J. Membrane Biol. 12, 287–299 (1973)

Mitochondrial Permeability for Alcohols, Aldoses, and Amino Acids

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Received 4 December 1972

Summary. Mitochondria isolated from potato tubers were placed in solutions containing various alcohols, aldoses, or neutral amino acids. Based on the osmotic responses in the different media, the reflection coefficients and hence the relative permeabilities of the nonelectrolytes could be determined. The reflection coefficients (σ_j's) of the potato tuber mitochondria for alcohols became progressively larger as hydroxymethyl groups were added to the molecule, *viz.* methanol ($\sigma_j = 0.07$), ethylene glycol (0.25), glycerol (0.44), *meso*-erythritol (0.71) and adonitol (0.98). This increase in σ_j (decrease in permeativity) with increasing chain length parallels the decreasing lipid-water partition coefficients of the solutes. The reflection coefficients of D-sorbitol (1.02) and of D-mannitol (0.99) indicate that these six-carbon polyhydroxy alcohols are relatively impermeant and hence they would be suitable osmotica in which to suspend mitochondria. The σ_j's varied from 0.96 to 1.02 for D-ribose, D-xylose, D-lyxose, D-arabinose, α-D-glucose, β-D-glucose, D-galactose, D-mannose, glycine, L-alanine, L-threonine, L-phenylalanine, L-methionine and L-cysteine, indicating that these sugars and amino acids do not readily diffuse across the pair of membranes surrounding potato mitochondria. By contrast, the σ_j's of liver mitochondria for glycine and of pea chloroplasts for most of the same aldopentoses and amino acids are close to zero. Thus, different organelles can vary widely in their permeability properties for nonelectrolytes.

If we increase the osmotic pressure in a solution bathing cells or organelles, water will flow out of them. Such osmotic responses are often described by the Boyle-Van't Hoff relation, which refers to the equilibrium situation when the internal osmotic pressure (π^i) equals the external osmotic pressure (π^o). By the Boyle-Van't Hoff relation, the osmotic pressure (π^o or π^i) times the volume of the aqueous compartment within the cell or organelle is constant at a given temperature. If we increase the concentration of some solute in the bathing solution and thereby increase π^o, water will leave the internal aqueous compartment so that π^i again becomes the same as π^o. The classical Boyle-Van't Hoff relation presupposes that the membranes are permeable only to water [15].

Although the classical treatment is correct for impermeant internal and external solutes, it is not adequate for describing the case of penetrating solutes. To remove the inadequacy of the Boyle-Van't Hoff expression, we must then turn to irreversible thermodynamics to discuss osmotic responses. This leads to the introduction of the reflection coefficient of species j (σ_j), which can conveniently be defined as the ratio of the external osmotic pressure of species j effective in causing a water efflux divided by the actual external osmotic pressure of that neutral species [10, 15]. The reflection coefficient is a dimensionless parameter which is unity for an impermeant solute and equals zero for freely permeant compounds. Therefore, σ_j is a measure of the relative permeability of a membrane for neutral species j.

Use of reflection coefficients to study the permeability of organelles has recently been successfully applied to chloroplasts [16, 17, 23]. Here we will use this new approach based on irreversible thermodynamics to examine mitochondria. Not only is such information currently unavailable but also knowledge about mitochondrial permeability is important for a more complete understanding of subcellular compartmentation. In the present experiments we added an alcohol, an aldose, or a neutral amino acid to the external solution and then measured the resulting mitochondrial volume V after the initial rapid efflux of water. Upon subtracting the nonaqueous volume b from V, the volume of the water within the mitochondria ($V-b$) is obtained. This volume of internal water is inversely related to the effective osmotic pressure of the external solution [15]. A compound which enters the mitochondria easily (low σ_j) is not effective in causing an osmotic efflux of water, and so the change in $V-b$ is then relatively small for a given increment in the external concentration of that species. On the other hand, an impermeant solute ($\sigma_j=1$) will lead to the maximal osmotic efflux of water. By observing the effects on $V-b$, we could readily investigate the relative permeability of mitochondria for alcohols, sugars and neutral amino acids.

