiment	Solute	Concentration in one space (m*M*) External	Concentration in one space (m*M*) Internal[b]	Deviation from one-space model (%)	Concentration in hypothetical sucrose-impermeable space (m*M*)	Deviation from two-spaces two-spaces model (%)[c]
1	Sucrose	264	200	12	129	51 (24)
	K^+	1.2	24			
	Cl^-	0.1	7			
	Total	265	231			
2	Sucrose	302	236	12	134	55 (30)
	K^+	2	23			
	Cl^-	0.1	6			
	Total	304	265			
3	Sucrose	361	254	20	113	69 (50)
	K^+	1	27			
	Cl^-	0.1	7			
	Total	362	288			
4	Sucrose	496	354	20	147	70 (53)
	K^+	1	31			
	Cl^-	1	8			
	Total	498	393			

[a] Data selected from Amoore and Bartley (1958, Table 10).

[b] Mg^{2+} was not considered in our calculations since it is not likely to be free. These preparations contain a high concentration of acid-soluble phosphates.

[c] Values in parentheses calculated from the maximum estimate of ion concentration, on the assumption that $[K^+]$ = [anion].

the two-space model leads to an osmotic pressure from the K^+ and maximum anionic concentration that is well below the actual osmotic pressure of the medium. This discrepancy can be resolved only if sucrose is also assumed to be present in the inside compartments.

It is clear from these simple considerations that the results are not consonant with the two-space model but can be explained by the one-space model. The one-space model also explains an old observation (Amoore and Bartley, 1958, Table 2) that the internal mitochondrial K^+ level is inversely proportional to the internal concentration of sucrose. This finding cannot be easily explained by the two-space hypothesis but follows logically from the assumption of osmotic equilibrium and the one-space model. (This observation could be explained by the assumption that the sucrose-permeable volume increases as the sucrose-permeable space decreases and that K^+ leaks from the internal compartment. In these preparations, however, the sucrose-permeable space remained essentially constant with variations in volume.) Figure 1 shows this reciprocal relationship. According to the slope of the line, 1.8 moles of sucrose are equivalent to 1 mole K^+. If the K^+ were accompanied by a monovalent anion, the moles of ions would correspond to 2. The results are similar when internal K^+ and sucrose are plotted as a function of time (Amoore and Bartley, 1958, Fig. 1). Similar results were obtained recently by Gamble and Garlid (1970, Fig. 3).

D. Osmotic Behavior

In a number of experiments, the osmotic pressure of the medium has been varied and the mitochondrial volume and various spaces estimated experimentally. According to the one-space model, deviations from perfect osmotic behavior (e.g., osmotically inactive volume) would be accounted for quantitatively by the net change in osmotically active solute in the total mitochondrial space. According to the two-space model, however, only the sucrose-impermeable space would be osmotically responsive, with solutes in the sucrose-permeable space behaving similarly to those in the medium. Since the predictions differ in some cases, the two models can be analyzed in light of the available data.

When mitochondria are placed in a concentration of sucrose that differs from the original suspension medium, they either shrink or swell. In an ideal semipermeable system, the new osmotically active volume (V_2) would be a simple function of the initial osmotically active volume (V_1) and of the external concentrations of the medium. This relationship is described

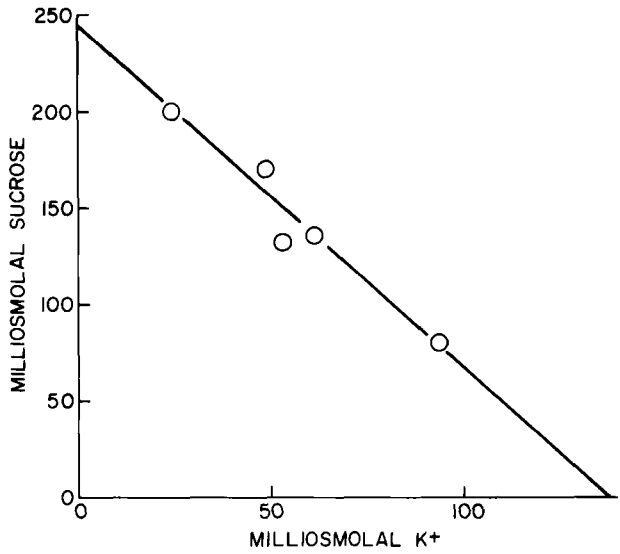

FIG. 1. Reciprocal relationship between mitochondrial K^+ and mitochondrial sucrose. Each point represents a determination carried out on a different preparation. From Amoore and Bartley (1958).

by Eq. (1), where C_1, is initial and C_2 the new concentration of nonpenetrant in the medium:

$$V_2 = (C_1/C_2)V_1 \tag{1}$$

In the two-space model, however, with one space bounded by a semipermeable membrane and the other by a membrane completely permeable to sucrose, the behavior would be according to Eq. (2):

$$V_2 = (C_1/C_2)V_p + V_s \tag{2}$$

where V_p represents the sucrose-impermeable volume and V_s the sucrose-permeable volume.

