SOME OBSERVATIONS ON THE PERMEABILITY OF MITOCHONDRIA TO SUCROSE

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ABSTRACT

In experiments carried out with sucrose-isolated rat liver mitochondria, C^{14}-sucrose seems to penetrate most of the mitochondrial space rapidly, whereas it seems to penetrate the remainder relatively slowly. However, taking into account the wide size distribution found in isolated preparations, these rates have been found consistent with the kinetics expected from the assumption of a single compartment per mitochondrion. These data cannot be readily interpreted on the basis of two mitochondrial spaces, one highly permeable, and another relatively impermeable to sucrose.

INTRODUCTION

It has been reported in a number of works that isolated mitochondria are rapidly permeable to sucrose (1–5). Sucrose seems to penetrate most of the solute space rapidly, whereas it seems to penetrate the remainder relatively slowly (*e.g.*, references 1, 3). These findings have been interpreted by some investigators to mean that two spaces may be present in mitochondria, a space freely permeable to sucrose, and a space relatively impermeable to it (2–5). This interpretation has been lent support by the observation that, under the conditions used, only the space not penetrated by C^{14}-sucrose seems to respond osmotically (4).

In this work, this question has been re-examined experimentally by studying the kinetics of penetration of C^{14}-sucrose. Under these conditions, the experiments confirm that the permeability to sucrose is relatively high, approximately 3 to 6 $\times$ 10^{-5} cm/hour. In addition, initially the sucrose is taken up (moles/unit time) rapidly and subsequently more slowly. However, taking into account the wide size distribution found in isolated preparations, the rates have been found consistent with the kinetics expected from the assumption of a single compartment per mitochondrion.

MATERIALS AND METHODS

Isolation of Mitochondria

Male rats of the Holtzmann strain ranging in weight from 200 to 300 gm were starved overnight for 15 to 20 hours. They were then killed by a blow on the head, followed by cervical fracture of the spinal cord. The livers were immediately removed, cut into large pieces and washed in chilled 0.4 molal sucrose (pH 6 to 8). In a typical experiment, 8 to 9 livers were used. After mincing with scissors, the livers were homogenized in 0.4 molal sucrose in a glass and Teflon homogenizer of the Potter-Elvehjem type. The temperature was maintained between 0 and 4°C. Approximately 5 gm of liver was homogenized in approximately 20 cc of sucrose. The total homogenate was diluted to about 40 cc per 5 gm of liver and centrifuged at 600 *g* for 15 minutes in a LRA-1 Lourdes angle centrifuge at 0°C. The supernatant, recovered by suction, was then centrifuged at 8,500 *g* for 15 minutes (0°C). The resulting pellets were homogenized with a loose homogenizer of the Potter-Elvehjem type into 160 cc of cold 0.4 molal sucrose. 20 cc mitochondrial suspensions were layered on 20 or 25 cc of 0.55 molal sucrose, 0.02 M Tris (tris(hydroxymethyl)aminomethane) at pH 7.5 at 0°C. After centrifugation at 10,300 *g* for 20 minutes at a cham-

ber temperature of −2°C, the supernatant layers were removed by suction. The pellets were resuspended in 0.55 molal sucrose, 0.02 M Tris, pH 7.5 (10 cc/ 5 gm of original liver).

Radioactive Substances Used

The labeled substances used were obtained from New England Nuclear Corporation (Boston, Massachusetts); they consisted of C^{14}-sucrose (uniformly labeled, approximately 5 mc/mmole), C^{14}-glycerol (1,3 C^{14}, approximately 0.06 mc/mg) and C^{14}-carboxydextran of a molecular weight of 15,000 to 17,000 (approximately 0.55 mc/gm). Some experiments were carried out with C^{14}-carboxydextran of a molecular weight of 30,000 to 40,000 (1.1 mc/gm) and 60,000 to 90,000 (0.81 mc/gm). Results of these experiments were identical with those obtained with the lower molecular weight compound. The C^{14}-glycerol solutions used contained unlabeled 0.1 molal glycerol. All labeled solutions were in 0.55 molal sucrose, and 0.02 M Tris, at a pH of 7.5 at 0°C. In one group of experiments, it was necessary to determine whether unlabeled carboxydextran competed with the labeled variety of identical molecular weight (15,000 to 17,000). The unlabeled compound was prepared by New England-Nuclear Corp. In these experiments, the unlabeled carboxydextran used was at a concentration of 0.018 gm/cc and the labeled compound at a concentration of 0.00018 gm/cc.

Procedures

Initially, aliquots of the suspension were placed in heavy glass tubes which were centrifuged at 33,000 to 36,000 *g* for 50 minutes. At these speeds, the chamber temperature of the centrifuge was maintained at −8°C. These conditions were found to approximate a preparation temperature of 0°C (1 ± 1°C). After the centrifugation, the tubes were drained by suction and the sides of the tube dried with absorbent paper.

In the experiments, 0.200 cc of the labeled sucrose solutions was added to the pellets. The lambda pipettes and their contents were chilled in ice for 5 to 10 minutes before addition. The pellets were approximately 0.15 to 0.2 gm in wet weight. The added solutions and the pellets were then mixed (glass stirrer and magnetic stirrer for 5 minutes, see Results) and incubated for varying periods of time. C^{14}-carboxydextran suspensions were usually incubated for 20 minutes at 0°C., the C^{14}-sucrose suspensions for variable times at 0°C., and the C^{14}-glycerol suspensions for 10 minutes at room temperature. The preparations were centrifuged at 33,000 to 36,000 *g* for 12 minutes (top speed) at a chamber temperature of −8°C (sample temperature of about 0°C). Approximately 0.1 cc was removed for counting. For the determination of C^{14}-carboxydextran spaces and dry weights, approximately 0.2 cc was removed after centrifugation for 20 minutes. For counting, 0.050 cc of 1:10 dilutions of the radioactive solutions were placed evenly on Whatman No. 540 paper, 2.1 cm in diameter and counted while wet with a D-47 counter (Nuclear-Chicago) operated with a micromil window, and a 181 A or 192 A scaler of Nuclear-Chicago. Although no systematic experiments were carried out to test these points, it was noticed that the results were more reliable when the sucrose solutions containing the label were diluted, possibly because of the lower viscosity after dilution. After prolonged storage in the cold (usually frozen), followed by prolonged centrifugation, the radioactivity of the stock solutions of C^{14}-carboxydextran was found to be slightly lowered (about 1 to 2 per cent). For this reason, all C^{14}-carboxydextran stocks were centrifuged for 60 minutes at 33,000 to 36,000 *g* at −8°C chamber temperature before use.

