The Structure of the Mitochondrial Membrane:* Inferences from Permeability Properties‡

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(Received for publication, March 24, 1959)

ABSTRACT

Mitochondria possess a semipermeable membrane with properties similar to the cell membrane. Despite the presence of a limiting membrane, mitochondria swell approximately 4 to 5 times their original volume without lysis or loss of internal solute. For this reason, it has been argued that the membrane might be convoluted. The present kinetic study of the permeability of isolated mitochondria was undertaken to clarify this question. A photometric method described previously was used.

In the case of highly lipid soluble penetrants, the results suggest that neither the permeability nor the surface area available for penetration varies significantly during considerable swelling. These results may be interpreted to mean that the mitochondrial membrane is convoluted. For highly polar compounds, the permeability of the membrane also remains unchanged during swelling, but the surface area available to penetration increases. These results may be interpreted to mean that in this latter case, the surface of the convolutions becomes available only after they are unfolded by swelling.

The simplest model that can explain the permeability properties of this membrane consists of a bimolecular lipid layer where the inner monomolecular layer is convoluted.

INTRODUCTION

Electron microscope studies of mitochondria fixed with OsO_4 reveal a limiting structure (24, 34) consisting of two electron-dense layers with a relatively electron-transparent layer in between. An analogous layering occurs in the lamellae (cristae) within the lumen of the mitochondria, and some investigators consider the lighter layer of the surface membrane continuous with the one present in the cristae (*e.g.* 25), while others consider it to be a separate structure (*e.g.* 35).

Sjöstrand has proposed that the electron-transparent space corresponds to a lipid bimolecular layer and the two denser layers to two protein layers (35). The model follows closely that proposed for the cell membrane (see 9, 10). Each denser layer can be resolved into two thinner dense layers enclosing a lighter internal space (28, 12, 30). It has been suggested that this morphological arrangement could be the consequence of the presence of two bimolecular lipid layers (see 29, 30). The two double layers would enclose a space, the nature of which is still undefined. This postulate is supported by a number of observations on natural and artificial lipid systems (*e.g.*, 30, 27).

This view, a membrane composed in part of a lipid layer, is in accord with classical studies where a lipid or lipoprotein surface was postulated for mitochondria on the basis of chemical composition and staining reactions (8, 3, 4). It is supported strongly by osmotic and permeability data. Mitochondria obey osmotic law and their permeability is directly dependent on the oil-water partition coefficient of the penetrant; the permeability constant of each non-electrolyte studied parallels closely those encountered for typical cells (36).

Despite the presence of a limiting membrane, mitochondria can undergo considerable increases

* The term membrane has been used in this paper to denote the composite structure bordering mitochondria.

‡ Aided by a grant from the American Cancer Society, Inc.

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in volume without lysis (17, 6, 7, 36, 37), loss of internal protein (13) or loss of other internal solutes (36, 37). For these reasons, it was proposed that the mitochondrial semipermeable membrane might be convoluted. Accordingly, it was suggested that the cristae might represent folds (36).

In the present study, this possibility has been examined more critically. The permeability of the membrane at various degrees of swelling was measured. The development of a quantitative photometric technique to estimate the volume of mitochondria has made this approach feasible (37), since from measurements of volume changes with time, it is possible to calculate permeability constants (see 16, 37). The analysis of the results also permitted estimation of the surface area available to penetration.

Methods

Mitochondria were isolated from liver, kidney, or heart by methods based on that of Hogeboom, Schneider, and Palade (14). Male rats of the Sprague-Dawley strain, weighing approximately 300 to 400 grams, were killed by a blow on the head followed by cervical fracture of the spinal cord. When liver mitochondria were isolated, the rats had been starved for 16 to 20 hours.

The organ used was excised and stripped of extraneous tissues. In the case of the kidney, the renal capsule and the renal pelvis were removed. The remaining portions were cut into large pieces which were immediately placed into chilled isolation medium consisting of 0.25 M sucrose. In some cases (see legend of Fig. 2), 0.01 M sodium citrate at pH 7.4 was added to this solution.[1]

The remainder of the isolation procedure was conducted at 2–6°C. After repeated washings with the isolation medium, the tissue was minced with scissors and homogenized in a large volume of solution by means of a motor-driven teflon and glass homogenizer of the Potter-Elvehjem type.

Liver and heart homogenates were centrifuged at 600 *g* for 15 minutes; kidney preparations were centrifuged at 600 *g* for 20 minutes. An International, size 1, type SB centrifuge was used. The supernatant was decanted, and centrifuged in a Servall angle centrifuge at 8,500 *g* for 10 minutes.