The assumption of a single volume that is partially leaky in relation to sucrose can be shown to be identical with the situation described by Eq. (2). Equation (3) describes V_2 as a function of V_1, C_1, C_2, and of the net amount of osmotically active solute (ΔS) that has either entered or left during the incubation period.

$$\frac{C_m V_1 + \Delta S}{V_2} = C_2 \quad \text{or} \quad V_2 = \frac{C_1 V_1}{C_2} + \frac{\Delta S}{C_2} \tag{3}$$

C_m corresponds to the concentrations of osmotically active material inside the mitochondria when they are suspended in a sucrose solution of concentration C_1. Since the system is in osmotic equilibrium [as is also assumed in

Eqs. (1) and (2)], $C_m = C_1$. Since $\Delta S/C_2$ has dimensions of volume, it can be expressed as V_s and Eq. (3) then becomes identical to Eq. (2). Even though in this model V_s has no true physical meaning, it nevertheless behaves as an osmotic dead space as long as ΔS represents the net amount of osmotically active solute taken up during the incubation period.

A precise evaluation of osmotic behavior requires a complete accounting of internal solutes. Such data are not usually available. In addition, deviations from predicted osmotic behavior can be brought about by considerably more subtle effects than by penetration of external solute as assumed in Eq. (3). For example, leakage of internal solute at more dilute external concentrations can also produce a large osmotically inactive space since in dilute solutions the volume would be less than predicted. A number of recent experiments have suggested the existence of a substantial osmotically inactive volume that does not correspond to the sucrose-permeable space (Bentzel and Solomon, 1967; Klingenberg and Pfaff, 1966; Harris and Van Dam, 1968). This space could be the water of hydration of the mitochondrial matrix, as proposed by Bentzel and Solomon (1967). It could also be attributable to leakage of internal solute in more dilute solutions. These aspects will be discussed in the rest of this section.

1. Experiments in Which Total Sucrose and Solutes Have Been Estimated

Some experiments that present a complete or almost complete balance prove to be very revealing. The *total amount of sucrose* is used to estimate the sucrose-permeable space. If the one-space model applies, then in some cases osmotic changes should lead to changes in the apparent sucrose-permeable space. Any sucrose that penetrated prior to the incubation period should behave as any other internal solute. For this reason, the one-space model does not rule out osmotic volume changes involving the hypothetical sucrose-permeable space. Changes in the sucrose-permeable space, however, would be inconsistent with the two-space model.

Experiments that permit a critical evaluation of the two models have been carried out (Bartley, 1961), and some of them are summarized in Table VI. In Table VI the different volumes are listed under the different experimental conditions. In addition, the loss or gain in sucrose is listed. Data for K^+ and Cl^- are also available but the exchanges are slight. In Table VI the second column indicates the experimental result. The next two columns present the predictions of the two models. In experiment 1, the mitochondria are transferred from 0.25 *M* to 0.54 molal sucrose. Either model has good predictive value. When the transfer is from 0.54 to 0.28 molal sucrose (experiment 2), however, the two-space model is in conflict with the experimental results.

TABLE VI

OSMOTIC BEHAVIOR AND SOLUTE EXCHANGES

	Result	One-space model	Two-space model
Experiment 1 (Experiment 2 of Bartley, 1961, Table 1)			
Initial condition: 0.25 *M* sucrose			
Sucrose-permeable space (liters/kg)	1.40	—	—
Total water (liters/kg)	2.04	—	—
Final condition: 0.54 molal sucrose			
Sucrose-permeable space (liters/kg)	1.35	1.37	1.40
Total water (liters/kg)	1.62	1.74	1.72
Solute taken up (moles/kg)	0.336	—	—
Experiment 2 (Experiment 3 of Bartley, 1961, Table 1)			
Initial condition: 0.54 molal sucrose			
Sucrose-permeable water (liters/kg)	1.35	—	—
Total water (liters/kg)	1.62	—	—
Final condition: 0.28 molal sucrose			
Sucrose-permeable water (liters/kg)	1.96	1.90	1.35
Total water (liters/kg)	2.27	2.44	1.89
Solute leaving (moles/kg)	−0.200	—	—