Materials and Methods

Mitochondrial Isolation

Mitochondria were isolated from tubers of *Solanum tuberosum* L., cv. Idaho Russet potato, Burbank (generously provided by Dr. Herman Timm, Department of Vegetable Crops, University of California, Davis). After cutting the ends from three potatoes, a cylindrical core 2 cm in diameter was removed from the center of each tuber. One hundred grams of such tissue was comminuted using a pre-chilled Oster Automatic Juice Extractor Model 357 (Oster Mfg. Co., Milwaukee, Wis.) with a single layer of Miracloth (Chicopee Mills, Inc., New York, N.Y.) as a liner to filter out cellular debris [11]. The tissue was extracted into 200 ml of 290 mM sucrose, 5 mM 2-(N-morpholino)

ethane sulfonic acid (MES)-KOH (pH 6.2). This extract was centrifuged for 2 min at 5,000 × *g* at 0 °C to remove starch grains and large cellular debris; the supernatant fluid was strained through 1 layer of Miracloth and then recentrifuged for 5 min at 40,000 × *g* to obtain a mitochondria-containing pellet. (Increasing the time for the second centrifugation to 60 min increased the yield of mitochondria by only about 40%). After decanting the supernatant fluid, the mitochondria-containing pellets were combined with 2 ml of isolation medium and gently but thoroughly resuspended by drawing up and down in a Pasteur pipette.

The isolation medium had the same osmotic pressure and pH as sap expressed from the tubers. In particular, 100 g of tuber were ground in a mortar and the macerated tissue was then centrifuged for 10 min at 10,000 × *g*. The resulting cell sap had a pH of 6.2 at 0 °C and an osmotic pressure equivalent to 326 milliosmolal as determined with an Advanced Instruments Osmometer Model 31 LAS. Although such sap is not cytoplasm, as a first approximation its osmotic pressure was assumed to represent that experienced by mitochondria *in vivo*. The rate of endogenous respiration under the conditions of these experiments was extremely low, being less than 0.1% of the value for the same mitochondria incubated at 25 °C in a conventional medium containing 5 mM citrate [11, 12]. The isolated mitochondria were not swollen or otherwise atypical as judged by phasecontrast microscopy, consistent with the osmotic responses to be presented below.

Determination of Mitochondrial Volume

To measure the osmotic responses of mitochondria, 0.2 ml of the mitochondrial suspension was placed in 5.0 ml of a test solution composed of the isolation medium (290 mM sucrose, 5 mM MES-KOH, pH 6.2) plus various concentrations of alcohols, aldoses, amino acids, or additional sucrose as indicated below (chemicals were purchased from Calbiochem, Los Angeles, Calif., or Sigma Chemical Co., St. Louis, Mo.). The test solution was in a 50-ml centrifuge tube that contained an aluminum foil disc 1.3 cm in diameter which was closely appressed to the region where the pellet would form (a thin layer of vaseline proved effective in holding the disc in place). The tube was then centrifuged for 5 min at 40,000 × *g* at 0 °C and the supernatant fluid decanted. The foil with its mitochondrial pellet was carefully removed with tweezers, dried around the pellet with absorbent lint-free tissue, and then weighed to within 0.01 mg (pellet weights averaged about 20 mg, while the foil discs weighed approximately 13 mg). The great precision obtainable for weight determinations compared with Coulter counter or optical methods for measuring mitochondrial volume made the above technique suitable for the present studies.

Experiments using light scattering by mitochondrial suspensions indicated that the initial rapid efflux of water when the mitochondria were placed in a solution of higher osmotic pressure was complete within a few seconds, in agreement with previous findings [22]. Consequently, the centrifugation step for measuring the packed weight was begun a few seconds after adding the mitochondria. Subsequent swelling that occurs as external solutes enter the mitochondria will be discussed below.

To analyze osmotic responses we need to know the mitochondrial volume, while the above measurements gave the weight of the mitochondria plus that of the supernatant fluid trapped in the interstices of the pellet as well as remaining on it. Certain of the pellets were therefore resuspended in the test solutions, whose densities had been determined to 1 μg/ml with a 10-ml Weld pycnometer. The density of the resuspended pellet was measured to within 10 μg/ml and the density of the original pellet was then calculated [14]. Using the pellet density, pellet packed weights could be converted to pellet packed volumes (this conversion had very little effect on the relative values; e.g.,

the pellet densities for 100 mM of the various aldopentoses ranged only from 1.062 to 1.064 g/cm^3 at 0 °C).