For estimating water, the pellets in all experiments were dried at 60°C in a vacuum oven for 48 hours or more. These conditions were found sufficient to bring the pellets to constant weight.

RESULTS

Rationale of the Methods

The study of the permeability of isolated mitochondria by direct estimation of uptakes requires the incubation of the suspended particles in the presence of the solute under study. After the desired exposure, the mitochondria can be separated out from the incubation medium by centrifugation or filtration (*e.g.*, reference 19). The penetration of the substance can be determined after the separation by estimating the amount of solute present in either the mitochondria or the supernatant. This can be accomplished chemically, or, in the case of radioactively labeled components, by conventional counting techniques.

The method of making estimates of the penetration from the amount remaining in the supernatant following centrifugal separation has the advantage of not requiring packing into a tight pellet; therefore, sedimentation need not be prolonged. It is the method followed in most of this work. However, this procedure has decided disadvantages; the differences between the various experimental samples is small because of the large volume necessary in the resuspension medium.

Measurement of meaningful kinetics requires complete mixing of the particles in the pellets with the suspending medium. Alternatively, a thoroughly mixed mitochondrial suspension can be mixed with the solution in question. The latter

procedure has the advantage of removing any doubts as to the thoroughness of the mixing. However, for the concentrated suspensions used in these experiments, thorough resuspension in a small volume, such as that obtained by gentle homogenization of the preparation with a Potter-Elvejhem homogenizer, occasionally results in extensive damage. That damage has taken place is shown by increases in C^{14}-carboxydextran space and sucrose space. In some cases, the C^{14}-sucrose space measured in the shortest possible time (12 minutes after mixing) corresponded to the total space available to the solute (the space occupied by either C^{14}-glycerol or C^{14}-sucrose after equilibration). In two cases where this was not the case, the kinetics of penetration corresponded to those of the experiments described here.

After addition of 0.200 cc of labeled medium, mixing was carried out with a conventional glass rod, followed by 5 minutes' mixing with a magnetic microstirrer (approximately 80 RPM). After this procedure, the suspensions appeared homogeneous. Ideally, mixing should be continued throughout the incubation to avoid formation of concentration gradients in the external medium. Unfortunately, prolonged mixing results in extensive breakdown. This is reflected in the appearance of protein in the supernatant, a systematic increase with time in the C^{14}-carboxydextran space and a decrease in the dry weight upon resedimentation. With 5 minutes' mixing, the amount of breakdown is minimal but probably still occurs. Some of the results that follow (Fig. 1, *A* and *B*) are expressed as ratios of either volume (cc) or weight (gm) per unit weight of the original wet pellet (gm) before mixing. Therefore, the small but significant weight of the disrupted particles has not been excluded from the wet pellet weights used in the calculations. Since these are only used as reference values, and the ratio varies but little from experiment to experiment, this procedure is justified.

The results presented in this paper are the means of several experiments (usually 4 or 5). Although the interpretation of each individual experiment is in agreement with the interpretation presented, an analysis based on a single typical experiment, with single or duplicate determinations, is generally unsatisfactory since some doubts always remain about the actual magnitude of the experimental error. The pooling of several experiments is considerably simplified by the fact that the results are reproducible from experiment to experiment (*e.g.*, see Fig. 1 and legend of Fig. 3). The deviations given in this discussion are standard errors.

The magnitude of intramitochondrial volume (V_m) and hence extramitochondrial volume (V_e) are necessary for evaluating the kinetic results (Fig. 3). Experiments were used in this analysis when V_m and V_e were approximately the same (differences were about 10 per cent). Results of other experiments with different V_m or V_e values are comparable. The errors given (*e.g.*, Fig. 3) are likely to include (*a*) small variations in V_m or V_e, (*b*) the small differences in the properties of the preparations and, of course, (*c*) the experimental errors of the measurements *per se*.

Estimate of the Volumes

A quantitative evaluation of the results requires a knowledge of the volume of the mitochondria (V_m). Estimating V_m requires the determination of the volume external to the mitochondria.

The volume external to the mitochondria can be determined by using a substance which does not penetrate significantly and is not bound to the particles. High molecular weight substances such as proteins or polysaccharides are presumed not to penetrate. In the past, C^{14}-carboxypolyglucose (2), C^{14}-carboxydextran (6) or I^{131}-iodinated albumin (1) have been used. Lack of binding or penetration has been inferred from the correspondence of the polymer space to the space predicted theoretically as extramitochondrial from the assumption of tightest possible packing of perfect spheres. An additional validation used has been the failure of this apparent space to decrease with increasing external concentration. Such a decrease would be expected in the presence of binding (3), if the concentration is sufficiently high to saturate binding sites. Alternatively, lack of penetration has been assumed from the failure of this space to increase markedly with time (*e.g.*, references 1, 3). C^{14}-carboxydextran has been found to distribute in accordance with the interpretation that it serves well as an extramitochondrial marker (6). It is for this purpose that it was used in the present experiments. In the present work the space occupied by C^{14}-carboxydextran remains constant with time for the period tested (Fig. 1, curve *A*) and is therefore likely to be restricted to the external space in agreement with previous observations (6). The measurements reported are expressed as *R*, the ratio of volume

penetrated (cubic centimeters) per unit weight of wet pellet (grams). A binding of C^{14}-carboxydextran is not likely since the addition of unlabeled carboxydextran (100 times the amount of labeled material) is without effect. The ratio of the C^{14}-carboxydextran space in the presence of unlabeled carboxydextran to the space determined without the addition of unlabeled material is 0.98 ± 0.05 (4 independent experiments, the deviation is the standard error).

The analysis of the data would be considerably simplified if it were possible to demonstrate that during the experimental period no swelling, and therefore no variation in mitochondrial volume, has occurred. This is shown by the constancy of the dextran space with time (Fig. 1) and the corresponding constancy of pellet weight (not shown). It is conceivable, however, that swelling may remain concealed by a breakdown of the particles. A fragmentation or lysis of the particles should be detectable as a decrease in the dry weight of the pellets. However, these remain unchanged after the incubation (Fig. 1, curve *B*).

The constancy of volume with time is somewhat puzzling in view of the high permeability to sucrose of these preparations (see following discussion). This is probably the result of (*a*) a matching of the sucrose penetration with the exit of an internal solute, and/or (*b*) an almost complete equilibration with sucrose during the isolation procedure. An exit of potassium has been reported in preparations where the volume does not significantly change (3).