The hypothetical sucrose-permeable space increases in volume when mitochondria are transferred from 0.54 to 0.28 molal sucrose, but the sucrose-impermeable space does not. This is inconsistent with the two-space model but not with the one-space model. Nevertheless, the one-space model cannot explain the exit of sucrose without a favorable concentration gradient. A transfer of the mitochondria from a high to a low concentration of sucrose cannot lead to a sucrose concentration inside the mitochondria higher than that in the medium as long as the osmotic response is rapid. A gradient favorable to the exit of sucrose can occur only if some other osmotically active component is present in the suspending medium.

A more recent study (Pfaff, 1967) obtained similar results. Osmotic volume changes were found in whole or in part in the hypothetical sucrose-permeable space.

Experiments by Klingenberg and Pfaff (1966) have been interpreted as substantiating the two-space model. In their work the mitochondrial water, the total pellet sucrose, and the carboxypolyglucose-^{14}C–ether space were determined after a 3-minute incubation in media of different external osmotic pressures. The polyglucose–ether space was used as an index of the extramitochondrial space. The total mitochondrial sucrose space accounted for most of the mitochondrial space that seemed to be osmotically unresponsive. This contradicts the one-space model which predicts that the os-

motically unresponsive volume corresponds only to the sucrose that entered during the incubation period. In this experiment the osmotically unresponsive space was determined by extrapolation of the mitochondrial fluid volume to infinite concentration of external medium (i.e., 1/[sucrose] = 0). Only four sucrose concentrations were measured, however, one at approximately 0.05 *M* sucrose. In our experience changes in mitochondrial volume at this concentration are frequently not typical of mitochondrial responses at higher concentrations even after very short exposures (Tedeschi and Harris, 1955, Fig. 3). Tarr and Gamble (1966) found a loss of internal K^+ at comparable concentrations of sucrose after 10 minutes at 25°. The results of Klingenberg and Pfaff at the three remaining concentrations do not permit a meaningful conclusion since they could also be used to argue for the absence of any osmotic behavior. Moreover, later results published by the same laboratory (Pfaff, 1967) do not confirm the results at the higher osmotic pressures.

2. Experiments Determining the Sucrose-^{14}C Space

A number of experiments have been carried out in which the space permeable to sucrose or mannitol has been measured by adding sucrose-^{14}C or mannitol-^{14}C at one or several osmotic pressures. In these situations both models should have equal predictive value provided the isotope is added initially. From the point of view of the one-space hypothesis, the ^{14}C-solute space should serve as a measure of penetration into the single internal space. It should therefore provide an estimate of $\Delta S/C_2$ [Eq. (3)], which has the appearance of an osmotically unresponsive space. Since experiments carried out at a single osmotic pressure do not contribute to the resolution of this question, they are not discussed here.

In the experiments of Bentzel and Solomon (1967), the label was added before the mitochondria were exposed to media differing in osmotic pressure. In these experiments the major fraction of osmotically inactive space corresponds to the space penetrated by the sucrose-^{14}C. As we have seen, however, on the basis of Eq. (3) these experiments can be interpreted according to the one-space model. The space penetrated by sucrose would appear as an osmotically unresponsive space with either the two- or the one-space model. A similar experiment has been carried out by Hunter and Brierley, making use of mannitol-^{14}C rather than sucrose (Hunter and Brierley, 1969).

In experiments in which sucrose is not the major component of the medium (Harris and Van Dam, 1968) or in which the label is introduced after a period of preincubation (Malamed and Recknagel, 1959; Tarr and Gamble, 1966), the two models should lead to different predictions.