The mitochondrial packed volumes calculated from the measured packed weights include a certain amount of test solution in and on the pellet [17]. As we will discuss below, sucrose did not enter the mitochondria to any appreciable extent over the time period of interest in these experiments. Hence, the fraction of pellet space occupied by the medium could be determined by adding [^{14}C] sucrose (obtained from Amersham/Searle Corp., Arlington Heights, Ill.) to the test solution before centrifugation and then comparing the radioactivity in the pellet with that in an equal volume of supernatant fluid. Specifically, the volume fraction of the pellet composed of medium was the counts/min per ml of pellet divided by the counts/min per ml of supernatant fluid. After correcting for such solution, the mitochondrial volume was obtained.

Results

The volumes of mitochondria isolated from potato tubers were determined for various sucrose concentrations from 250 to 590 mM. Fig. 1

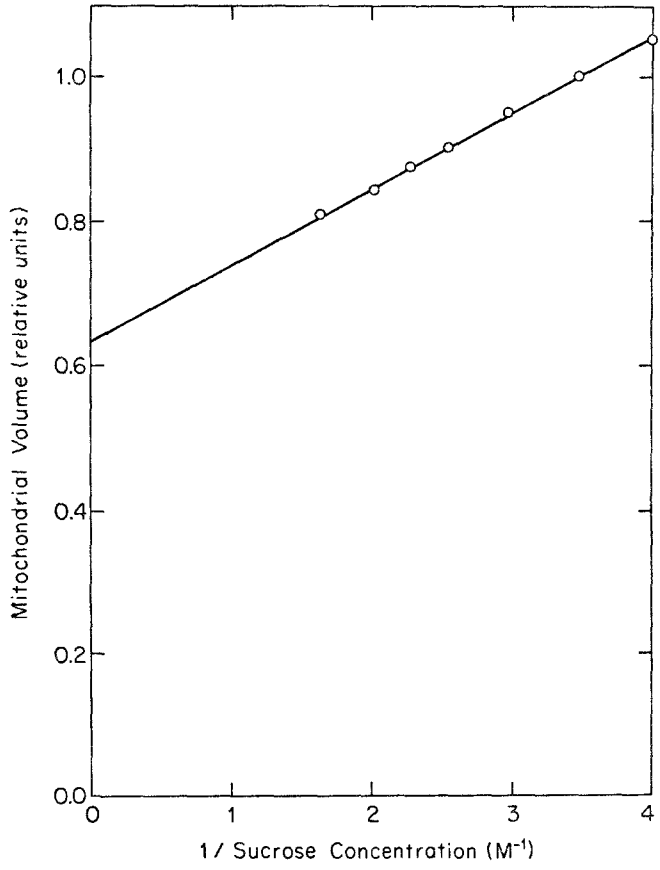

Fig. 1. Volumes for mitochondria isolated from potato tubers and suspended in various concentrations of sucrose. Five mM MES-KOH (pH 6.2) were present throughout. Nine determinations are averaged for each data point