The volume available to the penetration of sucrose is somewhat in question. The volume of water in the mitochondrial phase can be easily calculated from the weight of the pellets before and after drying (with a correction for the extramitochondrial phase and sucrose trapped in the extramitochondrial phase). The extramitochondrial volume can be estimated from the C^{14}-carboxydextran space. However, the volume of the internal solution is not likely to correspond to the water space. The latter can be easily calculated by making assumptions in relation to the composition and specific gravity of the internal medium. However, these calculations leave a considerable margin of doubt. For this reason, the volume available to the penetration of C^{14}-glycerol was determined (incubated for 10 minutes at room temperature). Under these conditions, assuming a permeability constant of approximately 1×10^{-2} cm/hour (see reference 7), equilibrium in relation

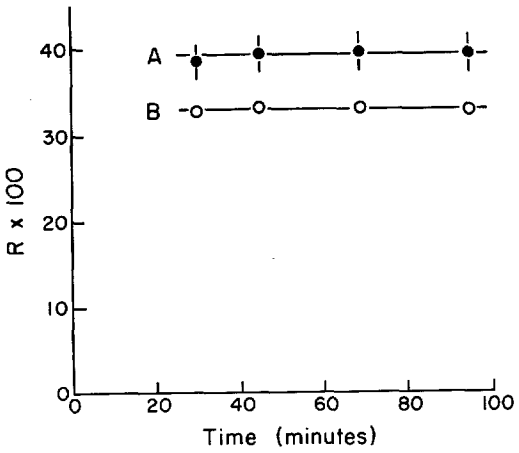

FIGURE 1 Curve *A*, constancy of the C^{14}-carboxydextran space. *R* is the volume penetrated by dextran per wet weight of pellet, cc/gm. See text. The deviations recorded are standard errors. The results presented are the means of 4 experiments.

Curve *B*, constancy of the dry weights after incubation with C^{14}-carboxydextran. The weights are not corrected for extramitochondrial sucrose. They are expressed as dry weight per wet weight of pellet. See text. The results presented are means of 4 experiments. Deviations from these values are less than 1 per cent,

to glycerol should occur in a few seconds. Although this volume is usually somewhat lower (10 to 15 per cent) it agrees at least roughly with the calculated volumes of internal solution (these assume 20 to 30 per cent of mitochondrial protein to be soluble (see references 20, 27), 70 per cent of the solids to be protein (see reference 21) and a volume to weight ratio of 0.75 (22) for protein in solution). The glycerol space is reproducible from experiment to experiment and it is a fixed percentage of the total pellet weight. Expressed as C^{14}-glycerol volume (cubic centimeters) per initial weight of wet pellet (gram) it was measured as 0.695 ± 0.034 (4 independent experiments, the deviation is the standard error). This fixed proportion was used to estimate mitochondrial volume (V_m) from the weight of wet pellets. Direct estimates of water weight were also carried out for each experimental sample and the calculated experimental volumes were approximately in agreement with the C^{14}-glycerol distribution volumes (10 to 15 per cent).

Distribution of Particle Size

The quantitative interpretation of data on the kinetics of the penetration of a solute requires a

knowledge of the size of the particles. This is particularly important for a population which has been found in previous work (*e.g.*, see reference 8) to vary considerably in size.

That size plays a significant role is readily apparent from theoretical considerations, well recognized in the study of the permeability of cells (*e.g.* see reference 13). The rate of diffusion of an uncharged solute should be approximated by Equation 1 which is a statement of Fick's Law. In this equation k is the permeability constant, A is the surface area available to penetration, V, the volume, C_i, the internal concentration of penetrant, C_o, the external concentration of penetrant, and t is time. Thus, the apparent permeability (which for purposes of discussion can be defined as kA) of the volume occupied by any particular population or class size is a function of the surface area.

$$\frac{dC_i}{dt} = k\frac{A}{V}(C_o - C_i) \qquad (1)$$

The measurements of size distribution already available (*e.g.* reference 8) make it clear that this is by no means a negligible factor. For example, in the swollen mitochondria measured (8), there are sizable populations, 0.5 and 2 μ in diameter. The difference between the two corresponds to a 16-fold difference in surface area and hence a 16-fold difference in apparent permeability. Estimates of the size distribution of the particles bearing succinic dehydrogenase activity (9), and the different sedimentation properties of the particles bearing succinic-cytochrome C reductase activity (10) are in general agreement with this concept. Similar differences are found in the size distribution of the present preparation (see below, Fig. 2). It is possible that these measurements are overestimates, since it is difficult not to bias the estimates in favor of the large populations, particularly since the light microscope is used close to its limit of resolution (approximately 0.2 μ). In our preparation, for example, the distribution of sizes (see Fig. 2, the curve) suggests that a significant portion of the population is below a diameter of 0.2 μ but is not detected. If this inference were correct, as much as a 30-fold difference in the apparent permeability of two significant mitochondrial populations, one of maximal and the other of minimal size, would be possible.

In this study, in order to obtain an estimate of the size distribution of the particles, a sample

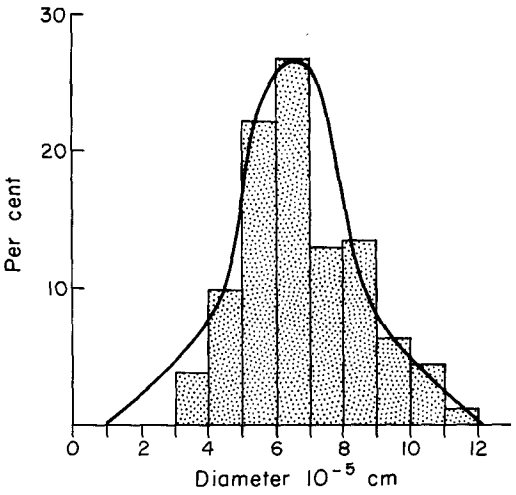

FIGURE 2 Sizes of the mitochondrial population; the mean diameter is 6.8×10^{-5} cm (see text). By volume rather than number, the population distributes in this fashion:

Diameter (cm $\times 10^{-5}$)	3.5	4.5	5.5	6.5	7.5
V_m per cent	0.4	2.3	9.6	19.3	14.3
Diameter (cm $\times 10^{-5}$)	8.5	9.5	10.5	11.5	
V_m per cent	21.4	14.2	13.4	5.0	

preparation was photographed with an American Optical Company phase microscope with positive contrast and oil immersion. An exposure of 1/125 of a second was used. The particles were suspended in the incubation medium in the presence of 13 per cent dextran to slow down Brownian motion. The total enlargement, including the photographic magnification, was 2,000. Many of the particles were not perfect spheres, and in these cases, as a simplification, an average of the major and minor axes was used (these are shown in Fig. 2). These measurements can only be considered an approximation because of the limited resolution of the light microscope in this size range, and the fact that some of the particles are not perfect spheres. However, these measurements are in good agreement with the mean values previously obtained (8, 11). For example, the mean diameter obtained previously (11) at the corresponding osmotic pressure was 6.4×10^{-5} cm (see reference 12, Fig. 1), indistinguishable from that of these experiments.