In the experiments of Harris and Van Dam (1968, Fig. 6), mitochondria were incubated in a medium containing 50 m*M* sucrose, 5 m*M* potassium chloride, 2.5 mM tris–chloride, 5 m*M* phosphate, 2 m*M* EDTA, 10 m*M* magnesium chloride, and 1 μg rotenone per milliliter. In these experiments water was determined from the distribution of tritiated water and the sucrose volume from sucrose-^{14}C. The labels were added in trace amounts before the osmotic pressure of the medium was raised by adding potassium chloride to the external medium. The total mitochondrial volume was found to *increase* with increasing osmotic pressure, in contrast to the findings of several other studies (see Table II). The space that appeared to be inaccessible to sucrose was found to be small and to decrease with increasing osmotic pressure, as expected from the two-space hypothesis. Since not all solutes were accounted for, it is difficult to decide whether or not the results refute the one-space model, in which, in accordance with Eq. (3), the osmotically inactive space should correspond to the volume penetrated by the suspending solute (mostly potassium chloride and sucrose). The fact that a large portion of the sucrose-impermeable space is not osmotically active may represent volume changes brought about by the penetration of solutes other than sucrose (e.g., potassium chloride). Alternatively, this space could be the hydrated matrix space proposed by Bentzel and Solomon (1967) in agreement with the two-space model.

In the experiments of Malamed and Recknagel (1959) and of Tarr and Gamble (1966), mitochondria were exposed to media of varying osmotic pressures, with the radioactive marker (sucrose-^{14}C, and in the experiments of Tarr and Gamble ^{36}Cl also) added afterward. The penetrated space was estimated from the label in the pellet (Tarr and Gamble, 1966) or the dilution of the label remaining in the supernatant (Malamed and Recknagel, 1959). In the experiments of Tarr and Gamble, only two or three concentrations of sucrose were used (osmolality 0.16 and 0.63, and in some experiments 0.34 also). The sucrose- (or Cl^--) inaccessible space increased with decreasing osmotic pressure. For example, the inaccessible volume at a 0.16 molal concentration was 1.40 ml/gm of mitochondria, whereas at 0.6 molal it was 0.51 (Tarr and Gamble, 1966, Table I). Theoretically, with the two-space model [Eq. (1)], it should be to 2.04 ml/mg at 0.16 molal. The discrepancy is not necessarily serious and can be explained away [e.g., it is possible to postulate that the sucrose-impermeable unresponsive portion at the higher osmolalities corresponds to the water associated with the mitochondrial matrix postulated by Bentzel and Solomon (1967)]. Neither are the results necessarily in conflict with the one-space hypothesis. As discussed, the penetration of solute would account for the osmotically unresponsive volume. The portion of the osmotically unresponsive volume not penetrated by the label may be attributable to the

volume penetrated by the solute before the introduction of the isotope. This explanation requires that the permeability to Cl^- be approximately the same as that to sucrose. The rate of penetration into the hypothetical sucrose-impermeable space was found to be approximately the same for Na^+, Cl^-, and sucrose (Gamble and Garlid, 1970, Fig. 2), as was also suggested by earlier experiments (Amoore and Bartley, 1958).

In the work of Malamed and Recknagel (1959), four different medium concentrations were used. The sucrose-^{14}C space corresponded closely to the osmotically inactive volume. These data have been interpreted as evidence for the two-space model. The sucrose-^{14}C space was estimated after an initial exposure to different osmotic pressures. With the assumption of the one-space model, the osmotically inactive space should correspond to the space penetrated by sucrose from the beginning of the experiment. In other words, it should include the penetration of sucrose that preceded the addition of the sucrose-^{14}C. The agreement between the osmotically inactive space and the sucrose-^{14}C space is probably fortuitous since the calculations have not taken into account the mitochondrial volume occupied by the sucrose as pointed out by others (Bentzel and Solomon, 1967). In addition, the volume changes are likely to be determined by a balance between osmotic swelling and the entrance or exit of solutes. At least one of the external concentrations of sucrose and perhaps two are in the range in which typical osmotic behavior is not observed (Tedeschi and Harris, 1955) and considerable K^+ leakage has been reported (Tarr and Gamble, 1966). More significantly, the lower the external concentration the greater the leakage, even at short exposures (Tarr and Gamble, 1966), and some of these exposures are in the range of sucrose concentrations used by Malamed and Recknagel. That the pepentration of sucrose-^{14}C should vary little or decrease slightly with mitochondrial volume (to account for the constant or near-constant sucrose-permeable space) has been argued since the area/volume is changed by swelling (Tedeschi, 1965). Consequently, the conclusions from these experiments can be considered inconclusive at best.

E. The Energy Expended in Transport and the Size of the Internal Space

The energy necessary to transfer K^+ into the sucrose-impermeable space can be calculated readily from the data of Cockrell, Harris, and Pressman (1966). On the basis of the values of Harris and Van Dam (1968) for a sucrose-impermeable space, the calculated internal concentration of K^+ would be too great to account for the measured transfer of 7.9 moles of K^+ per mole of high-energy phosphate hydrolyzed.

