Bioenergetics (1970) **1**, 193–213.

The Mechanism of Mitochondrial Swelling. V. Permeability of Mitochondria to Alkali Metal Salts of Strong Acid Anions

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Abstract

Mitochondria do not swell appreciably when suspended in media containing the chlorides or bromides of alkali metal or ammonium ions. On the other hand, extensive swelling takes place when mitochondria are suspended in ammonium or sodium acetate. These findings have been widely interpreted to mean that the mitochondrial membrane is impermeable to chloride and bromide ions. However, the resistance of the mitochondria to volume changes is not necessarily a valid criteria of impermeability to a given ion pair. Such a conclusion presumes the as yet untested assumptions that (1) permeability to the ion pair is always the rate-limiting step in swelling, and (2) permeability to the ion pair is equivalent to the driving force for water influx. We have conducted experiments addressed to the question of mitochondrial permeability by methods (tracer exchange diffusion) which are independent of volume changes. Our findings indicate that the mitochondrial membrane is very readily penetrated by alkali metal chloride and bromide salts. Further, we have concluded that the resistance to swelling in such media is associated with a lack of driving force.

Introduction

The passive permeability of various biological and synthetic phospholipid membranes to anions of strong acids and of low mass has been the subject of numerous investigations. For example, the depolarization studies of Eccles[1] suggest that post-synaptic membranes are rapidly permeable to chloride and bromide ions; Tosteson[2] has elegantly demonstrated that chloride and bromide ions enter red cells with half-times ranging from a fraction of a second to several seconds; the data of Conway[3] suggest that muscle cells are permeable to chloride and bromide ions; and Bangham, Standish and Watkins[4] have shown that chloride ions penetrate artificial phospholipid membranes very rapidly. The picture which emerges from these studies is that anions such as chloride and bromide penetrate natural or synthetic phospholipid-containing membranes with ease.

However, studies on the behavior of the mitochondrion suspended in media containing the chlorides or bromides of alkali metal or ammonium ions have been interpreted in terms of the impermeability of the *mitochondrial membrane* to these anions. Such a view has enjoyed an unusually wide acceptance in view of the restricted nature of the supporting evidence. This evidence is principally of two kinds: (1) osmotic responses

of the mitochondrion suspended in decimolar salt media, and (2) analyses of the ion-distribution volume in mitochondrial particles separated from salt-containing media by centrifugation.

Chappell and Crofts[5] made extensive use of the osmotic response of the mitochondrion as a determinant of permeability. Isolated mitochondria show little tendency to swell when suspended in isoosmolar ammonium chloride, whereas they do swell extensively when suspended in ammonium acetate media. On the basis of these observations Chappell and Crofts concluded that mitochondria were permeable to ammonium acetate but impermeable to ammonium chloride and more specifically to chloride ions. On the basis of similar experiments Chappell and Crofts postulated that the mitochondrial membrane is impermeable to sodium and potassium ions[5,6] since isolated mitochondria show little tendency to swell in sodium or potassium acetate whereas they swell extensively in ammonium acetate. The latter results have been challenged by several authors (e.g., Brierley,[7] Blondin,[8] Mitchell[9]), since sodium supports extensive pseudoenergized* swelling of the mitochondrion when present as the acetate salt. The picture which has emerged as a direct result of studies on mitochondrial osmotic responses is that the mitochondrial membrane is permeable to acetate and other weak acid anions, and to the cations, sodium and ammonium; and impermeable to chloride and bromide anions and to the potassium cation.

While we do not argue with the view that the extensive pseudoenergized swelling of the mitochondria is a valid criterion of permeability to a given salt, we must emphasize that to conclude that the lack of swelling is tantamount to proof of impermeability is without justification. The latter conclusion presumes the as yet untested assumptions—first, that permeability to the salt is always the rate-limiting step in the swelling process and, second, that equilibration of externally added salt between the inside and outside of the mitochondrion is all that is required to provide the driving force for swelling. With regard to the first reservation, Chappell and Crofts[5,6] have themselves presented evidence that conditions which lead to swelling in alkali metal salt media (e.g., in the presence of gramicidin) lead to an apparent increase in the permeation of the membrane not only by the alkali metal ion but also by the hydrogen ion. But they produce no evidence whereby the reader can judge as to whether the apparent alteration in permeability to hydrogen or alkali metal ion is the key to the induction of swelling. The second reservation is grounded in the view that the mitochondrial membrane bears more resemblance to a fixed-charge system that to an inert and uncharged polymeric barrier. Given this property of the membrane the possibility has to be considered that an unequal distribution of ions across the mitochondrial membrane systems can develop as a function of the microenvironmental pH. As a result of such a Donnan distribution of ions, the mitochondrion should be capable of maintaining a higher concentration of ions inside as compared to outside at any value above and below the isoelectric pH. Such a distribution of ions in theory can provide a major driving force for swelling and, indeed, such a role for the membrane has been postulated in the case of chloroplasts[10] and mitochondria.[8] For these reasons we do not view the use of the resistance to osmotic response as a reliable measure of membrane impermeability to a given ionic species.

The determination of permeability properties by volume distribution analysis initiated by Amoore and Bartley[11] and Amoore[12] and later taken up by Gamble[13] and Brierley[14]

* For a definition of the term "pseudoenergized" the reader is referred to ref. 8.

is equally ambiguous. When mitochondria are sedimented from a decimolar solution of alkali metal salt, the resulting pellet can be analyzed for total water content and total salt content. If the resulting concentration of either anion or cation in the pellet is less than that in the original suspending medium then it is concluded that the membrane is impermeable to that species. Such a conclusion, however, does not take into account the possibility of Donnan exclusion of ions. Data of this kind are also often complicated by the possibility of an unknown amount of the ion being already present endogenously or that an active process can lead to the exclusion of the ion in question. Furthermore, permeability has a clear connotation of rate, whereas in the majority of the ion-distribution studies this parameter is completely ignored.

Despite the clear shortcomings of the data by which the permeability properties of the mitochondrial membrane system have been assessed, investigators have had no difficulty in rationalizing mitochondrial swelling data within this widely accepted framework. This apparent success had undoubtedly contributed to the popularity of the currently accepted mitochondrial permeability principles. However, there are several serious deficiencies of such tenets with respect to the explanation of mitochondrial water movements. The presentation of these deficiencies forms a major part of the present communication. In addition, we have taken the view that unambiguous measurements of permeability must be determined under conditions of near zero-volume change, since the rate and extent of volume change may be regulated by factors other than the permeability of the ion in question. This limits possible procedures to a study of the rate of exchange of isotopically labelled permeant ionic species across mitochondrial membranes under equilibrium conditions. The second part of the present communication deals with this approach.

Methods

Preparation of Mitochondria

Standard suspensions of beef heart mitochondria were prepared by the method of Crane *et al.* except that the isolation medium was buffered with 0·01 M Tris-HCl, pH 7·8, in the place of potassium phosphate.[15] Heavy beef heart mitochondria (HBHM) were isolated from these standard suspensions according to the method of Hatefi and Lester.[16] Fractionation was carried out twice at pH 7·8 in a medium 0·25 M in sucrose and 0·005 M in Tris-HCl; the mitochondria were sedimented for 20 min at 30,000 rev/min in the No. 30 rotor of the Spinco ultracentrifuge. The fractionated mitochondrial pellet was finally suspended and adjusted by dilution with the above medium to a final protein concentration of 50 mg/ml. Freshly prepared mitochondrial suspensions were routinely used in all the experiments to be described.