[1] Isolation in citrate was found to prevent a sudden and somewhat unpredictable deterioration frequently occurring in both heart and kidney mitochondria. This deterioration might be associated with the presence of calcium ion (see 7). Versene, used previously as a chelating agent, was found to interfere with the kinetics of the processes studied.

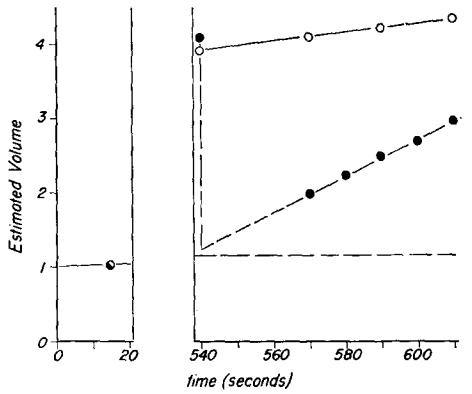

FIG. 1. Osmotic reversal of the swelling of mitochondria in mannitol. The mitochondria were suspended at zero time in 10 cc. of 0.33 molal mannitol in 0.001 M tris, pH 7.4. Open circles: relative osmotically active volume of control; average of 8 determinations. After 9 minutes of exposure 3 cc. of 0.33 molal mannitol were added. Closed circles: relative osmotically active volume of experimental; average of 10 determinations. After 9 minutes of exposure 3 cc. of sucrose were added bringing the concentration of sucrose to 0.920 molal. The dashed horizontal line represents the reversal volume predicted by osmotic law. The standard error of each point is approximately the diameter of the circles.

The resulting mitochondrial pellet was suspended in a solution of 0.3 osmolal sucrose and 0.02 M tris (trimethylol amino methane) at pH 7.4. The method of suspension was found to be of some importance since excessive shaking of the suspension reduces the optical density and the osmotic response of the particles considerably. In this work, after a preliminary suspension with a glass rod, the large pieces were homogenized in a glass and teflon homogenizer. The suspension was kept at 0°C. until used.

In a typical experiment, 10 cc. of the experimental solution were added to 0.1 cc. of the suspension. The calibration measurements necessary to convert the optical density readings into relative osmotically active volume (see 37) were carried out after resuspending in raffinose-potassium chloride solutions of the required concentrations and refractive indexes. In one case (Fig. 1), propylene glycol was added to some of the calibration solutions in order to reach the appropriate range of refractive index. All calibration solutions were in 0.02 M or 0.01 M tris, pH 7.4.

The optical density of the final suspensions was measured at suitable time intervals at a wave length of 520 mμ by means of a Coleman Junior spectrophotometer, model 6A. For kinetic experiments, with the exception of those carried out with mannitol, the output of the photocell of this instrument was continuously recorded by a Sanborn recording system

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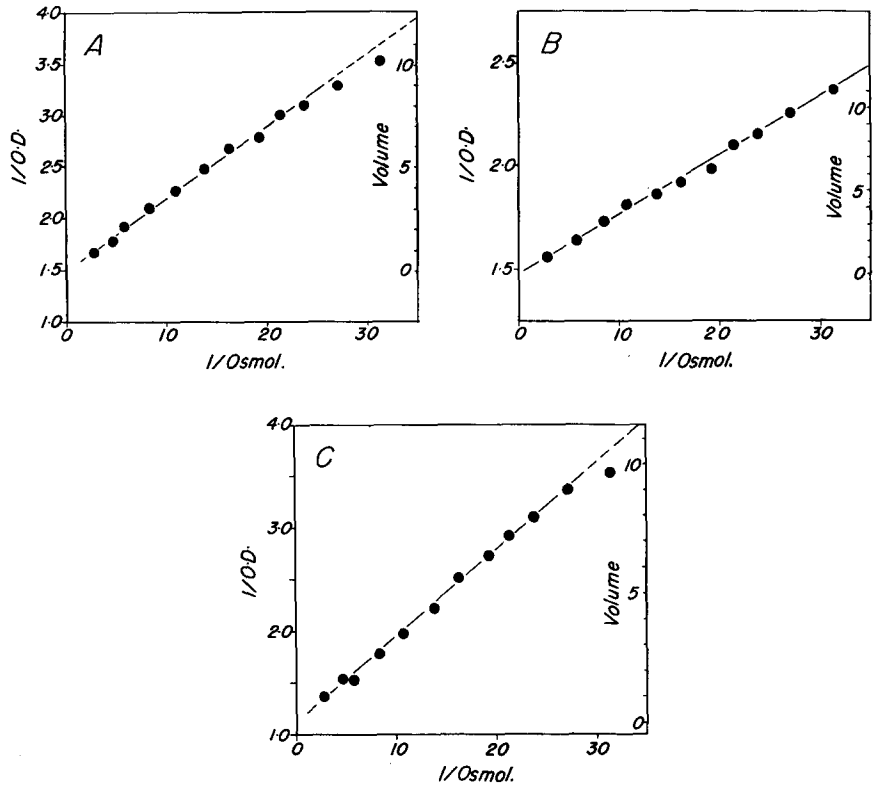

FIG. 2. The dependence of the optical density and the osmotically active volume on the concentration of nonpenetrant. Potassium chloride solutions in 0.01 M tris, pH 7.4, were added to the suspensions. The standard error of each point is less than the diameter of the circles.