The sucrose-inaccessible volume shown in Table IV (calculated from Harris and Van Dam, 1968, Table III), is 22 ± 4% of the total volume. In the work of Cockrell *et al.* (1966), up to 7.9 moles of K^+ were translocated per ATP hydrolyzed, at an external K^+ concentration of 2.5 m*M*, with an internal concentration of K^+ at 100 mmoles/gm of protein. If it is assumed that the maximum water volume is 3.35 ml/gm of protein (Gamble, 1957, Table 1), the sucrose-impermeable space would be about 0.74 ml/gm of protein. The K^+ concentration would then be approximately 135 m*M* and the energy required to transfer 7.9 moles of K^+ would require 18.6 kcal ($7.9 \times 2.3\ RT \log 135/2.5$), that is, more than that available from 1 mole of ATP hydrolyzed. The use of higher values for the sucrose-impermeable space as reported by other workers would lower this figure. For example, if the sucrose-impermeable space were 50% of the mitochondrial fluid volume, the energy yield needed from each terminal phosphate of ATP would be approximately 14.7 kcal. This figure would also be improbable unless the efficiency of the system approached 100%. It should also be noted that these estimates of the necessary energy expenditure are minimal since they do not take into consideration the outflux of K^+ which must accompany the uptake. These calculations add to the serious objections that have been leveled against the two-space model.

F. Hydrated Spaces Unavailable to Solutes

Bentzel and Solomon (1967) have suggested the presence in a 0.272 osmolar sucrose medium of a hydrated, osmotically inactive volume of about 50% of the sucrose-impermeable space or about 15% of the total volume. This alternative is in accord with present understanding of the behavior of macromolecules (e.g., see Bobo, 1967), either in solution or inside cells. The presence of an osmotic dead space of this kind would be analogous to that found in the red blood cell. The internal content of the red blood cell is apparently responsible for an osmotic dead space well in excess of that predicted from the volume of the solids (e.g., see Kwant and Seeman, 1970). As mentioned, it is also interesting to note that the 15% reported by Bentzel and Solomon (1967) is not too far from the 20% reported by Harris and Van Dam (1968) or Tarr and Gamble (1966) for the sucrose-impermeable space which could therefore correspond in large part to a "hydrated volume."

Although there may well be a volume that is unavailable to internal solutes, it is likely to be small. Measurements of the spaces penetrated by glycerol-^{14}C (Tedeschi, 1965), sucrose-^{14}C (Tedeschi, 1965), glycine-^{14}C (Garfinkel, 1963), EDTA-^{14}C (Settlemire *et al.*, 1968), and 2-oxoglutarate

(Chappel *et al.*, 1968) reveal essentially a complete penetration of the mitochondrial space. Some of the experiments, however (Jackson and Pace, 1956; Tedeschi, 1965; Chappel *et al.*, 1968), may support the concept of a small, inaccessible internal water volume.

It has been proposed (Harris *et al.*, 1966a, 1967) that valinomycin induces volume changes in mitochondria that either are larger than the volume changes attributable to ion penetration or do not parallel ion uptake. This conclusion was reached on the basis of several observations:

(1) When the light scattered by a suspension is monitored simultaneously with K^+ uptake, these two parameters occasionally do not vary in parallel.

(2) Mitochondrial volume changes as estimated from changes in the medium Na^+ concentration (on the assumption that Na^+ is excluded from the internal space) are larger than the volume changes estimated from K^+ uptake.

(3) The magnitude of the changes in the light scatter observed at various medium K^+ concentrations is the same, even though the amount of K^+ accumulated varies.

(4) It can be deduced from the energy cost of active K^+ transport that the internal K^+ concentration cannot be that required to reach the osmotic pressure of the medium.

(5) It can be estimated by extrapolation of the data of Cockrell *et al.* (1966, Fig. 10) that when the external K^+ concentration is 80 m*M* transfer of K^+ cannot be measured with the K^+ electrode. This may be because at this K^+ concentration in the medium K^+ is taken up along with water from the medium. If this were the case, more water would be taken up than necessary to maintain equality between internal and external osmotic pressures.