Measurement of Swelling

Mitochondrial volume changes were monitored in either of two ways. In the first method changes in absorbance at 520 mμ were determined in a Beckman DK-2 recording spectrophotometer equipped for time-drive operation. The final mitochondrial protein concentration was 0·33 mg/ml in cuvettes with a 1 cm path length (total volume 3·0 ml). Whenever reagents were introduced into the medium during a recording, small volumes of concentrated solutions were added to minimize dilution effects. Water-insoluble

reagents were added in the form of ethanolic solutions; the additions were usually 20 μl or less. A fall in absorbance was taken as a measure of the increase in volume of the mitochondria, an interpretation confirmed by previous work.[17,18] The second method involved the determination of the water content of mitochondrial pellets obtained by centrifugation of the reaction medium. The weight of the centrifuge tube plus the pellet was determined before and after drying (overnight in a vacuum oven at 60°). The centrifuge tubes were cut with a razor in such a way that only the portion of the tube containing the pellet (eyeball) was used in the gravimetric analysis. These experiments were usually carried out in duplicate at 25°, each in a total volume of 5·0 ml; the final mitochondrial protein concentration was 1·0 mg/ml. The period of incubation is defined as the reaction period exclusive of the time of centrifugation. Centrifugation was carried out in a Spinco No. 40 rotor equipped with inserts to accommodate 6·0 ml centrifuge tubes (Beckman No. 3022327). Centrifugation was carried out for 3 min after acceleration to 20,000 rev/min. Because of the considerable time required for full acceleration in the rotors of the Spinco ultracentrifuge, kinetic studies involving gravimetric determination of the water content required the use of a high-speed Misco centrifuge capable of reaching full speed (22,000 rev/min) in less than 10 sec and of effecting complete sedimentation of the mitochondria in 45 sec (inclusive of acceleration time). The time from the initiation of deceleration (manual braking) to decanting of the supernatant was less than 30 sec. The minimum time required for introduction of a reagent or mitochondria into a suspension medium, and of effecting complete mixing and transfer to centrifuge tubes (polyallomer, Sargent No. S-17882-C), was 15 sec. In these experiments the time of incubation is defined as the period inclusive of the time of centrifugation (45 sec) but exclusive of deceleration time and separation of suspending medium from the pellet (30 sec). The latter period is expressed as minus values on the time scale in the figure depicting exchange diffusion data (Fig. 3, p. 205). The protein concentration in these experiments was 2·0 mg/ml and the total incubation volume was 3·0 ml. In both procedures used for the estimation of mitochondrial water content, the osmotically active volume (sucrose-free water) was determined according to previously published procedures.[8,19] Whenever extended incubation periods were necessary (i.e., 90–120 min), trace quantities of sucrose-^{14}C were added 2·0 min prior to centrifugation.

Measurement of the Concentration of Cations and Anions

The sodium, rubidium, and bromide ion content of mitochondrial pellets was determined by counting the gamma-emitting isotopes ^{86}Rb, ^{22}Na, and ^{82}Br in a Packard Model 5019 Auto-gamma spectrometer coupled to a Packard Model 3003 Tri-Carb scintillation spectrometer. Discriminator settings were adjusted to allow assay in the range 0·44–0·54 MeV for both ^{22}Na and ^{86}Rb, and between 0·40 and 0·60 MeV for ^{82}Br. The pellets obtained from incubations in the presence of ^{22}Na and ^{82}Br were counted directly by insertion of the "eyeball" into the gamma-counting tubes. Aliquots of the supernatant fluid (1 ml) were counted in order to determine the specific activity of sodium in the suspension medium. Suitable controls were performed to insure that there was no loss in assayable activity by virtue of the difference in counting geometry between pellet and supernatant. Because of the considerable absorption by aqueous systems of the radiation of ^{86}Rb, the above-mentioned procedure was found to be unsuitable for the determination of the rubidium content of the mitochondrial pellets.

The following procedure was used for this special case. The dried pellets containing ^{86}Rb were solubilized in 3·0 ml of 0·01 M cetyl pyridinium chloride and the resulting clear solution was acidified with 1·0 ml of 0·4 N HCl. Aliquots of this acidified solution (1 ml) were introduced into the gamma-counting tubes for assay. Aliquots of the acidified (0·1 N final) supernatant (1 ml) were counted for determination of the specific activity of rubidium in the suspending medium.

Results

Isolated beef heart mitochondria show little tendency to swell when suspended for short periods of time (5–20 min) in decimolar chloride or bromide salts of alkali metal or ammonium ions.[7,8] However, when the period of observation is extended it becomes apparent that swelling is proceeding, but at an extremely slow rate. For example, the following observation is implicit in the data described by Fig. 1. Beef heart mitochondria swell rapidly and extensively in 0·15 M sodium acetate even in the absence of an energy supply. In terms of the optical method used in compiling the data for Fig. 1, mitochondria swell to one-half of their maximum size in approximately 4 min when suspended in sodium acetate. However, when mitochondria are suspended in 0·15 M sodium chloride, with or without an energy supply, approximately 130 min are required for the same volume increment, i.e., it takes approximately thirty-two times longer for mitochondria to attain one-half their maximum volume in a chloride-containing medium as opposed to an acetate-containing medium.

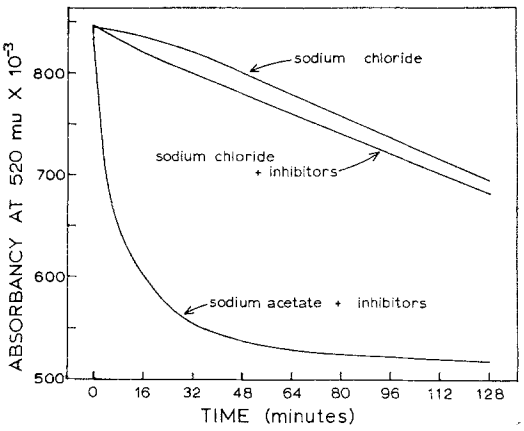

Figure 1. Time course for the decrease in absorbance at 520 mμ of mitochondria incubated in alkali metal salts. Heavy beef heart mitochondria (0·1 ml of a suspension containing 1·0 mg of mitochondrial protein) were added to 2·9 ml of incubation medium containing 0·15 M sodium acetate, pH 7·4, or 0·15 M sodium chloride and 30 μmoles of Tris-Cl, pH 7·4. The inhibitors, when present, were antimycin and rotenone, each at a concentration of 2·0 μg/mg protein. Absorbance was measured at 25°, as described in the section on methods.

The fact which we wish to emphasize at this point is that mitochondria do swell in alkali metal chloride media, although very slowly, and therefore the mitochondrial membrane must be viewed as permeable to chloride ions.