A. Kidney mitochondria. Each point corresponds to an average of 6 determinations after 15 seconds of exposure. The mitochondria were isolated in the presence of citrate (see Methods).

B. Heart mitochondria. Each point corresponds to an average of 4 determinations after 15 seconds of exposure. The mitochondria were isolated in the presence of citrate (see Methods).

C. Liver mitochondria. Each point corresponds to an average of 6 determinations after 15 seconds of exposure.

consisting of a low level preamplifier, model 150–1500, a power supply, model 150–400, and a single channel recorder, model 151–100. The experiments were carried out at 20 ± 1°C.

Osmotically active volumes were calculated from the measured optical densities by a method previously described (37). The optical density changes represent primarily osmotic volume changes. This was demonstrated in an earlier study by osmotic reversal techniques (36, 37) for all the substances used in these experiments except mannitol. The instantaneous reversal technique previously described (37) can also be used in this case (Fig. 1). 0.33 osmolal mannitol was added to the suspension at zero time. After 9 minutes of exposure, a calculated amount of sucrose was added (closed circles). The upper curve (open circles) represents a control. The horizontal dashed line represents the reversal volume predicted from osmotic law. There is no question that the process is reversible. The rapid apparent swelling following the reversal is probably the consequence of a damaging effect of the high concentration of sucrose.

RESULTS

Changes in the volume of mitochondria can be followed photometrically; the volume of the particles is directly proportional to the reciprocal of the optical density of the suspension (36, 37). Results obtained by this method show that isolated mitochondria follow osmotic law very closely over a wide range of concentrations of external medium (36, 37, and Fig. 2). These results can be interpreted to mean that mitochondria can swell considerably without a leakage of internal solute. In extremely hypotonic solutions, the volume of the particles appears less than that predictable from osmotic law. The mitochondria apparently have

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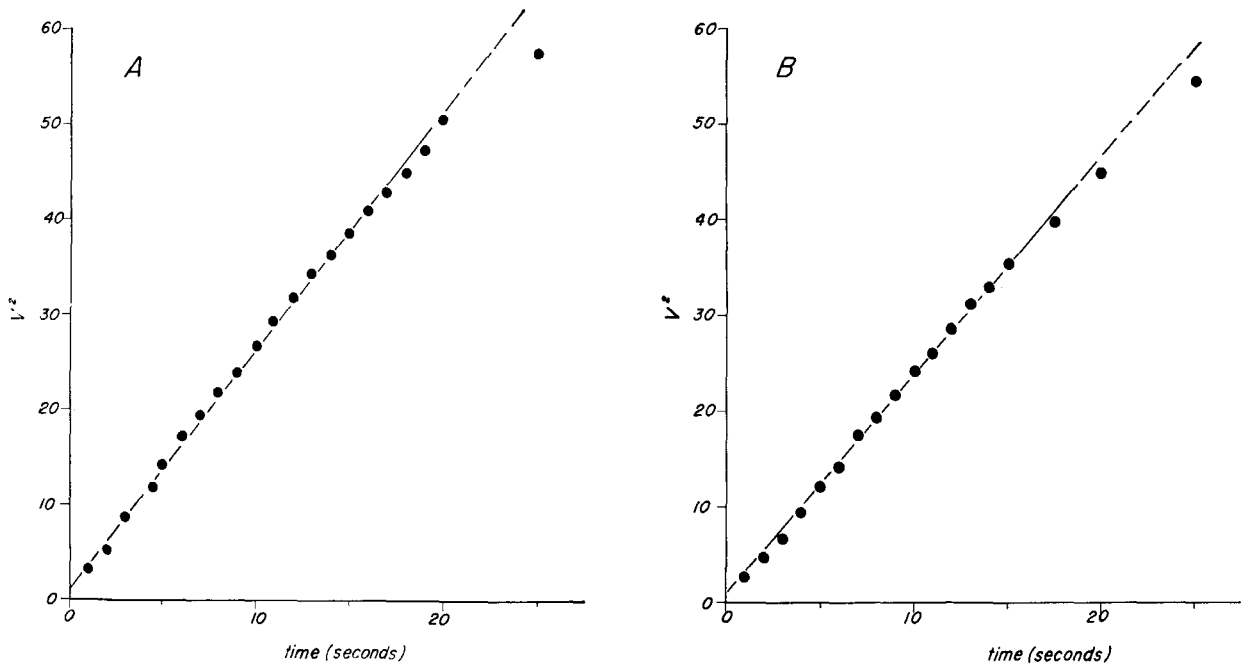