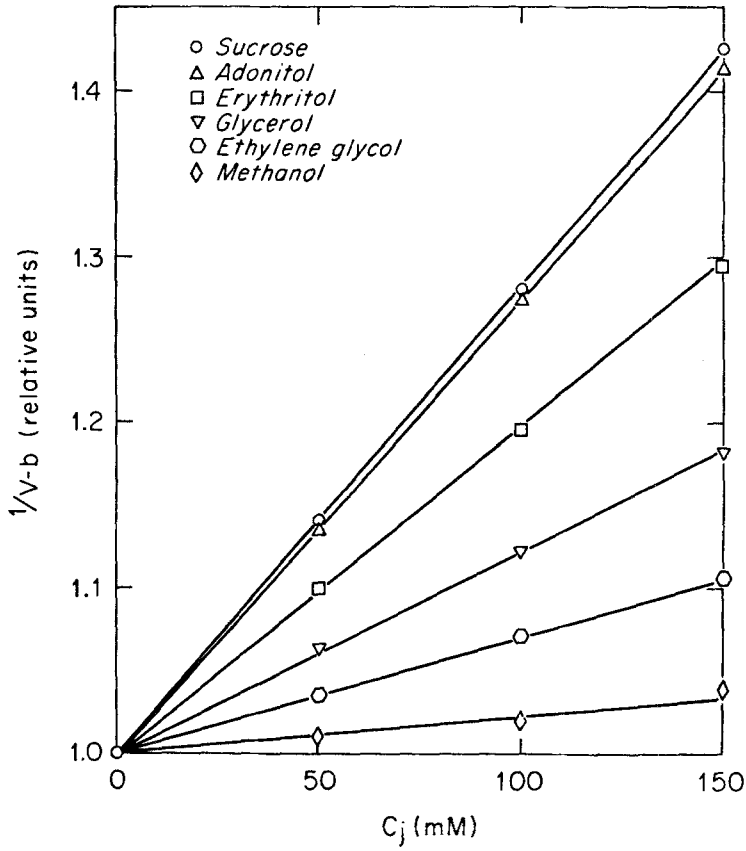

Fig. 2. Osmotic responses of mitochondria to polyhydroxy alcohols. Six experiments are averaged under each condition

illustrates that the mitochondrial volume V was inversely proportional to the external sucrose concentration and hence to $\pi^o(\pi^o \cong c_{\text{sucrose}}\ RT)$, as predicted by the Boyle-Van't Hoff relation [15]. The intercept on the ordinate ($1/c_{\text{sucrose}}=0$) in Fig. 1 represents the mitochondrial volume at an infinite external concentration of sucrose. This volume, which is obtained by extrapolation, corresponds to the nonaqueous volume b, since an infinite c_{sucrose} would remove all of the osmotically responding water from the mitochondria. Of particular interest is the rather high value for b, *viz.* 63% of the volume for mitochondria that are suspended in 290 mM sucrose, 5 mM MES-KOH (pH 6.2), a solution with the same osmotic pressure as the sap expressed from the potato cells.

Osmotic responses of mitochondria to various concentrations of alcohols are presented in Fig. 2. The abscissa, c_j, is the concentration of species j

added to mitochondria suspended in 290 mM sucrose, 5 mM MES-KOH (pH 6.2). The quantity $V-b$ represents the volume of water that responds osmotically in mitochondria of total volume V and thus an increase in the ordinate, $1/(V-b)$, indicates an efflux of water from the mitochondria as the external osmotic pressure is raised. When an alcohol penetrates more readily than does the rather impermeant sucrose, the effectiveness of the external osmotic pressure in causing a volume change is reduced, and thus the slope of a plot of $1/(V-b)$ *vs.* c_j is then less; the reflection coefficient of species j is proportional to the slope, and σ_j reaches a maximum value of essentially unity for sucrose.

As Fig. 2 indicates, σ_j becomes larger along the series of polyhydroxy alcohols of increasing chain length. Methanol is extremely permeant and leads to only 7% as much water efflux as does an equal concentration of sucrose. The next member of the series, ethylene glycol, has a larger reflection coefficient, *viz.* 0.25, while σ_j for glycerol is 0.44 and that of the four-carbon polyhydroxy alcohol, *meso*-erythritol, is 0.71. Adonitol is even less permeant ($\sigma_j=0.98$), causing nearly the same osmotic efflux of water as does sucrose. Not shown in Fig. 2 (but also summarized in Table 1) are the reflection coefficients of a pair of six-carbon polyhydroxy alcohols. Specifically, σ_j was 1.02 for sorbitol and 0.99 for mannitol. For purposes of calculation, we are assuming that the reflection coefficient of sucrose is 1.00.

Solutes with reflection coefficients less than unity enter the mitochondria. This subsequently leads to an osmotic influx of water and thus the mitochondria swell in time. To investigate the magnitude and the time scale of such volume changes, the mitochondria were kept in the test solutions for various periods before the centrifugation step in the packed weight determination. Fig. 3 summarizes such observations which were carried out using a c_j of 100 mM for sucrose ($\sigma_j=1.00$), adonitol (0.98), glycerol (0.44) and methanol (0.07). After only 15 min of storage in the medium containing the very permeant solute methanol, the mitochondrial volume was indistinguishable from that of the control (the volume in 290 mM sucrose, 5 mM MES-KOH, pH 6.2). The rate of increase in volume toward that of the control was considerably slower for the less permeant glycerol in the external solution than it was for methanol (Fig. 3); however, after 30 min glycerol achieved nearly the same concentration inside the mitochondria as outside, since the mitochondrial volume V then became almost as large as the volume in the control solution. Some penetration of adonitol was evident after 15 min and the volume change became 74% of the initial value after 30 min. Very little sucrose entered for a storage period of 30 min