The data of Fig. 2 show the change in osmotically active volume (sucrose inaccessible space) of the mitochondrion as a function of time when incubated in 0·15 M sodium or potassium chloride in the presence of electron-transport inhibitors. The presence of inhibitors of electron transport insures against any volume change arising by the active concentration of any minor ionic species. Therefore, any change in volume must be accounted for in terms of the penetration of alkali metal salt. The data show that 120 min are required for the osmotically active volume of mitochondria to increase to values of 2·83 μl and 2·30 μl/mg protein, respectively, when suspended in sodium and potassium chloride, whereas osmotically active volumes in excess of 3·0 μl/mg protein are common

in media containing sodium acetate after less than 5 min of incubation.[20] The point may again be made that mitochondria swell in alkali metal chloride media but at a much slower rate than in acetate media. More importantly, in the present case some estimate of salt penetration can be made. The time interval between the earliest measureable volume (1·0 min) and the final measurement of volume (120 min) is 119 min. During this time the osmotically active volume has increased by 1·65 and 1·23 μl/mg respectively, in sodium chloride and potassium chloride media. Since the external salt concentration is 150 mμmoles/μl in both cases, we may assume that the salt equivalent of the increased matrix water is 247 and 184 mμmoles/mg protein, respectively. Assuming that the entry of water must have been at or near the external concentration of salt, this amount of sodium and potassium chloride must have penetrated the inner membrane. Little more can be said about the maximal rate of penetration of the inner membrane by alkali metal salt, since, again, to calculate rate we would have to assume that the penetrability of alkali metal salt is the rate limiting factor in the swelling process. On the basis of this and the previous experiment we wish only to emphasize that it is fallacious to conclude that mitochondria are completely impermeable to alkali metal chloride salts.

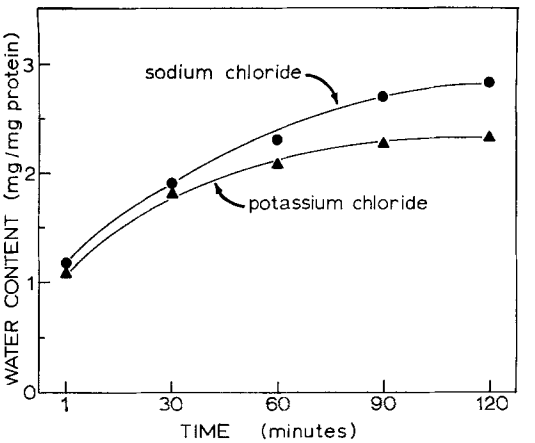

Figure 2. Time course for the increase in osmotically active volume of mitochondria incubated in media containing sodium and potassium chloride. Heavy beef heart mitochondria (0·5 ml of a suspension containing 20 mg of mitochondrial protein) were added to 9·4 ml of incubation medium containing potassium or sodium chloride at a final concentration of 0·15 M and Tris-Cl (100 μmoles) at pH 7·4. Sucrose-^{14}C (0·1 ml containing 1·0 μCi) was added either initially (1·0 min value) or 2·0 min prior to centrifugation. After incubation at 25° for the times indicated, duplicate 3·0 ml aliquots were sedimented and the pellets analyzed as described in the section on methods. The water values expressed refer to sucrose-impermeable water. Antimycin and rotenone were present in all incubations, each at a concentration of 2·0 μg/mg protein.

Comparison of the Behavior of Mitochondria in Media Containing Chloride and Bromide Salts of Alkali Metal Ions

All of the measurements of anion distribution and exchange diffusion which are described in the present communication have been carried out with bromide salts of alkali metal ions because of the technical advantages in the use of ^{82}Br as a tracer rather than ^{36}Cl.* The data of Table I show that the swelling behavior of mitochondria in chloride and bromide media is indistinguishable, as has been previously shown in experiments of shorter time periods.[8] The increase in osmotically active water between 1 and 120 min is almost identical in all cases where chloride and bromide salts are compared under identical conditions.

We have also chosen to use ^{86}Rb as a tracer rather than ^{42}K. Aside from the much longer half life of ^{86}Rb, the use of rubidium salts was also preferred to those of potassium because of the large endogenous potassium ion content of the mitochondria (e.g.,

* Since the isotope ^{82}Br is a strong gamma emitter, radioassay was easily accomplished without protein solubilization as would be required in the case of ^{36}Cl.

TABLE I. Increase in osmotically active volume of mitochondria after incubation in 0·15 M sodium chloride or bromide

Salt in incubation medium	Addition of antimycin and rotenone	Increase in sucrose-free water between 1 and 120 min (mg/mg protein)
Sodium chloride	–	0·68
	+	1·65
Sodium bromide	–	0·74
	+	1·58

Heavy beef heart mitochondria (0·5 ml of a suspension containing 20 mg of mitochondrial protein) were added to 9·4 ml of incubation medium containing sodium chloride or bromide at a final concentration of 0·15 M and the Tris salt of the same anion (100 μmoles) at pH 7·4. Sucrose-^{14}C (0·1 ml containing 1·0 μCi) was added after 118 min of incubation at 25°; after 120 min 3·0 ml aliquots were sedimented and the pellets analyzed for water and sucrose-^{14}C content as described in the section on methods. Antimycin and rotenone were each present at a concentration of 2·0 μg/mg protein.

TABLE II. Increase in osmotically active volume of mitochondria after incubation in 0·15 M potassium or rubidium bromide

Salt in incubation medium	Presence of antimycin and rotenone	Increase in sucrose-free water between 1 and 120 min (mg/mg protein)
Potassium bromide	–	0·79
	+	1·40
Rubidium bromide	–	0·67
	+	1·34

Heavy beef heart mitochondria (0·5 ml of suspension containing 20 mg of mitochondrial protein) were added to 9·4 ml of incubation medium containing potassium or rubidium bromide at a final concentration of 0·15 M and the Tris salt of the same anion (100 μmoles) at pH 7·4. Sucrose-^{14}C (0·1 ml containing 1·0 μCi) was added after 118 min of incubation at 25°; after 120 min 3·0 ml aliquots were sedimented and the pellets analyzed for water and sucrose-^{14}C content as described in the section on methods. Antimycin and rotenone were each present at a concentration of 2·0 μg/mg protein.

100–120 mμmoles/mg protein). Table II provides evidence that the behavior of rubidium and potassium salts is also indistinguishable, as has previously been shown.[8,21]

Distribution of Ions in Mitochondrial Pellets Isolated from Media 0·15 M in Sodium Bromide

In the previous experiments the assumption that alkali metal salt has penetrated the mitochondrion was based on the fact of water movements in media containing these salts. The data of Table III provide direct evidence in support of this assumption. These data were obtained by allowing mitochondria to incubate in 0·15 M sodium bromide in the presence of either ^{22}Na or ^{82}Br. After isolation of the pellets by centrifugation the total water content and total amounts of sodium and bromide ions were determined as described in the section on methods. There should be no ambiguity with respect to the determination of bromide by radioassay; however, sodium determination is potentially

TABLE III. Sodium and bromide ion content of pellets obtained by sedimentation of mitochondria incubated in 0·15 M sodium bromide

Tracer Ion	Additions	Water content of pellet (mg/mg protein)	Pellet ion content		Non-equilibration content of labelled ion*	
			Total mμmoles	mμmoles/ mg pellet water	mμmoles/ mg pellet water	mμmoles/ mg protein
^{22}Na	None	4·73	759·2	160·5 ± 5·4†	+10·5	+49·7
^{82}Br	None	4·62	657·5	142·5 ± 2·6	− 7.5	−34·6
^{22}Na	Gramicidin	9·88	1600	162 ± 3	+12	+121·8
^{82}Br	Gramicidin	10·27	1493	145·3 ± 4·2	−4·7	−48·3

Heavy beef heart mitochondria (0·5 ml of a suspension containing 12 mg of mitochondria protein) were added to 11·5 ml of incubation medium containing sodium bromide at a final concentration of 0·15 M and Tris-Cl (60 μmoles at pH 7·4). Antimycin and rotenone were contained in all incubations each at a concentration of 2·0 μg/mg protein. The media also contained approximately 0·5 μCi of either ^{22}Na or ^{82}Br and, where indicated, 20 mμmoles of gramicidin D. Incubations were carried out for 30 min at 25°; 5·0 ml aliquots were centrifuged and the pellets analyzed as described in the section on methods. Sucrose-^{14}C (1·0 μCi) was used for the evaluation of sucrose-free water in separate but identical experiments. The values are quoted in the text.