FIG. 3. The kinetics of permeability to fast penetrants: liver mitochondria. 0.33 osmolal concentration of the penetrant in 0.001 M tris, pH 7.4, was added to the suspension. Each point represents an average of 10 determinations. For calculation of P (see Equation 2), it was assumed that $A = 4.01 \times 10^{-8}$ sq. cm. The value was chosen arbitrarily and includes the area of the postulated folds. *A*. Penetration of urea: $P = 0.011$ cm./hr. *B*. Penetration of glycerol: $P = 0.0099$ cm./hr.

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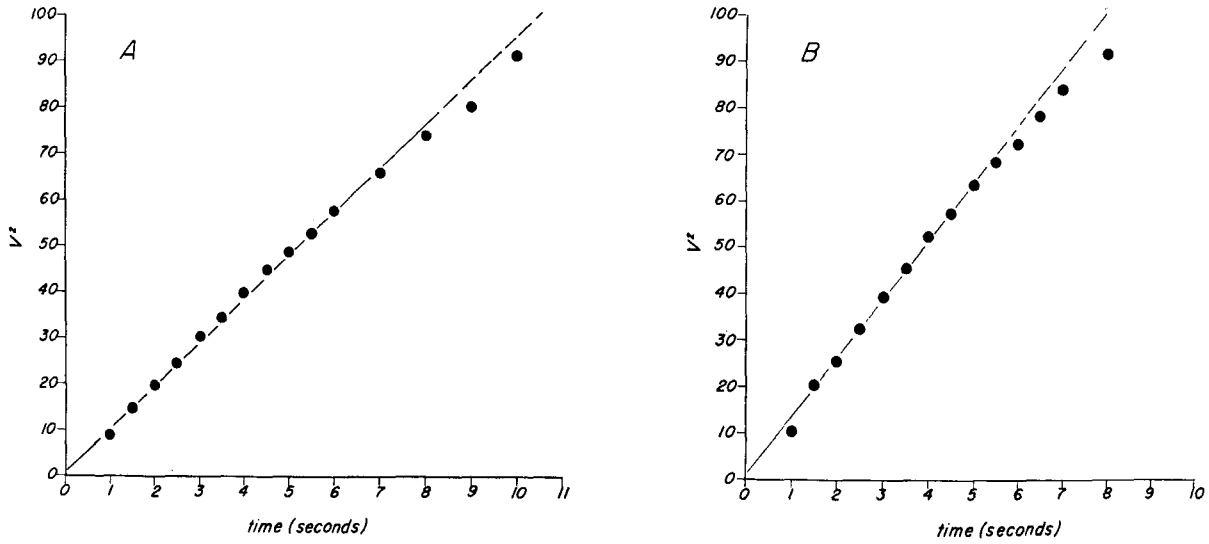

FIG. 4. The kinetics of permeability to fast penetrants: liver mitochondria. 0.33 osmolal concentration of the penetrant in 0.001 M tris, pH 7.4, was added to the suspensions. Each point represents an average of 11 determinations. For calculation of P (see Equation 2), it was assumed that $A = 4.01 \times 10^{-8}$ sq. cm. (see Legend of Fig. 3). *A*. Penetration of thiourea: $P = 0.040$ cm./hr. *B*. Penetration of methylurea: $P = 0.053$ cm./hr.

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become leaky with consequent loss of internal solute. This, however, does not occur until the osmotically active volume has become 7 to 10 times that in isotonic solutions. The change corresponds to a 4 to 5.5-fold increase in total mitochondrial volume and a 2.5 to 3.1-fold increase in apparent surface area as calculated from direct measurements of mitochondrial size (36). These results occur not only with liver mitochondria (36, 37, and Fig. 2 *C*), but also with kidney (Fig. 2 *A*) and heart mitochondria (Fig. 2 *B*). Therefore, they can be considered characteristic of mitochondria.

It is difficult to see how such a large change in volume and apparent surface area can occur without stretching or damaging the mitochondrial membrane. For example, cell membranes, which have comparable permeability properties, can withstand only moderate stretching. It has been estimated that in the case of the erythrocyte an increase in surface area of as little as 8 per cent results in an increase in permeability sufficient to induce hemolysis (26, 15). Exact data for other cells is somewhat scant. In the case of Arbacia eggs, it can be calculated from published data (22) that the permeability to water increases sharply after the eggs swell 44 to 66 per cent of their original volume. This corresponds to an increase in surface area of 28 to 40 per cent.