The presence of dextran (molecular weight

60,000 to 90,000) is not likely to have altered the mean values significantly since these are in good agreement with those published previously (8, 11), where dextran was not used.

In order to check whether accurate results can be obtained with this technique, measurements were carried out with latex particles. Particles 0.87 μ in mean diameter were measured as 0.83 μ with the light microscope (88 particles counted). Particles 0.37 μ in diameter were measured as 0.35 μ (50 particles counted). It is therefore likely that the results obtained are at least approximately correct. However, it should be recognized that the contrast and sharpness of image is greater in the case of the latex particles.

Estimate of the Time of Exposure

The times of exposure to the label during the centrifugation are not known exactly. This is of little significance for the longer incubations; most of the exposure consists of the incubation itself. However, for shorter incubations the centrifugation time becomes significant. Since a significant amount of mitochondria is not sedimented from the top 0.1 cc sampled until after approximately 2 minutes at top speed, this time was included, along with 1 minute of manipulation and 2 minutes required to reach top speed, in the total time. These times can only be an approximation since (*a*) setting the time arbitrarily in this fashion can only be an oversimplification, and (*b*) some diffusion of the label to the bottom layer undoubtedly occurs. However, although altering these estimates does alter the calculated permeability constant, it has little effect on the pattern of the kinetics. For example, changing our estimates of the centrifugation time to 15 minutes (a maximal estimate since it includes the time span from the introduction of the label to the removal of the sample) lowers the permeability constant from 6×10^{-5} to 3×10^{-5}. For purposes of comparison, the data were analyzed making use of either our minimal estimate (Fig. 3 *a*) or our maximal estimate (Figure 3 *b*). However, the minimal estimate is probably the more realistic of the two.

Kinetics of Penetration

The mean values of 5 separate experiments, expressed as the amount of penetrant in the pellet, are shown in Fig. 3 (the large circles).

Diffusion of an uncharged solute into a spherical granule should be approximated by the following

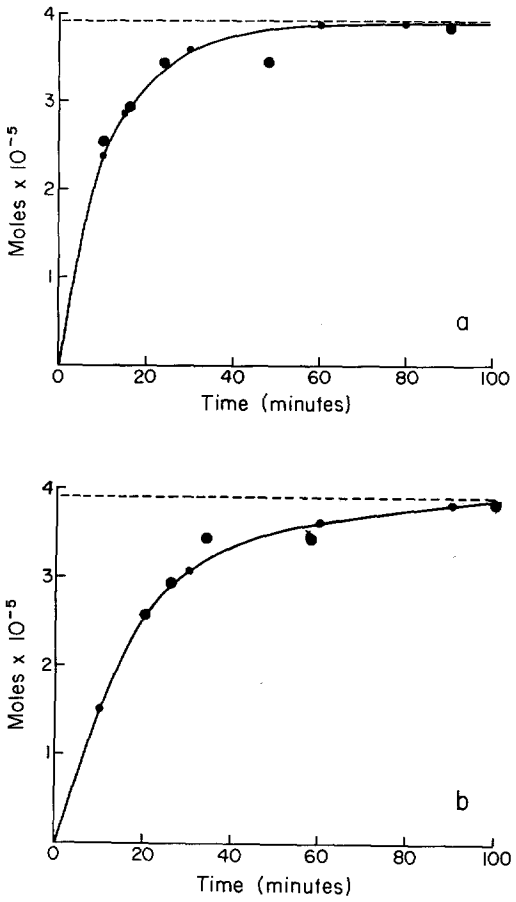

FIGURE 3 Kinetics of penetration of sucrose. The amount of sucrose which has penetrated at any particular time ($S(t)$) has been calculated from Equation 6. The small circles are calculated values. The large circles are experimental values. Although the estimates were for C^{14}-sucrose, the penetration of the total sucrose has been calculated and included in the estimate of $S(t)$. The constants used were $V_m = 0.089$ cc, $V_e = 0.340$ cc. The dashed line represents the theoretical equilibrium. In chronological order, the standard errors were: 0.32×10^{-5}, 0.22×10^{-5}, 0.22×10^{-5}, 0.13×10^{-5}, 0.30×10^{-5}.

For Fig. 3 *a*, the time of exposure for the earliest point was considered to be 10 minutes. Subsequent points followed the same assumptions. This is probably a minimal estimate. k in this case is 6×10^{-5} cm/hour. See text.

For Fig. 3 *b*, the time of exposure for the earliest point was considered to be 20 minutes. Subsequent points followed the same assumptions. This is the maximum possible estimate since the sample was removed from the supernatant at this point. k in this case is 3×10^{-5} cm/hour. See text.

expectations. The rate of penetration should follow Fick's Law as expressed in Equation 1. For spherical granules, this equation assumes the form of Equation 2, where C_i is the internal concentration of the substance in question; k, the permeability constant; C_o, the external concentration, t, time; and r, the radius of the particles. Where the assumption is made that C_o remains constant, the equation readily integrates as shown in Equation 3. In the presence of a heterogeneous population, the amount of solute which has penetrated at any given time is represented by Equation 4, where

$$\frac{dC_i}{d_t} = 3\frac{k}{r}(C_o - C_i) \tag{2}$$

$$C_i = C_o\,(1 - e^{-3kt/r}) \tag{3}$$

$S(t)$ is the amount of penetrant inside at time t, and $N(r)$ is the function describing the number of mitochondria as a function of radius. The constant k can be estimated approximately at very early times (t approaching 0). Under these conditions,

$$S(t) = \frac{4}{3}C_o\,\pi\int_0^\infty N(r)r^3(1 - e^{-3kt/r})\,dr \tag{4}$$

$1 - e^{-3kt/r}$ approaches $3kt/r$. After this initial determination, the constant can be more accurately determined by successive approximations. This procedure was followed making use of Equation 4 and integrating the relationship arithmetically, using the distribution of Fig. 2. The constant k was found to be 6×10^{-5} and 3×10^{-5} cm/hour, as the maximal and minimal estimates, respectively (see above discussion).