* This designation refers to the difference between the total measured ion content of the pellet and the product of the total pellet water content and the external salt concentration. A + designation means that the ionic concentration of the pellet water is greater than that in the suspending medium.

† Average error of five determinations.

complicated by the presence of endogenous sodium ions. However, even if the endogenous sodium ions do not freely exchange with tracer, this complication could not alter our results significantly. The following calculation will illustrate this point. The smallest value for total sodium ion assayed/mg protein from Table III was 759 mμmoles. Our mitochondria contain between 15 and 25 mμmoles/mg of endogenous sodium, which is only 2–3% of the total assayable sodium by the isotopic technique. Therefore, even if all of the endogenous sodium ions were to resist free exchange with tracer, the maximum possible error would be in the range of 2 or 3%, which is in the range of our experimental accuracy. The data of Table III show that when the total pellet sodium (759 mμmoles/mg protein) is divided by the total pellet water (4·73 mg/mg protein) the resulting sodium ion concentration in the pellet is 160·5 mμmoles/μl pellet water, as opposed to 150 mμmoles sodium/μl in the suspension medium. The excess pellet sodium of 160·5 – 150 (or 10·5) mμmoles/μl is equivalent to an excess of 49·7 mμmoles/mg protein, which may for the moment be accounted for either as sodium ion bound to the mitochondrial membranes or as sodium ion in excess as dictated by the Donnan ion distribution. In any case, it is apparent that sodium ions have completely equilibrated with all aqueous phases of the mitochondrion. To claim that the matrix volume excluded sodium ions would require adding the sodium ion equivalent of the matrix volume to the value for bound or Donnan distributed ions. The matrix volume in the sodium ion experiment was found to be 1·94 μl/mg protein, which accounts for 291 mμmoles sodium ion/mg protein. This, together with the excess of 49·7 mμmoles, amounts to 341 mμmoles of sodium ion which one would have to say is bound or Donnan distributed were the matrix compartment impermeable to sodium ions. This is clearly impossible, since it is almost three times more bound sodium than is accountable in mitochondria isolated from sodium bromide media in the presence of gramicidin—conditions in which the mitochondrion is unambiguously permeable to bromide. However, again this tells little about permeability, since only 290 mμmoles of sodium ion penetrated in a period of 30 min, or 9·7 mμmoles/min/mg protein if one assumes linearity throughout the incubation period. The same arguments can be applied to the results of the ^{82}Br experiment. Admittedly bromide ion concentration in the pellet is less than that in the suspending medium by 5%; however, this result is to be expected in a system where a Donnan ion distribution applies. If bromide were indeed impermeable, the concentration in the pellet should have been at least ten times smaller to be consistent with the matrix volume. The amount of bromide ion which would have to be bound were it completely excluded from the matrix volume would be approximately 270 mμmoles/mg protein. Such a value is clearly unrealizable, since there is evidence of only limited binding of bromide even in the presence of gramicidin—a potent facilitating agent of swelling in sodium bromide media.

Additional evidence in support of the view that sodium bromide penetrates the mitochondrial membrane is provided in Table IV. The rationale of the experiments with ^{22}Na as a tracer is the following. A total of 730 mμmoles of sodium ion/mg protein was found in mitochondria isolated from 0·15 M sodium bromide. The matrix water was 1·90 μl and the non-matrix water 2·63 μl/mg protein. We have two options by which to account for the distribution of the 730 mμmoles of sodium ion.

(1) *Sodium is excluded from the matrix water.* The non-matrix water accounts for 2·63 μl, which may contain 2·63 × 150 (or 395) mμmoles of equilibrated sodium ion. We must

TABLE IV. Sodium and bromide ion content of mitochondrial pellets after incubation in 0·15 M sodium bromide in the presence or absence of 0·25 M sucrose

Additions	Ion assayed	Water content of pellet (mg/mg protein)		Pellet ion content		Non-equilibration content of labelled ion	
		Total	Sucrose-free	Total mμmoles	mμmoles/ mg pellet water	mμmoles/ mg pellet water	mμmoles/ mg protein
None	Na-22	4·53	1·90 ± 0·2*	730	161 ± 2·5*	+11	+49·8
Sucrose	Na-22	2·45	0·39 ± 0·06	432	176·2 ± 4·6	+26·2	+64·2
None	Br-82	4·94	2·30 ± 0·17	706	142·8 ± 0·05	−7·2	−35·2
Sucrose	Br-82	2·33	0·42 ± 0·12	348	149·5 ± 3·7	−0·5	−11·65

Heavy beef heart mitochondria (0·5 ml of a suspension containing 12 mg of mitochondrial protein) were added to 11·5 ml of incubation medium containing sodium bromide at a final concentration of 0·15 M and Tris-Cl (60 μmoles) at pH 7·4. Antimycin and rotenone were contained in all incubations, each at a concentration of 2·0 μg/mg protein. The media also contained approximately 0·5 μCi of either sodium-22 or bromine-82 and, where indicated, sucrose at a final concentration of 0·25 M. Sucrose-C^{14} (1·0 μCi) was used for the evaluation of sucrose-free water in separate but identical experiments. Incubations were carried out for 30 min at 25°; 5·0 ml aliquots were centrifuged and the pellets analyzed as described in the section on methods.

* Average error of the three determinations.

therefore account for 730 – 395 (or 335) mμmoles of sodium, which is either specifically bound or held as a counter ion by virtue of the Donnan distribution.

(2) *Sodium equilibrates with all of the mitochondrial aqueous phases.* The total mitochondrial water is 4·53 μl, which should contain 4·53 × 150 (or 680) mμmoles of equilibrated sodium ion. We must therefore account for 730 – 680 (or approximately 50) mμmoles/mg protein of sodium ion as either specifically bound or held as a counter ion by virtue of the Donnan distribution.

If the same experiment is carried out in the presence of 0·25 M sucrose, the matrix volume is decreased from 1·90 to 0·39 μl/mg protein, i.e., by 80%, whereas the total mitochondrial water is only decreased from 4·53 to 2·45 μl/mg protein, i.e., by 46%. Therefore, the relative proportions of matrix and non-matrix water are shifted under conditions which should not effect ion binding. We are therefore in a position to predict the total sodium ion concentration on the basis of the two options described for the case in the absence of sucrose.

If only the non-matrix space were equilibrated with sodium ions then the total sodium content would be 2·06 × 150 (or 309) mμmoles. This, in addition to 335 mμmoles which are bound, would come to a total of 644 mμmoles/mg protein. If all the aqueous phases were equilibrated, we should expect 2·45 × 150 (or 367) mμmoles. This, plus the 50 mμmoles which are bound, would come to a total of 417 mμmoles/mg protein. As shown in Table IV, we found 432 mμmoles, which is in excellent agreement with theory based on total equilibration—an agreement as close as the accuracy of the experiments would allow.