One can resolve this difficulty by postulating that the mitochondrial membrane is convoluted, hence, not actually stretched, but unfolded during swelling (see 36). A more critical analysis is possible by following the permeability of the membrane during the swelling of mitochondria. Any stretching and damaging of the membrane should find reflection in an increase in permeability. The photometric technique for estimating mitochondrial volume offers a convenient technical approach. It permits the use of well understood osmotic principles and a simple theoretical analysis (16).

When mitochondria are suspended in a solution of penetrant, permeability constants can be calculated directly from the rate of volume change. The relationship can be expressed as in Equation 1 (see 16). In this equation, P is the permeability constant, t is time, and A, the surface area. V refers to the osmotically active volume and V_o to the initial osmotically active volume. Equation 1 integrates readily and takes the form of Equation 2. In this paper, V, V_o, and A are expressed in relative units where the osmotically active volume of mitochondria, suspended in a 0.335 osmolal solution of a non-penetrant, is taken as unity.

$$\frac{V}{A}dV = PV_o\,dt \tag{1}$$

$$Pt = \frac{V_o}{2A}\left[\frac{V^2}{V_o^2} - 1\right] \quad \text{or} \quad V^2 = 2AV_oPt + V_o^2 \tag{2}$$

Equation 1 and 2 can be used when the permeability to the penetrant is much lower than that to water and in the relative absence of non-penetrant (16). These conditions are fulfilled in these experiments.

A plot of V^2 with time should give a straight line with V_o^2 as the intercept and $2AV_oP$ as the slope, as long as the permeability and the permeable area remain constant. On the other hand, any increase in area or permeability should increase the slope of the line, and, therefore, result in deviations from a straight line.

Experiments of this kind were carried out with fast penetrants and suspensions of both liver (Figs. 3 and 4) and kidney mitochondria (not shown). The values of V^2 obtained vary linearly with time, in the way predicted (Figs. 3 and 4), over a wide range of swelling (up to V = 7–10). Deviations, however, finally occur. At these critical swellings, the magnitude of the volume is less than that predicted from the equation and this effect can be interpreted as a loss of internal solute. This critical volume coincides with that producing leakiness in the experiments discussed earlier (Fig. 2). Similar results were obtained with kidney mitochondria suspended in urea and thiourea.

The simultaneous occurrence of a process tending to reduce the volume (*e.g.* leakiness) could conceivably mask an increase in permeability or available surface. Therefore, it could bring about a spurious interpretation. However, such coincidences are highly unlikely since leakage is time dependent and relatively independent of the penetrant used. The increase in either surface area or volume will vary differently with time for each penetrant and a coincidental relationship could not hold in all cases.

This question can be examined in more detail with the same penetrant and in the same experiment. A systematic variation of the initial volume (V_o) should correspondingly alter the rate of volume change (see Equation 1). It is not likely to alter the rate of leakage or other independent processes accordingly. An experiment of this kind

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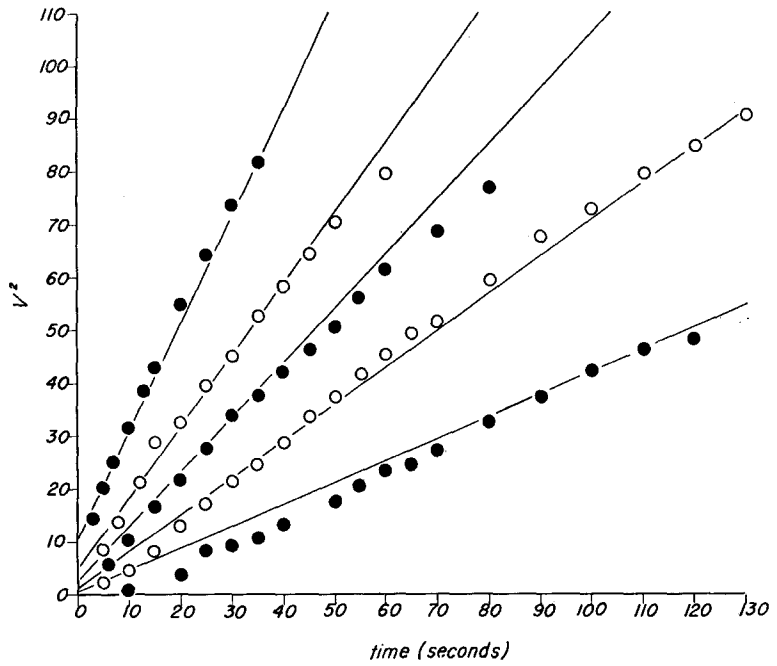