Equations 3 and 4 can only be approximate since C_o is not constant with time. Fortunately it does not vary much with time and it follows the empirical Equation 5. The measured values of C_o deviate from those predicted by this relationship by no more than 2 per cent. In our faster estimate of penetration, the constant a is 0.439 molal, b is 0.120 molal and h, 5.886 per hour. In our slower estimate, h becomes 3.361 per hour.

$$C_o = a + be^{-ht} \tag{5}$$

Substitution of Equation 5 into Equation 2, followed by the appropriate manipulations leads to Equation 6, which was used in preparing Figs. 3 *a* and 3 *b*. In Fig. 3, the small circles represent values from Equation 6; the line was drawn through these points.

$$S(t) = \frac{4}{3}\pi\int_0^\infty N(r)r^3\left[a + \frac{3bk/r - e^{-ht}}{(3k/r) - h} - \left(a + \frac{3bk/r}{(3k/r) - h}\right)e^{-3kt/r}\right]dr \tag{6}$$

From these results (Fig. 3, large circles), it is clear that the kinetics of sucrose uptake conform rather well to our expectations for a single space in each mitochondrion and uniform permeability constants throughout the mitochondrial sizes.

It may be argued that all the granules may not be uniform in relation to their permeability constant. Although the present results lend no support to this idea, it is possible and even likely that some differences are present (*e.g.*, the cell population in the liver is not uniform; the pellets do not appear uniform (see reference 1)). The error in the determinations does not permit distinguishing between small differences in permeability constant (the error ranges approximately between 4 and 12 per cent; see the legend of Fig. 3). It should be noted, however, that if we were to assume an error considerably larger than that obtained, the results would still validly reject the possibility of compartments differing significantly in their permeability constant. For example, for the analysis of Figure 3 *b*, if after 60 minutes of exposure the amount of sucrose penetrating was 10 per cent below that given in Fig. 3 (significantly higher values are not theoretically possible), the constant k would be 30 per cent below the calculated value (the standard error for this point was about 4 per cent). In the case of earlier points (*e.g.*, 12 minutes), assuming an estimate of sucrose approximately 25 per cent above or below the curve, the constant would also differ by about 30 per cent. Therefore, it would seem unlikely that we are dealing with compartments differing radically in their permeability constants.

It is also clear that these experiments cannot exclude the presence of a very small volume which is either instantaneously penetrated by sucrose or not penetrated at all. However, this volume must be very small and within the experimental error of these determinations.

DISCUSSION

The results presented above are not consistent with the interpretation that two compartments differing radically in their permeability constants

are present. Some of the reservations inherent in this interpretation are discussed above (see Rationale of Methods, in Results). A number of questions are raised by this work, particularly in relation to other studies which seem to be at variance with this interpretation. These difficulties will be discussed below.

Have the Conditions of These Experiments Excluded a Sucrose-Permeable Space?

The concept of a sucrose-permeable space assumes the presence of a space essentially instantaneously permeable to sucrose, and, in addition, a space essentially impermeable to sucrose (2–4). Would it be possible that the experimental conditions of this work have excluded this hypothetical sucrose-permeable space?

The mitochondrial breakdown may differ significantly from that of other experiments because of the more thorough mixing carried out in these experiments. As discussed along with the Results, this procedure damages mitochondria and liberates some protein from them. Conceivably, the osmotic pressure of the protein of the supernatant could be sufficient to collapse, at least in part, a space impermeable to high molecular weight substances but permeable to sucrose. In the work of some authors (*e.g.*, reference 3), increasing the concentration of C^{14}-carboxypolyglucose (to a maximum of 1 per cent) decreases the intramitochondrial space substantially. This is not the case with the present preparations. In addition, in other works this effect appears minimal (*e.g.*, reference 2). For example, in the work of Werkheiser and Bartley (2), 0.9 per cent C^{14}-carboxypolyglucose decreases the volume only slightly (5 to 10 per cent, see p. 86). In addition, 7.3 per cent PVP and 6 per cent dextran seem to have no significant effect (Table 7 and 8, reference 2). Other considerations also fail to support this possibility.

From his experiments, Share (6) reports an intramitochondrial C^{14}-sucrose space of about 44 per cent after 10 minutes' centrifugation. Malamed and Recknagel (4) report an intramitochondrial C^{14}-sucrose space of 61 per cent in 0.30 M sucrose after 60 minutes' centrifugation (see Table I, reference 4). Although the present experiments were carried out with a higher concentration, the results are comparable. For example, after 5 minutes of mixing and 15 minutes of total centrifugation time, the sucrose penetration corresponds to a C^{14}-sucrose space of 66 per cent. It is, therefore, very unlikely that we are dealing with the penetration of a space which significantly differs in permeability from that studied by these other authors.

TABLE I

C^{14}-sucrose space after 2 to 3 hours incubation at 0°C compared to C^{14}-glycerol space (10 minutes at room temperature). The result is expressed as R, the C^{14}-sucrose volume/C^{14}-glycerol volume. The results are in each case the means of 4 determinations. The deviations are standard errors.

Sucrose concentration	R
molal	
0.55	0.96 ± 0.05
0.275	0.97 ± 0.03

Have the Conditions of These Experiments Excluded a Sucrose-Impermeable Space?

The results of these experiments are not consistent with the presence of a significant space totally impermeable to sucrose. However, it should be noted that in the present experiments the osmotic pressure is considerably higher than that used in other experiments (*e.g.*, references 2–4). If the hypothetical sucrose-impermeable space were small in relation to the total volume of the mitochondria as has been postulated, conceivably at the higher osmotic pressure it has become so small as to be undetected in these experiments. This, however, does not seem to be the case since after 3 hours of incubation the space occupied by C^{14}-sucrose is generally indistinguishable from the C^{14}-glycerol space, whether the particles have been incubated in 0.275 molal or 0.55 molal sucrose (Table I). It should be noted, however, that incubations longer than 3 hours are frequently required for equilibration with the C^{14}-sucrose in 0.275 molal sucrose.

Does the Permeability of the Preparation Differ From That of Other Works?