The same line of argument can be extended to the ^{82}Br experiment and, again, the only reasonable conclusion we have been able to draw is that sodium bromide has indeed equilibrated with the matrix water.

Exchange Diffusion of Sodium and Bromide Ions Under Conditions of Zero Volume Change

In the previous sections we have established (1) that mitochondria swell at a very slow rate in alkali metal chloride media, and (2) that ion analyses of mitochondrial pellets obtained at the termination of these swelling experiments are consistent with complete equilibration of alkali metal ions with all of the mitochondrial aqueous phases. The results presented so far are still consistent with the view that the slow rate of swelling in alkali metal salt is related to limited permeability of the mitochondrial membrane to sodium or bromide ions. Whether permeability is indeed limited can easily be tested for directly. Given sufficient time, sodium and bromide ions will penetrate the matrix volume in considerable amount. We can allow such an event to occur in the presence of unlabelled ions and then add a trace quantity of labelled ion and study the rate at which the unlabelled ions in the matrix exchange with externally added labelled ions. This approach has the advantage of being applicable to very short time intervals during which no volume change is occurring, and of being completely independent of volume change in the presence of suitable controls. Table V provides the data of one such experiment. Mitochondria were incubated in the presence of 0·15 M $^{22}Na^{82}Br$ for 27 and 30 min, respectively. Both the water content and ion concentrations of the two samples were identical within the limits of experimental accuracy. Most important, it is apparent that no volume change occurred between 27 and 30 min. An identical experiment was performed in the presence of unlabelled salt for 27 min, and at some

TABLE V. Exchange diffusion of ^{22}Na and ^{82}Br after incubation of mitochondria for 30 min in 0·15 M sodium bromide

	Exchangeable isotope			
	^{22}Na		^{82}Br	
Time of exposure to isotope (min)	mg pellet water/ mg protein	mμmoles ^{22}Na/ mg pellet water	mg pellet water/ mg protein	mμmoles ^{82}Br/ mg pellet water
1·0	4·44	165 ± 2·7*	4·66	137 ± 5·0*
1·5	4·36	166 ± 6·0	4·48	144 ± 2·5
2·0	4·62	158 ± 5·2	4·70	138 ± 4·1
2·5	4·50	162 ± 3·1	4·62	142 ± 0·8
3·0	4·35	167 ± 1·2	4·48	140 ± 3·8
27·0	4·48	164 ± 0·7	4·64	142·5 ± 1·2
30·0	4·54	164 ± 2·2	4·76	143 ± 3·3

Heavy beef heart mitochondria (0·2 ml of a suspension containing 10 mg of mitochondrial protein) were added to 4·75 ml of incubation medium containing 0·15 M sodium bromide, 0·005 M Tris-Cl, and antimycin and rotenone, each at a concentration of 2·0 μg/mg protein. Each incubation contained, in addition, 50 μl of a solution of ^{22}Na or ^{82}Br (approx. 10 μCi/ml). A 3·0 ml aliquot of each experiment was centrifuged after 30 min of incubation (apart from the one experiment terminated at 27 min) and the resulting pellet was analyzed as described in the section on methods.

* Average error of two determinations.

variable point between 27 and 30 min a trace amount of ^{22}Na or ^{82}Br was introduced into the incubation mixture and then the experiment was stopped at 30 min. The data obtained by such experiments can be used to compute the rate of isotopic exchange diffusion. We were very much surprised to discover that both sodium and bromide were completely equilibrated in the shortest time interval capable of experimental execution

TABLE VI. Exchange diffusion of ^{22}Na and ^{82}Br after incubation of mitochondria for 90 min in 0·15 M sodium bromide

	Exchangeable isotope			
	^{22}Na		^{82}Br	
Time of exposure to isotope (min)	mg pellet water/ mg protein	mμmoles ^{22}Na/ mg pellet water	mg pellet water/ mg protein	mμmoles ^{82}Br/ mg pellet water
1·0	6·73	163 ± 3·6	6·38	140·5 ± 1·2
1·5	6·66	161 ± 4·0	6·70	143 ± 6·0
2·0	6·89	167 ± 7·2	6·48	142 ± 4·3
3·0	6·80	163 ± 1·7	6·39	146 ± 0·7
87·0	6·72	162 ± 2·8	6·49	149 ± 2·6
90·0	6·81	164 ± 1·9	6·54	148·5 ± 3·0

Except for the time of incubation, the experimental details are identical with those described in the legend for Table V.

(1·0 min). Since the average matrix volume measured under identical conditions was 1·92 μl/mg protein, this would mean that an amount of internally contained sodium bromide equivalent approximately to 280 mμmoles/mg protein equilibrated in less than 1 min with externally labelled sodium bromide.

Table VI describes an identical type of experiment in which mitochondria were allowed to swell slowly for a much longer period of time in order to enlarge the matrix volume, and in this way a rate of exchange involving a much larger internal salt content could be determined. The matrix volume under the latter conditions was 3·48 μl and the salt equivalent 522 mμmoles/mg protein. Again, the sodium ion was completely exchanged in less than 1 min, but the bromide ion only 94% exchanged. However, even here the equilibration was too close to complete to make rate calculations possible.

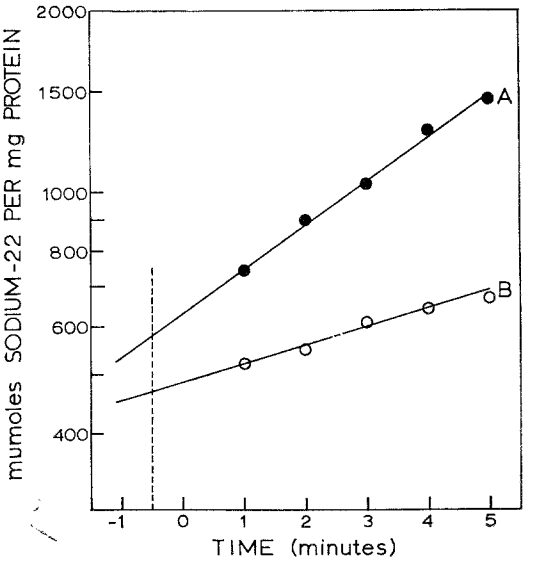

Figure 3. Kinetics of exchange diffusion of ^{22}Na at pH 5·60. Heavy beef heart mitochondria (0·2 ml of a suspension containing 10 mg of mitochondrial protein) were added to 4·75 ml of incubation medium containing 0·15 M sodium bromide and antimycin plus rotenone, each at a concentration of 2·0 μg/mg of protein. The medium in which the experiment described by curve A was carried out contained 25 μmoles of Tris-Cl, pH 9·1, in addition to the usual components, and the incubation proceeded for 15 min, followed by pH adjustment to 5·60. The medium in which the experiment described by curve B was carried out contained 25 μmoles of Tris-Cl, pH 7·4; the incubation was allowed to proceed for 30 min prior to pH adjustment to 5·60.