FIG. 5. The kinetics of permeability of liver mitochondria in the presence of various concentrations of malonamide. Solutions of 0.5, 0.3, 0.2, 0.15, 0.10 osmolal in 0.001 M tris, pH 7.4, were used. P = 0.0033 cm./hr. on the assumption that $A = 4.01 \times 10^{-8}$ sq. cm. (see Equation 2).

was carried out with various concentrations of malonamide (Fig. 5). The lines in this figure were calculated from Equation 2, with the assumption of no change in permeability or permeable area. The permeability constant used was the average of the calculated constants for each concentration. There is good agreement between the predicted values expressed by the lines and the experimental values. Some significant deviations occur at the lower volumes (see Discussion).

Since a plot of V^2 with time produces a straight line, the slope $2AV_oP$ remains unchanged and thus it would seem probable that the permeability and permeable surface also remain unchanged. Conceivably, it is possible that the permeability could in fact decrease to the same extent as the increase in surface area. However, such a systematic coincidence is highly unlikely over such a wide range of stretching (2 to 3-fold increase in apparent surface area). In the case of the cell membrane, moderate swelling results either in no significant change in permeability or in an increase in permeability (*e.g.* see 5, 31, 21), while more marked swelling invariably results in an increase in permeability or lysis (*e.g.* see 26, 15, 22, 31).

A swelling without apparent change in available surface area or permeability suggests that the surface membrane is convoluted. A bimolecular lipid layer would constitute the simplest model of a lipid semipermeable membrane. Accordingly, this bimolecular layer could be convoluted (Fig. 6, Model *A*). The convolutions of the proposed bimolecular lipid layer would be lined by the hydrophilic portion of the molecules; thus the space enclosed by the folds would be hydrated and therefore, readily accessible to the penetrant.

A second alternative is also evident (Fig. 6, Model *B*). The inner layer of this bimolecular layer could be the main barrier to diffusion (see Discussion). In this case, the folds would be lined by the lipophilic ends of the molecules and should be readily accessible to sufficiently lipid soluble penetrants. In artificial systems (*e.g.* soap micelles), the analogous space between two monomolecular layers (the lipophilic side) is accessible to nonpolar substances (*e.g.* oil) (32).

The two possibilities can be distinguished experimentally. In the first alternative (Model *A*), the permeable surface area should remain unchanged during swelling regardless of the substance used. In the second alternative (Model *B*), the

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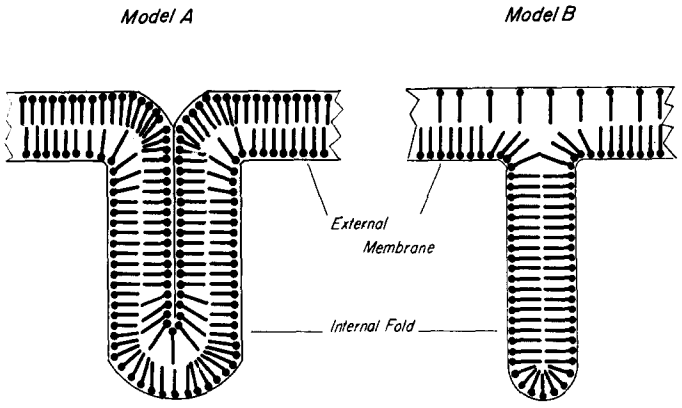

FIG. 6. Models proposed for the molecular structure of the lipid component of the mitochondrial membrane. The figures (Models *A* and *B*) represent a small portion of the proposed surface structure. The polar end of the molecules is represented by the circles, the non-polar portion by the rods.

Model *A*. A convoluted bimolecular lipid layer (see Text).

Model *B*. A bimolecular lipid membrane where the inner layer is convoluted (see Text).

space between the folds should not be readily accessible to highly polar and consequently relatively lipophobic compounds. For these substances, the penetrability should be a function of the surface area which is increasingly exposed during swelling. This turns out to be the case for erythritol and mannitol (Figs. 7 *A* and *B*).

A plot of V^2 with time, used in the previous analysis, shows a progressive increase in slope which is of the right order of magnitude to be accounted for by a progressive increase in accessible area. However, a more exact theoretical analysis is possible.