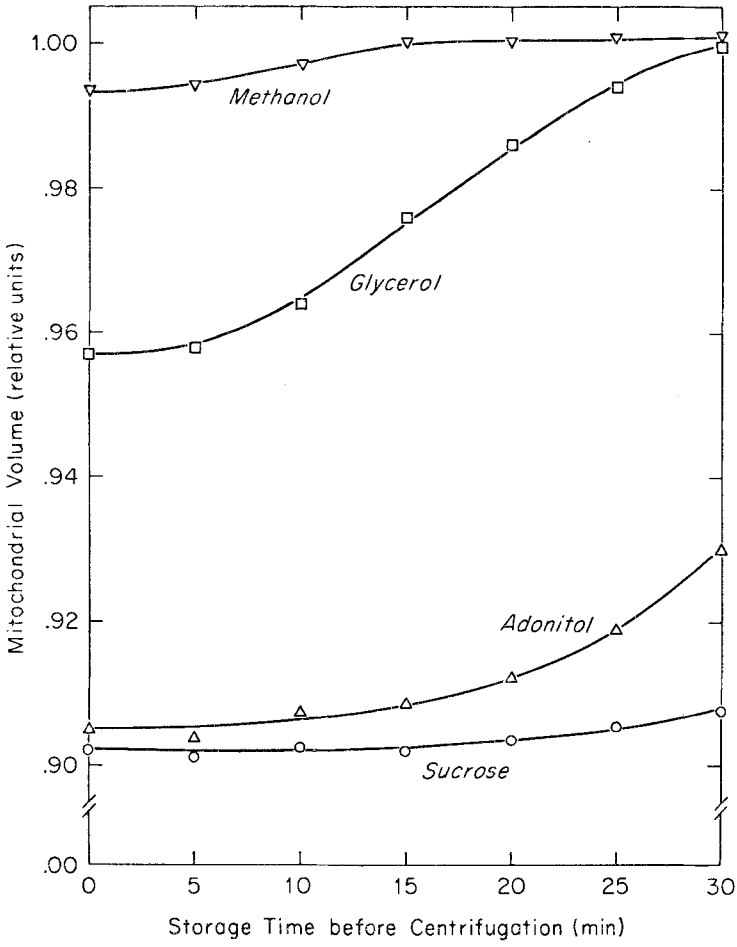

Fig. 3. Effect of storage time at 0 °C in the presence of various compounds on the mean mitochondrial volume. The external solution contained 290 mM sucrose, 5 mM MES-KOH (pH 6.2), and 100 mM of the compound indicated

(*cf.* Fig. 3), supporting the contention that sucrose is relatively impermeant when considered for the time scale of these experiments.

Next, the reflection coefficients of potato tuber mitochondria were determined for various five-carbon polyhydroxy aldehydes (the aldopentoses). As summarized in Table 1, the D isomers of ribose, xylose, lyxose and arabinose were all rather impermeant. Also, the four aldohexoses (α-D-glucose, β-D-glucose, D-galactose and D-mannose) that were tested all had reflection coefficients close to unity (Table 1). Thus, none of these sugars readily enter the mitochondria.

Reflection coefficients are defined only for neutral species [10, 15]. Since the proton which dissociates from the carboxyl group (low pK) of

Table 1. Reflection coefficients of mitochondria isolated from potato tubers[a]

Alcohol	σ_j
Methanol	0.07 ± 0.03 (18)
Ethylene glycol	0.25 ± 0.06 (18)
Glycerol	0.44 ± 0.05 (18)
meso-Erythritol	0.71 ± 0.04 (18)
Adonitol	0.98 ± 0.02 (18)
D-Sorbitol	1.02 ± 0.03 (17)
D-Mannitol	0.99 ± 0.02 (20)
Aldose	σ_j
D-Ribose	1.01 ± 0.02 (18)
D-Xylose	0.97 ± 0.05 (18)
D-Lyxose	1.00 ± 0.03 (18)
D-Arabinose	0.98 ± 0.04 (23)
α-D-Glucose	1.00 ± 0.01 (18)
β-D-Glucose	1.02 ± 0.05 (18)
D-Galactose	0.97 ± 0.04 (15)
D-Mannose	1.01 ± 0.02 (14)
Amino acid	σ_j
Glycine	0.98 ± 0.02 (23)
L-Alanine	1.02 ± 0.02 (21)
L-Threonine	0.96 ± 0.06 (18)
L-Phenylalanine	0.99 ± 0.05 (18)
L-Methionine	1.00 ± 0.03 (18)
L-Cysteine	0.97 ± 0.06 (15)