Estimation of Osmotically Active Volume from Exchange Diffusion Data

Permeability studies of phospholipid membranes[22] have shown that as the density of positive membrane charges is increased, the rate of penetration of the membrane by cations is inhibited, presumably because the increase in surface charge leads to electrostatic repulsion between the cation and the membrane. Such an effect, at least in theory, could be accomplished in mitochondria by lowering of the pH. There were two reasons for carrying out such an experiment: (1) it would serve as a control, in the sense that if sodium exchange could be made rate limiting we would like to know that our techniques are capable of demonstrating the phenomenon; and (2) it would serve as an independent means of determining the volume of the space surrounded by the rate-limiting membrane system, e.g., in this case the matrix volume. The evaluation of the volumes of a compartmented system by steady-state kinetics has been worked out by Kleinzeller and Knotkova.[23] The results of an experiment of this type are shown graphically in Fig. 3. The points depicted by line A were derived from an experiment where mitochondria were allowed to swell for 15 min in 0·15 M sodium bromide adjusted to pH 9·1. It has previously been established[8,24,25] that mitochondria swell more rapidly in alkali metal chloride or bromide salts at higher pH values. The pH of the suspension medium was then adjusted to 5·60 with HCl and the exchange diffusion rate of ^{22}Na was studied

for 5 min. Control experiments with ^{22}Na present from inception gave values of 1660 mμmoles sodium ion/mg protein present in the mitochondrion, while the corresponding water content was 10·22 μl/mg protein. Exchange-diffusion values gave good first-order kinetics as shown in the semi-logarithmic plot of Fig. 3. When the line described by points A is extrapolated to −0·5 min (the time that the pellet is separated from the suspending medium) a value of 580 mμmoles of labelled sodium ion is found on the intercept. This amount of exchange diffusion corresponds to the fastest flux rate according to Knotkova. It represents the amount of sodium in the pellet which underwent immediate exchange and is probably referable to the sodium contained in the extra-particulate and non-matrix water. The remainder of the exchangeable sodium (1080 mμmoles/mg protein) is seen to exchange at a much slower rate and may be viewed as being within a compartment bounded by a semi-permeable limiting membrane. The amount of sodium present in this compartment is 65% of the total sodium in the pellet. Since the matrix volume as determined with ^{14}C sucrose in an identical experiment was found to be 67·7% of the total pellet volume, the very close correspondence of these two numbers suggests that the sodium which resisted immediate exchange is indeed contained in the matrix compartment.

The curve described by points B is an identical experiment, except that only limited swelling was allowed to proceed for 30 min at pH 7·40 prior to acidification to pH 5·60. The total sodium ion content was 746 mμmoles/mg protein and the increment in the amount of sodium ion taken up with time was 271 mμmoles/mg protein, or 36·4%. The percentage of total pellet water which was found to be matrix water by sucrose-^{14}C exclusion was 41·5%, again in good agreement with the value determined by steady-state kinetics.

Exchange Diffusion Studies with Rubidium Chloride

Mitochondrial volume changes are indistinguishable in media containing sodium, potassium, and rubidium chloride.[8] We might infer from this that all of the results

TABLE VII. Exchange diffusion of ^{86}Rb and ^{82}Br after incubation of mitochondria for 30 min in 0·15 M rubidium bromide

	Exchangeable isotope			
	^{86}Rb		^{82}Br	
Time of exposure to isotope (min)	mg pellet water/ mg protein	mμmoles ^{86}Rb/ mg pellet water	mg pellet water/ mg protein	mμmoles ^{82}Br/ mg pellet water
1·0	4·58	162	4·44	139
1·5	4·49	158	4·36	141
2·0	4·56	154	4·62	140
2·5	4·61	166	4·50	142
3·0	4·44	162	4·35	144
27·0	4·53	161·5	4·48	141·5
30·0	4·59	159	4·54	142·5

Except for the use of ^{86}Rb, the experimental details are identical with those described in the legend for Table V.

presented are also applicable to potassium and rubidium chloride. The data of Table VII provide evidence in support of this view. Since rubidium and potassium ions behave in identical fashion in beef heart mitochondria under a large variety of conditions,[8,21] we chose to investigate only rubidium, because of the technical advantage cited in the section on methods. It is apparent from the data presented that rubidium bromide also undergoes immediate isotopic exchange.

The Alkali Metal Chloride Induced Reversal of Mitochondrial Swelling

Brierley *et al.*[26] have demonstrated that alkali metal chlorides added to suspensions of previously swollen mitochondria will effect a contraction of the osmotically active volume. This type of evidence is especially noteworthy, because in theory one can accurately control the driving force for the reversal of swelling by the addition of an impermeable non-ionic solute. Furthermore, the reversal of water intake under these conditions is very rapid and it is not immediately apparent that the water efflux is controlled by any rate-limiting step other than the osmotic pressure due to the impermeant solute. We have studied the ability of alkali metal chlorides and other solutes to effect a reversal of water uptake initially induced in a medium containing sodium acetate. Mitochondria were allowed to undergo pseudoenergized swelling in 0·176 M sodium acetate for 15 min, at which time the suspensions were diluted with water (final concentration of NaOAc was 0·15 M) or with various salts or polyhydroxylic solutes. The final concentration in the medium of the added solutes is given in Table VIII. After incubation for an additional 10 min the mitochondria were centrifuged and submitted to gravimetric analysis. The results as shown in Table VIII indicate that NaCl does indeed produce a contraction from 9·51 to 5·65 μl/mg protein. However, more important is the fact that NaOAc achieves the same contraction as does NaCl. This effect is to be expected with both salts, since the addition of salt has the effect of diminishing the driving force by its ability to swamp out the Donnan effect. On the

TABLE VIII. Reversal of swelling by addition of solute to mitochondrial suspensions exposed to 0·15 M sodium acetate

Additions	Final concentration	Water content of pellets after 20 min of incubation (mg/mg protein)
H_2O	—	9·51
Sucrose	0·25 M	2·54
Glycerol	0·25 M	9·27
NaOAc	0·30 M	5·86
NaCl	0·15 M	5·65
KOAc	0·15 M	3·60

Heavy beef heart mitochondria (0·3 ml of a suspension containing 12 mg of mitochondrial protein) were added to 9·9 ml of incubation medium containing 1·80 m moles of sodium acetate, pH 7·4, and antimycin and rotenone, each at a concentration of 2·0 μg/mg protein. After 15 min of incubation at 25°, 1·8 ml of a solution containing one of the solutes listed was added and the incubation continued for an additional 20 min. After centrifugation of duplicate 5·0 ml aliquots, gravimetric analyses were accomplished as described in the section on methods.

other hand, sucrose produces complete contraction, as one would expect, due to its impermeability. KOAc, which is an unambiguous example of an impermeable salt, produces considerably more contraction than does NaCl. We do not, therefore, view the NaCl-induced reversal of swelling as evidence of impermeability. On the contrary, the fact that the behavior of NaCl is indistinguishable in such a system from that of NaOAc is, to us, another argument in favor of the view that the mitochondrial membrane is permeable to NaCl.