The changes in light scattering brought about by a mitochondrial suspension usually parallel K^+ uptake (Harris *et al.*, 1966a, Fig. 14). The occasional deviations observed may be the result of uptake of some other medium component. In addition, the qualitative use of light scatter as an indication of volume change may not be justified. Light scatter and mitochondrial volume are related in a complex manner. Scatter, for example, is a function of the refractive index of the medium, and this varied in at least some of the experiments of Harris *et al.* (1967, Fig. 10). The extrapolation to 80 m*M* for the external K^+ concentration at which no apparent transfer occurs may be fortuitous.

Despite these objections, measurements of Na^+ concentration with a cationic electrode do argue in favor of the interpretation that the volume

changes exceed those attributable to K^+ uptake alone (Harris *et al.*, 1967, Fig. 8).

Nevertheless, as mentioned earlier, we can only explain the energy expenditure for K^+ transfer by assuming that sucrose penetrates a single mitochondrial space. The difficulty cannot be explained by assuming an increase in the water space in which K^+ cannot dissolve (for example, an increase in the water held by internal components such as hydrated molecules); for this case, the effective K^+ concentration would remain the same. Alternative explanations are unlikely, since the osmotic pressure of the internal medium would have to be lower than that of the external medium. As discussed, such a situation would not be consonant with the observed high permeability of mitochondria to water (Tedeschi and Harris, 1955; Bentzel *et al.*, 1966). Several other studies carried out in the absence (Jackson and Pace, 1956; Tedeschi, 1961; Blondin and Green, 1969; Hunter and Brierley, 1969; Hunter *et al.*, 1969; Rottenberg and Solomon, 1969) or presence (Rottenberg and Solomon, 1969) of valinomycin do not support the interpretation that water is taken up in excess of the osmotic requirement.

III. SUMMARY AND CONCLUSIONS

Two models are considered in relation to experiments involving the estimation of the volume of isolated mitochondria and the distribution of solutes. One model assumes that mitochondria are made up of two spaces, a sucrose-permeable and a sucrose-impermeable space. The other assumes that each individual mitochondrion is made up of a single internal space. Several results are inconsistent with the two-space hypothesis but can be explained by the one-space hypothesis.

The kinetics of sucrose penetration, discussed in Section II,A, can be explained by either model. The two-space model cannot account for the finding that some low-molecular-weight substances do not penetrate entirely into the sucrose-permeable space (Section II,B). The calculations based on the two-space model would predict gross osmotic imbalances between the mitochondria and the suspending medium, whereas mitochondria are in osmotic equilibrium (Sections II,C and D). In addition, the energy required to maintain the concentration gradient predicted by the two-space model would be insufficient to support the observed rates of active transport (Section II,E). Thus the one-space model appears to be compatible with most of the reported results. On the basis of this model, however, it is difficult to explain the exit of sucrose against an apparent

concentration gradient when mitochondria are shifted from a high to a low concentration of sucrose (see Section II,D).

IV. POSSIBLE NEW EXPERIMENTAL APPROACHES

Since no alternative models have been proposed, further experiments are needed either to allow a clear-cut choice between the two models or to propose an alternative. More attention to experimental variability and the complete reporting of more significant parameters will undoubtedly help to throw light on the questions analyzed here. Perhaps the most neglected parameter has been the internal space accessible to a penetrant. Another weakness is the frequent absence of statistical analyses. It seems rather sterile to continue with only the same experiments already reported in the literature, however. Since entirely new experimental approaches may throw light on these questions and at the same time lead to new proposals, we would like, for the purpose of stimulating further studies, to propose some additional approaches.

As already done by some investigators (Avers *et al.*, 1969), it should be possible by centrifugal techniques to fractionate mitochondria according to size. A test of the penetrability of the various fractions may reveal whether their permeability is uniform or whether the sucrose-permeable phase reflects the different mitochondrial sizes, as proposed by the one-space model.

The significance of the space enclosed by the two mitochondrial membranes in relation to the sucrose-permeable spaces could perhaps be investigated by using preparations from which the external membrane has been stripped off. Such preparations can be obtained by treating mitochondria with digitonin (Schnaitman and Greenawalt, 1968) or lubrol (Chan *et al.*, 1970). Provided such preparations are not too badly damaged, the presence in them of a sucrose-permeable space would not then be ascribable to a space present between the outer and inner membranes.

As already discussed, each model predicts entirely different intramitochondrial concentrations of ions. It is now possible to measure the ionic concentrations of compartments by means of ion-sensitive microelectrodes (Walker, 1971). Such microelectrodes could be inserted into large insect mitochondria (Tupper and Tedeschi, 1969) to permit the direct measurement of internal concentrations of ions.

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