[a] Osmotic responses to 50, 100 and 150 mM of each compound were determined as indicated in Fig. 2. Data are presented as the average ± SE (number of determinations). Each determination refers to the volume change produced by the solute compared with that caused by an equal concentration of sucrose, whose reflection coefficient was assumed to be 1.00.

an amino acid can be taken up by the amino group (high pK), certain amino acids are electrically neutral at pH's near 6.2, the pH of the test solution. This does not apply to aspartate, glutamate, arginine, lysine and histidine. Tyrosine, tryptophan and cystine are not sufficiently soluble in water for the osmotic responses of mitochondria to be determined. We tested certain of the remaining amino acids and the results are summarized in Table 1. All of the amino acids examined (glycine, L-alanine, L-threonine, L-phenylalanine, L-methionine and L-cysteine) had reflection coefficients close to unity for potato tuber mitochondria.

Discussion

Potato tuber mitochondria responded osmotically to variations in the external concentrations of sucrose, in agreement with numerous studies on mitochondria isolated from other tissues [1, 2, 9, 13, 18–20, 22]. The present approach was to add solutes to the external solution and then to determine the resulting volume after the initial rapid efflux of water. The water may come from the matrix surrounded by the inner of the mitochondrial membranes and/or from the possible aqueous compartment between the inner and the outer membranes. Since the mitochondria were used immediately after being isolated in a medium of the same osmotic pressure as the cell sap expressed from the tubers, it is reasonable to suppose that the compartment between the inner and the outer membranes is approximately the same size as it is *in vivo*. In this regard, the two membranes are found to be rather closely appressed for mitochondria in potato tuber [12] and thus the aqueous compartment between them is small. Using Fig. 1, we can calculate that 53% of the osmotically responding water is removed from the mitochondria when the sucrose concentration is raised from 290 to 590 mM; because of the small distance between the two membranes surrounding the mitochondria under the conditions of these experiments most if not all of this water comes from the matrix enclosed by the inner membrane.

Since the inner and the outer mitochondrial membranes are here closely appressed and are in series for water moving from the matrix to the external solution, the measured reflection coefficients describe the relative permeability for a solute diffusing *across both membranes*. If σ_j is unity for some compound, one of the membranes could be freely permeable while the other is relatively impermeable for that species. There is considerable evidence that the main barrier to the diffusion of sucrose into and out of mitochondria is provided by the inner membrane [2, 5, 7, 9, 13, 18, 20, 21]. Thus, the reflection coefficients measured here most likely represent the relative permeability of the inner membrane.

Based on the extrapolation to infinite osmotic pressure (*cf.* Fig. 1), we estimated that the nonaqueous components of mitochondria comprise 63% of the volume that occurs in 290 mM sucrose, 5 mM MES-KOH (pH 6.2). This volume fraction probably refers to the region surrounded by the inner membrane as we just mentioned. The determination that potato tuber mitochondria contain only 37% free water at the osmotic pressure that was estimated to occur in the cytoplasm indicates that mitochondria contain a large amount of internal membranes, soluble proteins and their bound

water of hydration, as well as other solutes. The relatively low fractional content of osmotically responding water in the matrix of potato tuber mitochondria agrees with studies on rat liver mitochondria [2, 7].

Sucrose has been extensively used as an osmoticum for mitochondria since 1948 [8]. An ideal osmoticum does not penetrate, in which case the isolated mitochondria would not change volume (assuming that no internal solute moved out or changed its concentration during isolation or subsequent storage). However, a neutral molecule of the size of sucrose would be expected to penetrate, even though slowly, and thus mitochondria suspended in a medium where sucrose is the osmoticum should gradually swell, as was indeed observed here. Very little sucrose entered over the first 15 min of storage of the mitochondria at 0 °C, while after 30 min about 6% of the initial osmotic response was lost (*cf.* Fig. 3). These results are in agreement with studies of Gamble and Garlid [5] who stored rat liver mitochondria in sucrose-containing solutions at 30 °C. They found that an insignificant amount of sucrose crossed the inner membrane in the first 5 min of storage, while sucrose enters the inner compartment over longer storage periods. Some penetration of sucrose into the matrix of rat liver mitochondria has also been observed within 30 min at 0 °C [1, 5, 21].