The Contraction by Sucrose of Mitochondria Swollen in Alkali Metal Chloride Media

The study of sucrose contraction has proven to be a valuable tool for the study of alkali metal chloride permeability by methods involving volume change. The beauty of the method, as previously discussed, is that the driving force for water efflux is straightforwardly related to the introduction of an impermeable solute. When mitochondria are swollen for 90 min in 0·15 M sodium chloride, a significant increase in osmotically active volume is found, as shown in Table IX. If sucrose is then added and the mitochondria harvested in 2·0 min and submitted to gravimetric analysis it can be seen that complete reversal is achieved, as shown in Table IX. If the mitochondrial membrane

TABLE IX. The effect of sucrose upon the reversal of alkali metal ion-induced swelling

Conditions	Sucrose	Water content of pellet (mg/mg protein)
90 min in 0·15 M NaCl, pH 7·4	−	6·56
	+	3·38
15 min in 0·15 M NaCl, pH 9·0	−	9·06
	+	3·56
15 min in 0·15 M NaCl, pH 9·0 followed by lowering of pH to 7·4	−	9·12
	+	3·92
15 min in 0·15 M KOAc, pH 7·4 followed by addition of antimycin + rotenone	−	9·41
	+	7·17

Heavy beef heart mitochondria (0·5 ml of a suspension containing 20 mg of mitochondrial protein) were added to 8·9 ml of incubation medium containing 1·5 m moles of either NaCl or potassium acetate, the latter adjusted to pH 7·4. Tris-Cl (0·005 M) was present in the NaCl experiments at the pH shown in the Table. Antimycin and rotenone, each at a concentration of 2·0 μg/mg protein, were added initially in the sodium chloride experiments and after 15 min of incubation in the potassium acetate experiment. After the conditions listed in the table had been satisfied, 0·6 ml of water or of 2·0 M sucrose were added, and after 3 min of incubation, 3·0 ml aliquots were removed and centrifuged. The resulting pellets were submitted to gravimetric analysis as described in the section on methods.

were in reality impermeable to NaCl, the salt which required 90 min to get in should require a comparable time to get out. This should have prevented rapid reversal of swelling. As a control for the theory involved, mitochondria were swollen for 15 min in KOAc in the presence of succinate. Antimycin was then added to block respiration and further swelling; the samples were isolated 2·0 min after the addition of sucrose. It is apparent that contraction was only partial in a case where an unambiguously impermeable alkali metal salt is involved.

The Ability of Gramicidin to Facilitate the Entry of Alkali Metal Ions in Organic Media

One of the inadequacies of the conventional view of the impermeability of the mitochondrion to alkali metal ions is that it offers no basis for rationalizing the effect of gramicidin-D, a polypeptide capable of forming a complex with sodium ions which is soluble in hydrophobic media.[27] Gramicidin-D will also cause mitochondria to swell extensively in NaCl media at neutral pH.[8] Since mitochondria were known to be permeable to NaOAc, the supposed impermeability of the mitochondria to NaCl was explained in terms of the inability of chloride ions to get in. It is impossible to rationalize the effect of gramicidin on swelling given the impermeability to chloride ions and the modulating effect of gramicidin on sodium ions. The data of Table X attest to this view. If a partition

TABLE X. Sodium and bromide ion content of toluene:butanol after partitioning with aqueous 0·05 M sodium bromide

Composition of organic phase	mμmoles of ion in organic phase	
	Sodium	Bromide
Toluene:butanol	0·8	7·50
Toluene:butanol + gramicidin	13·1	5·85

2·0 ml of toluene:butanol (70:30) were partitioned against 1·0 ml of 0·05 M ^{22}Na or ^{82}Br containing 20 μmoles of Tris base by shaking in a vortex for 30 sec. When present, gramicidin-D was introduced into the organic phase at a concentration of 25 mg/ml. The two phases were cleared by spinning for 5 min in a clinical centrifuge. Aliquots of both phases were assayed for radioactivity.

is made between 0·05 M aqueous $^{22}Na^{82}Br$ and a mixture of toluene:butanol, the amounts of anion and cation brought into the organic phase can be used as a measure of ability of a reagent to render a given ion soluble in a hydrophobic medium. In the absence of gramicidin the organic phase contained 0·8 mμmoles sodium ion, and gramicidin increased this to 13·1 mμmoles. At the same time the partition of bromide ion in the organic phase not only failed to increase but decreased significantly as gramicidin was added. It is clear from this study that while gramicidin is active with sodium, it appears incapable of inducing solubilization of bromide ion in the organic phase. This suggests that gramicidin would be incapable of accelerating swelling, if indeed chloride or bromide ions were impermeable species.

Discussion

Heavy beef heart mitochondria swell at a relatively slow rate in decimolar alkali metal chloride or bromide media. The absorbance decrements at 520 mμ which we have observed in our experiments is of the order of 0·002/min at pH 7·4 in 0·15 M sodium chloride. This is in very good agreement with the rate of passive swelling of rat liver mitochondria in 0·2 M KCl at pH 7·4 as published by Ogata and Rasmussen.[28] The latter authors described a rate of absorbance decrement of the order of 0·006 under

conditions very similar to those we have employed. Other authors have published rates of absorbance decrement for passive swelling in alkali metal chloride or bromide media which are either as much as ten-fold greater[24,29] or almost zero.[7,14] In the latter two cases, however, Hunter and Brierley[14] and Brierley *et al.*[7] followed swelling for only very short periods of time (3–5 min), during which measured rates of 0·001 or 0·002 would be too small for accurate determination. However, these same studies showed that the osmotically active volume increased by 20% in 0·12 M KCl in the presence of rotenone as compared with an identical swelling experiment carried out in 0·25 M sucrose.

The data from gravimetric experiments reported in the present communication are also in very good agreement with the thesis that mitochondria swell very slowly in alkali metal chloride or bromide media. The osmotically active volume of mitochondria after 1·0 min of incubation in 0·15 M KCl was found to be 1·07 μl/mg protein. Hunter and Brierley[14] have published a figure of 1·04 μl/mg protein for the osmotically active volume of mitochondria exposed for 3 min in 0·12 M KCl—a value in good agreement with ours. After 120 min of incubation in 0·15 M NaCl or KCl, we have found increments in the osmotically active volumes of only 1·65 and 1·23 μl/mg protein, respectively. Therefore, on the basis of these and other observations made previously[7,8,14,24,28,29] we feel justified in concluding that (1) our mitochondrial preparations are responding normally, i.e., they are not especially "leaky" in the conventional context to alkali metal chloride ions as judged by osmotic responses either optical or gravimetric; (2) mitochondria are not *completely* impermeable to alkali metal chloride or bromide ions as emphasized by other investigators[5,7,14]; and (3) mitochondria do, in fact, possess at least a low permeability to alkali metal chloride or bromide salts.

We have sought to concern ourselves with a more fundamental question, which is the relation between the rate of swelling in alkali metal chloride or bromide media and the apparent permeability to these salts; i.e., is the very slow rate of swelling the direct result of the limited permeability to these salts? To some extent, the swelling data above are inconsistent with this view. For example, the osmotically active volume of mitochondria after 1·0 min of incubation in 0·15 M NaCl was 1·18 μl/mg protein (Fig. 2). Prior to introduction of mitochondria into the sodium chloride media, and while they were still in 0·25 M sucrose (at a concentration of 2 mg protein/ml), the osmotically active volume was found to be 0·37 μl/mg protein, which is in good agreement with previously quoted values from this laboratory.[8] By extrapolation we can infer that the osmotically active volume increased at a rate of at least 0·81 μl/min/mg protein in a suspending medium (0·15 M NaCl) which possesses an osmotic pressure comparable in magnitude to that of the mother solution (0·25 M sucrose). Since the concentration of NaCl in the suspending medium is 150 mμmoles/μl, this rate is approximately equivalent to an influx of 121 mμmoles of sodium chloride/min/mg protein. If this is a valid extrapolation, it suggests that the rate of penetration of NaCl is not the rate-limiting step in the swelling process, but rather that mitochondria lack a driving force for rapid swelling in sodium chloride.