The permeability of mitochondria to sucrose was found in this work to vary but little from day to day under the conditions described. We have noted that approximately the same permeability is apparent in the experiments of Malamed and Recknagel (4) and Share (6), carried out with C^{14}-sucrose. In the work of other authors, how-

ever, incubations are for longer periods and, at least from superficial inspection of the data, the permeability to sucrose would seem to be considerably lower. It should be noted, however, that in these works, which follow the penetration of unlabeled sucrose, the mitochondria are already partially penetrated and presumably the fractions with higher surface area/volume ratios are already in part equilibrated. The kinetics followed would, therefore, be more representative of the larger, apparently less permeable, population.

Approximate permeability constants can be readily calculated. Direct integration of Equation 2 (see discussion of kinetics, above and reference 13) provides us with equation 3 *b* where C_{i1} is the internal concentration of sucrose at the beginning,

$$\ln \frac{C_o - C_{i2}}{C_o - C_{i1}} = \frac{-3kt}{r} \qquad (3b)$$

C_{i2} the concentration at time t, and C_o is assumed to be constant.

Calculations of the permeability constants from the half-times at 12°C. supplied by Jackson and Pace (1) give us constants ranging from 0.3 to 1.0×10^{-5} cm/hour, assuming a radius of 3.7×10^{-5} cm for mitochondria in 0.25 M sucrose, and 0.1 to 0.4×10^{-5} cm/hour in 0.88 M sucrose assuming a radius of 3×10^{-5} cm. These apparent constants are lower than those calculated from the present data. As mentioned above, this may be at least in part the consequence of the fact that the population not penetrated at the beginning of the experiment represents the larger mitochondrial population. However, in view of the higher temperature (12°C) used in the experiments under discussion, it is possible that the preparations of Jackson and Pace (1) are less permeable than those used in these experiments.

Approximate permeability constants can also be calculated from the data of Amoore and Bartley. In their paper (reference 3, p. 227) these authors report that the space inaccessible to sucrose was initially 0.81 liter/kg of dry trichloracetic acid insoluble material, and 0.55 liter/kg after 4 hours. The internal concentrations calculated from these figures and the volumes given (reference 3, Fig. 1) are approximately 0.119 molal initially and 0.148 molal after 4 hours.

Assuming the radius, r, to be 3.7×10^{-5} cm, k (Equation 3 *b*) becomes approximately 0.1×10^{-5} cm/hour. This estimate is considerably lower than that of the present experiments (an apparent 30- to 60-fold difference).

It is of some interest to note that the preparations of Amoore and Bartley (2, 3) are significantly different from those of other workers. After isolation in 0.25 molal sucrose at 2,700 *g* for 20 minutes, the residue is washed twice at 8,500 *g* for 10 minutes, and the fluffy layers discarded. The isolation procedure utilizes a speed considerably lower than that used by most other workers. In addition, they originally obtain a pellet of 0.275 gm intraparticulate wet weight (calculated from Table 3, reference 3); after the washings and removal of fluffy layers, only 0.027 gm remains, approximately 10 per cent of the original preparation. These particles differ grossly from the original suspension as indicated by the fact that they can be separated in this fashion from the rest of the particles and by the differences in their gross properties (*e.g.*, sucrose content and density) and probably size (and therefore lower apparent permeability). Since the pellets are also partially penetrated at the time of testing (in the sample used for the permeability calculations, approximately 48 per cent, see Fig. 1 and p. 227, reference 3), it is also possible that the mitochondria involved constitute but a fraction of this 10 per cent.

The question may be raised of whether only a contaminant is removed by these washings. This does not appear likely because of the high percentage of the preparation removed in the purification. For example, in another work (14), although a different isolation procedure was used (18,000 *g* for 10 minutes in 0.88 *M* sucrose) the whole isolated pellet was found to be constituted primarily by mitochondria, including the fluffy layer (see Table 1, reference 14). This view is also supported by other experiments where granules bearing enzymatic activities characteristic of mitochondria are found to differ considerably in their sedimentation properties (9, 10).

Other workers have used procedures significantly different from those of Amoore and Bartley. Share (15, 6) isolates at 7,000 *g* for 12 minutes in 0.25 M sucrose without subsequent washing. Jackson and Pace (1, 16), after isolating at 15,000 *g* (15 minutes in 0.25 M sucrose and 30 minutes in 0.88 M sucrose), wash three or four times, sedimenting at the same speed and removing the top layer mechanically. Malamed and Recknagel (4) wash only once after isolation (7, 250 *g* for 12 minutes, 0.3 M sucrose). None of these procedures is likely to re-

move as large of a proportion of granules as that removed by the procedures of Amoore and Bartley. For example, a single washing carried out by Amoore and Bartley (3) removes only slightly over half of the preparation. This is probably comparable to the single washing of Malamed and Recknagel (4). In the present work (see Materials and Methods), the isolated mitochondria are centrifuged through a denser sucrose layer once. In this procedure, only a small fraction of the preparation remains in the top layers above the pellet.

From these considerations, it would seem that a difference in the permeability between the preparations of Amoore and Bartley, and Jackson and Pace and those of others remains a definite possibility.

Is There an Osmotically Inactive Sucrose-Permeable Space?

The present experiments do not support the concept that there are two spaces, one rapidly permeated by sucrose, and another impermeable to it. Instead, the results are consistent with the hypothesis that all mitochondria have the same permeability properties, at least approximately, and that it is the difference in sizes which accounts for the differences in apparent permeability. According to this interpretation, all the intramitochondrial space is osmotically active.

On the basis of their data (2–5), a number of authors have introduced the concept of two spaces with radically different permeability properties. A two-compartment organization, such as that proposed, would necessarily lead to an osmotic behavior restricted to the sucrose impermeable space, as in fact proposed (see references 3, p. 235; 4; and 5, p. 56). Whether the two spaces are present in each individual mitochondrion or in two different kinds of particles should lead to the same results and formal theoretical treatment as recognized in those works (2–5). This section will discuss whether the data of these authors (2–5) are consistent with a two-space model, and whether their results can be reconciled with the model presently proposed.

Experiments have been carried out where, after suspension of the particles into a medium of a particular concentration of sucrose, the particles are resuspended in a different concentration (Table 1, Experiments 2 and 3, reference 5; Table 10, Experiments 1, 3 to 7, reference 3).

For the model where two spaces are hypothesized, the non-penetrated volume and only this volume should respond osmotically. The sucrose-penetrated volume should be totally unresponsive.