Our theoretical expectations are again expressed by Equation 1. However, in this case, the available area cannot be considered constant. After replacing V and A by the corresponding functions of the radius (Equation 3), on the assumption that the isolated mitochondria are spherical (see 36), the relationship integrates readily to the form of Equation 4. In these equations r corresponds to radius, b to the osmotically inactive volume, and G to the integration constant $([V_o^2 - 2bV_o - 3b^2]/A_o)$.

$$\frac{4\pi r^3\,dr}{3} - b\,dr = PV_o\,dt \qquad (3)$$

$$\frac{V^2 - 2bV - 3b^2}{A} = \frac{4}{3}PV_o t + G \qquad (4)$$

A plot of $[V^2 - 2bV - 3b^2]/A$ with time, in this case, should give a straight line with intercept at G and a slope of $4/3\ PV_o$. The experimental results (Fig. 7) are in good agreement with the derived equation. The relationships obtained approximate a straight line with intercept at G (which is negative).

The surface area was calculated from the photometric estimates of volume, making use of the measurements of osmotically inactive volume already published and assuming that isolated mitochondria are spherical (see 36).

The results are consistent with the interpretation that the surface area increases during swelling while the permeability remains constant. These results may be interpreted to mean that the proposed convolutions are not accessible to highly polar compounds and the surface becomes available to them only after unfolding during swelling. Therefore, we can tentatively consider the second model proposed (Fig. 6, Model *B*) as a better approximation of the structure of the lipid component of the mitochondrial membrane.

DISCUSSION

The results presented in this paper can be explained most simply by postulating the presence of a semipermeable membrane composed, at least in part, of a bimolecular lipid layer, in which the internal monomolecular layer is convoluted (Fig. 6, Model *B*). Although there is still considerable doubt about the interpretation of the information available from electron microscopy, it seems improbable that the mitochondrial surface structure is composed of a single bimolecular lipid layer.

Mitochondria are bound by composite mem-

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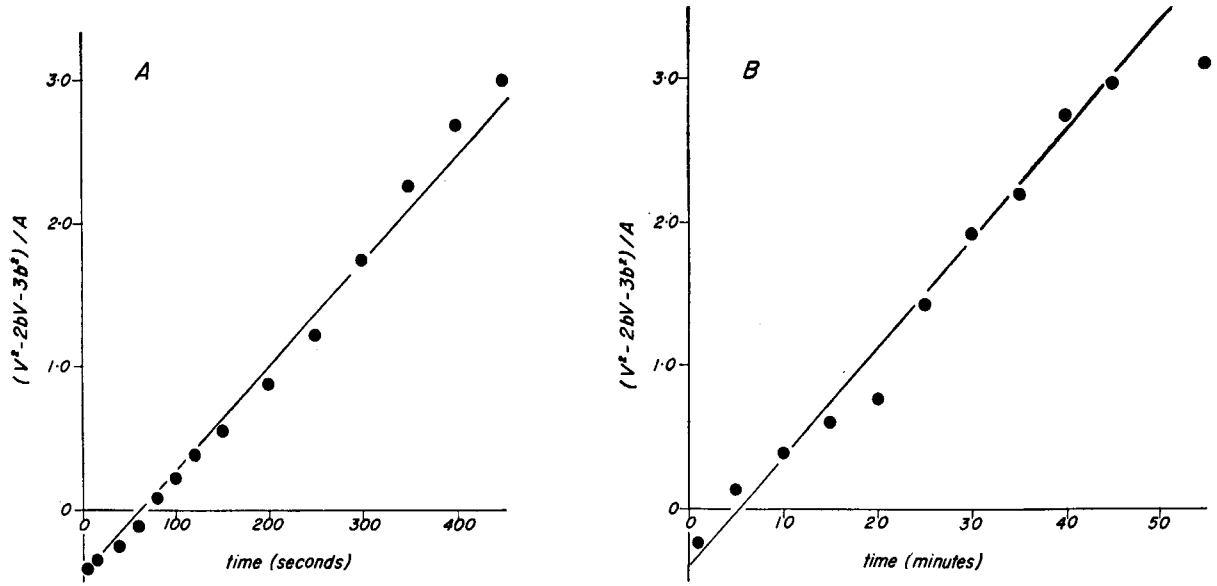

FIG. 7. The kinetics of permeability of liver mitochondria to highly polar compounds.

A. Penetration of erythritol: 0.33 osmolal in 0.001 M tris, pH 7.4. Each point represents an average of 12 determinations. $P = 0.00087$ cm./hr.

B. Penetration of mannitol: 0.30 osmolal mannitol in 0.001 M tris, pH 7.4. Each point represents an average of 5 determinations. $P = 0.00014$ cm./hr.

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branes. In these structures, two dense layers enclose a more transparent layer. A system of internal lamellae (the cristae) of equivalent morphology is in contact with these surface structures. Some authors consider the cristae discontinuous with the surface membranes (*e.g.* 35). However, it is generally agreed that the transparent space and the inner dense layer of the surface structures are continuous with the two equivalent layers of the cristae (*e.g.* 25, 20, 12).