The above arguments are, of course, dependent on the assumption that the external salt does actually penetrate the membrane systems and reaches an internal concentration approximately equivalent to that present externally. This view is intuitively obvious in a nonenergized system and has been abundantly substantiated in the present communication. Nevertheless, a number of authors have taken opposition to this view.[11–14]

For example, Hunter and Brierley[14] find that mitochondria isolated from a medium 0·12 M in KCl and with rotenone present to suppress electron transfer due to endogenous substrate contain a total of 550 mμmoles of potassium ion/mg protein and have a total water content of 4·10 μl/mg protein. The unambiguous fact of this finding is that the concentration of potassium in the total pellet water is 550 mμmoles/4·10 μl (or 134 mμmoles/μl) water, i.e., 14 mμmoles/μl greater than the external concentration of potassium. Hunter and Brierley suggest, however, that most of the potassium is bound, and therefore rationalize in favor of the impermeability of the membrane to potassium. If this were so, i.e., if no KCl penetrated the inner membrane, then only the mannitol-permeable water should contain KCl at an equivalent concentration. Their value for this space is 3·06 μl protein. The product of this value times 120 mμmoles/μl of protein (i.e., the concentration of external potassium chloride) equals 367 mμmoles/mg protein of equilibrated potassium ion. This would mean that 550 − 367 (or 183) mμmoles of potassium/mg protein are bound. The nonvalidity of this conclusion is adequately demonstrated by the same authors and in the same communication. They claim that mitochondria are permeable to potassium acetate in a medium in which respiration is proceeding. They find, under such conditions, 1000 mμmoles of K^+/mg protein in a total of 7·59 μl water/mg protein. Since the membrane is permeated by potassium under these conditions (0·12 M potassium acetate + respiration) the salt must be equilibrated and this equilibration would involve 7·59 μl × 120 mμmoles/μl of mitochondrial water, or 911 mμmoles potassium/mg protein. Since they found 1000 mμmoles/mg of potassium, then the amount bound must be 1000 − 911 (or 89) mμmoles, as opposed to 183 mμmoles in KCl. It is difficult to believe that the mitochondrion binds 100% more potassium under conditions where potassium does not penetrate the matrix space than under conditions where it does penetrate.

We therefore conclude that the chlorides and bromides of sodium and potassium penetrate the osmotically active space and this conclusion is based on three major points of experimentation and interpretation: (1) the studies on volume distribution presented in the present paper; (2) the evidence from osmotic responses that the inner membrane is not completely impermeable to these salts; and (3) the complete lack of cogent evidence to the contrary.

We have stated in the introduction that the only real test of permeability towards a given ion must be ascertained under conditions which are independent of volume change. This view is made abundantly clear in the present studies. Exchange diffusion of both sodium and bromide ions at neutral pH is instantaneous within the limits of measurement of our experimental techniques. A forthcoming communication[20] provides clear evidence of the adequacy of this technique for demonstrating when the impermeability to a given salt is rate limiting in the swelling phenomenon, as in the case of mitochondria exposed to potassium acetate in the presence of inhibitors of respiration. Furthermore, even in the present communication, experimental manipulation has made possible the demonstration of a measurable flux rate (Fig. 3) exhibiting first-order kinetics. The only logical conclusion that can be drawn from these facts is that (1) mitochondria are considerably permeable to alkali metal chloride and bromide salts; and (2) some other event is associated with the slow rate of swelling in such media. We have briefly alluded to the view that the mitochondria lack a driving force for swelling in such media. Although it is not within the scope of the present communication to discuss

this at length, a few words on the subject seem worthwhile. A fuller account of the driving force in mitochondrial swelling will appear elsewhere.*

The main thrust of the argument is based on studies initially carried out by Azzi and Azzone[24] and later extended by Blondin[8] and Brierley.[25] The principal observation is that the rate and extent of mitochondrial swelling is linearly increased in alkali metal chloride media as the pH is raised from 5·5 to 9·0. Since the isoelectric point of the mitochondria is believed† to be close to pH 5·5 this means that as the pH is increased the degree of ionization of fixed dissociable negatively charged groups is also increased. Increasing degrees of ionization of such groups would necessarily lead to changes in the amount of counter ions within the structure as the result of Donnan ion distribution, with concomitant changes (increases) in volume. The fact that mitochondria can be induced to swell in NaCl media in the presence of sulfhydryl reagents (e.g., *p*-hydroxymercuribenzoate) which add a negative charge[29,30] to the internal structure can also be viewed as an expression of this fact.

Although these observations have been known for some time, their interpretation has been completely within the framework of current permeability tenets. For example, Azzi and Azzone[24] view the mitochondrion as impermeable to chloride ions since isolated mitochondria resist passive swelling in sodium, potassium, or ammonium chloride media. However, they have established that considerable swelling occurs in such media under slightly alkaline conditions, and have inferred from these observations that considerable permeability to the chloride anion develops as a result of the pH increase. Although the logic of this interpretation seems unassailable within the existing framework of principles, the conclusion is preemptive of a large body of evidence[22,31] which in fact suggests that anion fluxes, through both synthetic and biological membranes, are decreased as the pH is raised.

Mitochondria swell spontaneously in sodium acetate media at neutral pH, whereas they resist extensive swelling when suspended in alkali metal chloride media adjusted to the same pH. Since permeability to salt can not be of a rate-limiting nature in the latter case, we may assume that the driving force for swelling is considerably less in NaCl than in sodium acetate media of the same external pH. Recently, Asai *et al.*[32] have shown that sodium acetate rapidly induces the extensive configurational changes which must precede large amplitude swelling, whereas sodium chloride does not. While the precise molecular nature of this configurational sequence is as yet undefined, it is almost certainly related to the driving force for swelling. The fact that accelerators (e.g., gramicidin, calcium, etc.) of swelling in NaCl media induce not only the configurational changes[32] but also an apparent internal alkalinization of the mitochondrion,[6,33] is consistent with this view.

It is apparent, therefore, that the internal R^- concentration (fixed negatively charged groups) must be considerably lower in mitochondria suspended in sodium chloride media than in mitochondria suspended in sodium acetate media at the same external pH. This view is consistent with the finding[8] that the pH optimum for rapid swelling is shifted, in chloride or bromide media, to the more alkaline range.

In conclusion, it is perhaps worthy of note that many of the above views have been postulated by Dilley and Rothstein[10] in relation to the mechanism of volume changes

* G. Blondin and D. E. Green in preparation.
† G. Blondin and G. Vanderkooi, unpublished results.

in chloroplasts. It is especially significant that the latter authors found it necessary to assume complete permeability of the chloroplast membranes (grana and stroma lamellae) to small anions (e.g., Cl^-) and cations (e.g., Rb^+).

Acknowledgements

The expert technical assistance provided by Mrs. Carole Funnell is greatly appreciated. Meat by-products were kindly furnished by Oscar Mayer and Company, Madison, Wisconsin. This work was supported in part by training grant GM-88 and Program Project Grant GM-12,847 from the National Institute of General Medical Sciences (USPHS).

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