Except for carrier-mediated fluxes, the rates of permeation of most nonelectrolytes through biological membranes are generally proportional to the partition coefficients of the solutes [4, 15, 24]. In particular, the concentration difference or "force" actually determining the flux of molecules across a membrane is the concentration just inside one side of the membrane minus that just within the other side, while the concentrations that are measured are those in the aqueous phases adjacent to the membrane. The partition coefficient links the measured concentration in an aqueous phase to the actual concentration existing just within the membrane.

The membrane-water partition coefficient of a solute is generally assumed to be closely approximated by the ratio of the equilibrium concentration of the solute in a lipid phase (such as ether) divided by the concentration in an adjacent and immiscible aqueous phase. As such ether-water partition coefficients of a series of solutes decrease from high values to low ones, the permeativity also usually decreases, and so the reflection coefficients for the same set of nonelectrolytes can go from zero up to unity [4, 24]. Since the partition coefficients of alcohols decrease as hydroxymethyl groups are added [3], we expect an increase in σ_j as the the chain length is increased in polyhydroxy alcohols. (Permeation also depends on the diffusion coefficient in the membrane which decreases about threefold from methanol to adonitol while the ether-water partition coefficient de-

creases about 1000-fold between these compounds, and hence the partition coefficient is the principal determiner of the variation in σ_j in this case.) Thus, the observed progressive increase in the reflection coefficient from 0.07 for methanol to 0.25 for ethylene glycol to 0.44 for glycerol to 0.71 for erythritol to 0.98 for adonitol is consistent with the concomitant decrease in the partition coefficient of these polyhydroxy alcohols as the chain length increases.

Osmotic responses can be used to evaluate the suitability of various osmotica for the isolation and the suspension of cells or organelles. We have already indicated that the σ_j's for sorbitol and mannitol were essentially unity for potato tuber mitochondria (*cf.* Table 1). Thus, neither of these compounds enter the internal matrix of the mitochondria easily and so they can function as suitable osmotica in which to suspend such organelles. Hunter and Brierley [9] have also found that mannitol does not enter rat liver mitochondria easily.

The four aldopentoses, the four aldohexoses, and the six amino acids used in the present studies all had reflection coefficients of about unity (Table 1). Thus, these compounds do not readily move from the external solution into the aqueous compartment surrounded by the inner membrane of potato tuber mitochondria. Garfinkel [6] has performed an interesting study of osmotic responses to glycine using mitochondria from various tissues. As for potato tuber mitochondria, glycine is essentially impermeant for mitochondria from guinea pig brain, heart, and kidney as well as from rat brain and kidney. However, mitochondria from guinea pig and rat *liver* are quite permeable to glycine below 65 mM [6]. In our present terminology, glycine then has a low σ_j. The reflection coefficients for certain aldopentoses and amino acids are also low for another organelle. Specifically, the σ_j's for pea chloroplasts are 0.00 for D-ribose, 0.43 for D-xylose, 0.47 for D-lyxose, and 0.01 to 0.06 for glycine, L-alanine, L-threonine, L-phenylalanine and L-methionine [16, 17, 23]. A carrier for aldopentoses and two others for amino acids occur in the limiting membranes of pea chloroplasts, which facilitate the penetration of these compounds and thereby keep their reflection coefficients low [16, 23].

In summary, the nonelectrolyte permeability of mitochondria from various tissues and of chloroplasts can be markedly different. Although the σ_j's of alcohols depend on chain length and the reflection coefficients for aldohexoses are near unity for both organelles, pea chloroplasts are much more permeable to the D-isomers of aldopentoses and the L-isomers of amino acids than are potato tuber mitochondria. Also, liver mitochondria are much more permeable to glycine than are mitochondria from the other

plant and animal tissues examined. Such differences in permeability for these subcellular compartments may well play an important role in metabolic control.

This research was supported by Public Health Service Research Grant No. GM 15183 from the National Institute of General Medical Sciences and Biomedical Sciences Support Grant No. 5-S05 RR 7009-06 from the National Institutes of Health. The mitochondrial isolation medium and the use of aluminum discs for collecting the pellets were devised by Mr. Philip J. Whitcome, who was supported by Training Grant No. GM 1531 of NIGMS. Competent technical assistance was provided by Miss Carol Hoelle.

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