For the model where a single space is assumed, the situation is more complex. In view of the high permeability to water (7, 11), the assumption that the total space behaves osmotically predicts that the relationship presented in Equation 7 is valid (see reference 13). ΔS is the amount of sucrose that penetrated during the experimental period; C_M is the total internal concentration of osmotically active material. C_M may include sucrose which penetrated before the experimental

$$\frac{C_M V_1 + \Delta S}{V_2} = C_2 \quad \text{or} \quad \frac{C_M V_1 + \Delta S}{C_2} = V_2 \qquad (7)$$

period. C_2 is the external concentration of solute (mostly sucrose), V_1 the initial volume, and V_2, the volume after resedimentation. The equation can be put in a somewhat different form (Equation 8). $\Delta S/C_2$ is the volume apparently penetrated during the experimental period (V_s). With this model, V_s is a purely conceptual volume and does not correspond to a physical entity.

$$\frac{C_M V_1}{C_2} + V_s = V_2 \qquad (8)$$

According to these considerations, V_s should not appear to respond osmotically. However, V_s does not include the volume penetrated in the period of time preceding the experimental period (before C_2 is introduced) since C_M is in part sucrose. Thus, with this model (single space), V_s (or ΔS) should account for the differences between $C_M V_1/C_2$ and V_2; in the alternate model (two spaces), the total apparent sucrose space should account for the osmotically unresponsive volume.

Some of the results of Amoore and Bartley (3) and Bartley (5) are inconsistent with the model which proposes the presence of two spaces. In some experiments, Amoore and Bartley find that it is the hypothetical sucrose-permeable volume which responds osmotically (*e.g.*, reference 3, Table 10, Experiments 3, 4, and 7), in others that it is the hypothetical sucrose impermeable space. Bartley finds that in one case it is the sucrose free space (reference 5, Table 1, Experiment 2) and in the other that it is the sucrose permeable space (Table 1, Experiment 3). Therefore, a significant portion of their results are not compatible with their model. All the results may, however, be ex-

plained completely or substantially by a model based on the hypothesis of single mitochondrial spaces.

Bartley finds a decrease in volume going from 0.25 molal sucrose to 0.54 molal sucrose (reference 5, Table 1, Experiment 2). The initial volume (V_1) is 2.04 liter/kg. of dry mitochondria and the final volume (V_2) is 1.62 liters/kg. Since the external space is not determined in these experiments, we may assume that it is about 20 per cent (see reference 2). A different estimate (*e.g.*, 30 per cent) would not significantly alter this argument. With this assumption, the volumes become 1.63 liters/kg. and 1.30, respectively. The predicted final volume (V_p), assuming perfect osmotic behavior and no solute transfer, would be given by the following relationship: $V_1C_1/C_2 = V_p$. In this equation C_1 is the initial external concentration ($C_1 = C_M$, Equation 7) and C_2, the final external concentration. In this case V_p is 0.75 liters/kg, or 0.55 liters/kg less than found. This discrepancy between predicted and actual volume can be accounted for by the penetration of 0.297 moles of sucrose/kg (ΔS). The uptake, experimentally measured, is 0.336 moles/kg. The shift in external volume and concentration, *i.e.* the change in amount of sucrose trapped, can account for the small difference between the two. For example, a 20 per cent external space (3) would result in more sucrose being trapped at the higher concentration, 0.071 moles/kg more. From these considerations, it is apparent that these results can be accounted for by the concept of a single, osmotically active volume presented in this paper. However, they can be accounted for equally well by the postulate that two spaces are present, one rapidly permeable and the other impermeable, as proposed by other workers. In this work (5), the reverse experimental design differentiates between these two models. In this experiment, the mitochondria are originally in 0.5 molal sucrose and are then placed in 0.281 molal sucrose (Table 1, Experiment 3). This procedure results in a swelling. The interpretation based on the two space model requires a swelling of the osmotically active volume and not that of the penetrated, osmotically inactive volume. However, the data indicate that it is the apparent sucrose permeable space which increased in volume, a finding inconsistent with the two space hypothesis. On the other hand, the results are consistent with the hypothesis of partially penetrated single osmotically active spaces as presented in this paper. In this case, the initial volume is 1.62 liters/kg and the final volume 2.27 liter/kg. From the above assumption (20 per cent external volume), V_1 becomes 1.30, and V_2, 1.82 liters/kg. Since C_1 is 0.50 molal and C_2, 0.281 molal, the predicted final volume should be 2.31 liters/kg. The difference between actual and predicted volume (0.49 liters/kg) could be accounted for by the loss of 0.138 moles/kg of solute. The shift from one concentration to the other in the extramitochondrial fluid and the change in size of the extramitochondrial space results in a loss of 0.034 moles/kg. The exit of the sucrose from the pellet would then be about 0.172 moles/kg. Bartley (3) reports a loss of 0.200 moles. It is clear that in contrast to the model which postulates two spaces of differential permeability, an osmotic model based on a single space per mitochondrion accounts for the data reasonably well. The exit of sucrose is of some interest. The actual transfer from an internal compartment may be smaller; as discussed above, this transfer must come, at least in part, from the extramitochondrial phase. Nevertheless, it cannot be explained readily. Since mitochondria may be considered to equilibrate instantaneously with the external medium, in relation to water, the total internal solute concentration could at no time be higher than that of the external medium. Since the latter is almost exclusively a sucrose solution, and the mitochondria contain solutes other than sucrose, it would seem necessary to invoke the postulate that sucrose is transferred against a concentration gradient. However, this is not necessarily the case. It is conceivable that a portion of mitochondria are approximately equilibrated in relation to sucrose (because of the higher A/V ratio, see above discussion). In that case, the internal sucrose can exceed the external concentration only where enough solute other than sucrose is present in the external medium. Considerable leakage of internal material is reported (*e.g.* potassium: 0.029 moles/kg). The total leakage could be in fact considerably higher if an anion accompanies the cation.

The experiments of Amoore and Bartley (reference 3, Table 10) are somewhat similar to these. Mitochondria originally in 0.25 molal sucrose are then transferred to a higher concentration of sucrose or sucrose-potassium chloride. For some of the experiments, either model has reasonably good predictive value (*e.g.*, experiments 8 and 9, Table 10; the volumes deviate from the single

space model by approximately 13 and 2 per cent, respectively and, from the two space model by 23 and 18 per cent). However, some of the experiments contradict the two space model (Table 10, Experiments 3, 4, and 7). It is not the non-penetrated space which responds, but the sucrose-permeable space. In these experiments, as in the ones discussed above, the volume obtained is higher than that predicted on the basis of osmotic behavior, uncomplicated by penetration or exit phenomena. For example, in Experiment 3, the deviation from V_p is 0.29 liters/kg; in Experiment 4, 0.49 liters/kg; and in Experiment 7, 0.46 liters/kg. The first can be accounted for by a penetration of 0.105 moles/kg, where 0.051 were measured, the second by 0.243 moles/kg where 0.155 were measured, and the third by 0.210 moles/kg, where 0.213 moles/kg were measured. Assuming that the differences are real, it would seem that the one space model may explain most of the effect.