Sjöstrand (34, 35) has proposed that the dense layers correspond to protein layers and the lighter layers to lipid bimolecular layers. This postulate is roughly in agreement with the present results if we consider the bimolecular arrangement of Model *B* (Fig. 6) enclosed by two protein layers. The convolutions in this case would correspond to the cristae, and would therefore, be continuous with the surface membrane. It is, however, unlikely that the dense layers correspond exclusively to proteins.

The dense layers observed in the earlier electron micrographs can be resolved into double layers enclosing a lighter layer (28, 12, 30). It has been proposed that these double structures represent, not protein layers, but bimolecular lipid layers perhaps in combination with other material (*e.g.* proteins) (30). Indeed, a number of studies with natural and artificial lipid systems support this view (30, 27).

This hypothesis would still leave unresolved the nature of the larger transparent space bound by these two composite layers. Conceivably, it could be lipid material, as argued by Sjöstrand (34, 35). The OsO_4 staining of lipids is considered to occur at unsaturated sites of the molecules (*e.g.* 1, 11). It is not unlikely from its similar fixing and staining action on membranes, that the action of permanganate, also used in some works (*e.g.* 30), is analogous. Apparently, about half of the fatty acids obtained from the lipids of mitochondria are saturated (18). If the saturated lipids were present preferentially in one layer, this portion would appear lighter with the electron microscope or would be altogether missing because of its solubility in organic solvents (see 11). The thickness of this lighter layer varies, depending on the tissue, from approximately 40 A to 80 A (see 35). It is, therefore, within the range of thickness of a bimolecular lipid layer. X-ray diffraction work with model lipid systems indicate a spacing between 65 and 78 A for wet preparations; shorter periods occur for dried preparations (*e.g.* 43 A for lecithin) (see 2).

The morphological data and the information from the kinetics of permeability can be explained by postulating a membrane composed of three bimolecular lipid leaflets. The enclosed leaflet would correspond to the large transparent space shown by electron microscopy, while the other two leaflets would correspond to the two double membranes. The inner monomolecular layer of the middle leaflet (the large transparent space) and the whole bimolecular lipid layer of the internal leaflet (the inner double layer) would be convoluted. This would constitute the semipermeable membrane studied in these experiments. In this model, the non-convoluted monomolecular portion of the middle leaflet and the outer leaflet (the external double layer) would be highly permeable. This need not be an intrinsic property of the layers since, as soon as these are stretched during swelling, their permeability would be highly increased. That this is probably the case is indicated by the lower permeability of the membrane at very small volumes (*e.g.* the deviations from linearity shown in Fig. 5). According to this interpretation, the cristae would correspond to the convoluted portions of the membrane and would be continuous with the surface membrane. The thickness and morphology of the cristae would roughly correspond to the outer membrane with which it would be continuous. This interpretation does not exclude a role for protein components which could be, for example, combined with the two enclosing leaflets (the double layers). However, the present data furnishes no information on this question.

It should be noted that these results cannot be readily explained by a model which postulates two bimolecular lipid layers (the double membranes) separated by a non-lipid interspace. For example, the postulate of the presence of convolutions of the inner double membrane would fail to explain the non-availability of the surface of the folds to highly polar compounds.

The possibility of the cristae constituting infoldings of the surface membrane was considered previously by Palade (24, 25). The alternative was considered unlikely at the time because variations in the tonicity of the medium were reported not to affect the length of the cristae. It would seem difficult, however, to predict local tonicity when mitochondria are fixed *in situ*. Considerable swelling could also occur in rod-shaped mitochondria

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without the need of increasing the surface area if the shape of the particles were changed simultaneously. A change of shape from a rod shape to a spherical shape, which does accompany swelling, could make available as much as 40 per cent of the surface area to a concomitant swelling without the need for drawing new material from the cristae. Although presently available results with *in situ* fixation might not support this point of view, there is some additional information from electron microscope studies of isolated preparations. Unquestionably, in these cases, a decrease in the number and length of cristae accompanies a reduction of the tonicity of the medium (see and compare 38, 39, 23, 33, 19).

The proposed model is presented as a working hypothesis. Undoubtedly, it will require modification in the future.

Alternate explanations of the present results cannot be excluded at this time; however, it should be noted that the model proposed explains: (*a*) the morphology and dimensions of the mitochondrial membranes as seen by the electron microscope, (*b*) the apparent constancy of the available surface area during swelling in the case of the penetration of fast penetrants, (*c*) the apparent increase in available surface area during swelling, in the case of slow penetrants, (*d*) the apparent constancy of the permeability during swelling, and (*e*) the shortening or disappearance of the cristae following considerable swelling of isolated mitochondria.

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