A failure to attain complete agreement between the degree of swelling and the penetration of solute need not conflict with the idea that volume parallels osmotic effects. Swelling, for example, could be brought about by the formation of osmotically active material within the mitochondria. Hydrolysis of endogenous organic phosphates, for example, has been noted in mitochondria at least under some conditions (2).

In the light of these considerations, it is difficult to see how the two space model can be preserved, since it has little predictive value.

The experiments of Malamed and Recknagel (4) differ in design from those described above. The suspensions are exposed to sucrose solutions 0.3 M or lower. After resedimentation, C^{14}-sucrose is added to the pellet. It was found that excluding a calculated external space, the C^{14}-sucrose–permeable space remains about the same at the various volumes and it coincides with the apparently osmotically inactive volume. We have seen that the data of other workers as well as those of the present work are not in agreement with this postulate. It is not clear whether the data of these experiments can be reconciled with a single space model. An attempt to answer this question requires discussion of the factors which (*a*) determine mitochondrial volume, and (*b*) control the diffusion of sucrose into the mitochondria at varying mitochondrial volume.

The volume attained by the mitochondrial pellets is likely to be a complex function of the penetration of sucrose, and the leakage of internal solute (see 1, 11 and 12, and above discussion).

That the permeability of mitochondria to sucrose is high and hence may induce volume changes is supported by the results reported in this paper as well as those of Jackson and Pace (1). The rate of swelling is also a function of initial volume and hence initial osmotic pressure (see references 1, 7).

What determines leakage of internal solute is not clear. It may in part depend on some unmeasured parameters, perhaps metabolic rate and amount of endogenous substrate present. In some cases, it would seem to occur significantly only after prolonged exposure (*e.g.*, see references 1, 12). In other cases, it seems to occur rapidly and significantly (3). It is also likely to be a function of initial volume (1, 12). Therefore, the apparent agreement with osmotic law found by Malamed and Recknagel (4) possibly represents a coincidence. Whether the equivalence between the C^{14}-sucrose permeable space and the apparent osmotically inactive volume is in fact fortuitous cannot be answered from the present information.

However, once the assumption is made that the volumes are not determined exclusively by the osmotic pressure of the medium, the constancy of the sucrose penetrated volume at different pellet volumes is possible (see Equation 3). The penetration of sucrose should decrease with increasing radius. In fact, since the change in radius is small, the decrease is probably minimal.

It is clear from these considerations that no new decisive argument can be presented at this time. However, the results of Malamed and Recknagel are not necessarily in conflict with those of the present experiments.

What is the Osmotic Dead Space?

Despite the high permeability to sucrose, it is clear that over a very short period of exposure mitochondria should show ideal osmotic behavior. This short period of exposure is realized either with the photometric method (*e.g.*, reference 17) or by photographing the preparations (*e.g.*, reference 11 and the present work). In fact, it is clear that under these conditions they approach ideal behavior (12). The osmotic dead volume is 43 per cent (12) when the medium is 0.335 osmolal. At the concentration used by many workers, 0.25 molal, this becomes approximately 36 per cent (12). The percentage of dry weight varies in the different

works. In fact, it is likely to vary considerably with differences in the preparatory procedure and the concentration of the isolation medium in a way which is not readily predictable (see factors influencing volume in discussion above). Amoore and Bartley (3) report figures ranging from 37 to 50 per cent (average approximately 41 per cent, see their Table 2). Werkheiser and Bartley report approximately 34 per cent. In this preparation, isolated with a method considerably different from that used previously in this laboratory (*e.g.*, reference 12), the solids correspond approximately to 43 per cent in 0.55 molal sucrose, 0.02 M Tris, or 27 per cent in 0.25 molal sucrose.

These calculations can offer only an approximate answer. However, it is clear that, regardless of what assumptions are made about the volume occupied by a given weight of mitochondrial solids, there is no reason to believe at present that the osmotic dead volume measured at short exposures corresponds to anything but the volume occupied by these solids. This would not be surprising, since it is the case for a number of other osmotic systems (18).

How Do Subcellular Particles Maintain Their Integrity in Sucrose Solutions?

In view of the very high permeability of mitochondria to sucrose, it becomes rather difficult to visualize how their integrity is maintained during isolation. This problem is not limited to mitochondria. It is likely, for example, that the permeability of microsomes (23) and catecholamine granules (24) is also very high. It would seem likely that a high permeability to the isolation medium would lead to a progressive swelling of the particles followed by their disintegration. Their preservation may stem from a number of factors. (*a*) The high permeability to the isolation medium may be a reflection of a progressive deterioration of the preparation and may not be operative during the isolation of the particles. The formation of a swelling factor in mitochondria is known (25). It is conceivable that the swelling may in this case be brought about by an increase in permeability. (*b*) During the isolation procedure, the internal solutes leaving mitochondria may parallel the penetration of sucrose with no drastic change in volume. There is evidence that leakage can take place (3). (*c*) The particles may have been isolated at a higher osmotic pressure than that present *in situ*, permitting a considerable amount of swelling during isolation without damage. This is not likely to be operative at more dilute concentrations. (*d*) The particles may be osmotically resistant. For example, internal material could be available for the formation of new surface membrane. In this fashion considerably more swelling could occur without drastic effects. There is some evidence of this phenomenon in mitochondria (7, 26). It is however unlikely in other subcellular particles.

CONCLUSIONS

The present results are consistent with the interpretation that sucrose diffuses into single mitochondrial spaces, where mitochondria vary in size according to the measured distribution.

The reports from other laboratories were examined in the light of two models: a single space model and a model proposed by others which has two mitochondrial spaces, one permeable and one impermeable to sucrose. At least part of the data of Bartley (5) and Amoore and Bartley (3) were found not to be consistent with the two space model. The data of Bartley (5) and Amoore and Bartley (3) were found to be reasonably consistent with the model which proposes a single mitochondrial space. In addition, it is concluded that the results of Malamed and Recknagel (4) do not necessarily conflict with the present view.

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