*THE MECHANISM OF MITOCHONDRIAL SWELLING**

By George A. Blondin† and David E. Green

INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN, MADISON

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The ability of mitochondria to swell under a wide variety of conditions is a property which has been the subject of intensive investigation for more than a decade.[1-3] The most baffling feature of mitochondrial swelling lies in the fact that it can take place in media of widely different osmolarities. Swelling of mitochondria in decimolar sucrose solutions generally does not occur unless coupled to energy-linked functions;[4-6] when such energized swelling is suppressed by addition of appropriate inhibitors, swelling is reversed.[7-9] However, the reverse relationship between mitochondrial volume and the generation of the energized state has been found to prevail under certain conditions in distilled water and in decimolar solutions of alkali metal chlorides;[10-12] i.e., swelling is reversed by coupling to energy-linked functions. These and many other observations sharply point up the fact stressed previously by Pressman[13, 14] and Lardy[15] that an explanation of changes in mitochondrial volume exclusively in terms of osmotic phenomena is far from adequate.

Data have been accumulated in our laboratory which provide the foundation for a radically different interpretation of mitochondrial swelling. Ultrastructural changes in the mitochondrial membrane appear to be central to the problem of swelling. The present communication is an interpretative account of the investigations which have provided the experimental foundations for relating mitochondrial swelling to ion-induced, ultrastructural rearrangement of the inner mitochondrial membrane.

Swelling as an Expression of the Inner Mitochondrial Membrane.—Swelling in solutions decimolar in alkali-metal salts[16] (and also in distilled water[17-19]) can be uniformly described in terms of the following ultrastructural changes in the inner membrane of beef heart mitochondria. As swelling proceeds, the tubular cristae are retracted and eventually disappear. The repeating units are withdrawn from the cristae and paid into the vesicular part of the inner membrane (see Fig. 1). Thus, as the cristae are pulled back into the peripheral membrane, the membrane expands in diameter.

The electron-micrographic evidence[16-19] for the ultrastructural changes that take place during swelling suggests that the conditions which lead to swelling modify the geometry of the repeating units in such a fashion that these are no longer stable in the tubular configuration of the cristae. The rearrangement in the membrane consequent to this alteration in the repeating units is expressed in the transition from a collapsed inner membrane with multiple tubular invaginations to an extended inner membrane with no invaginations.

The inner membrane alone is responsible for the changes in water content during swelling and for the various phenomena described in the present communication. As swelling proceeds, the outer membrane is perforated but this perforation does not affect either the continuity of the swelling process or the reversal of swelling induced either by sucrose or by inhibitors of respiration.

Ion Binding and Salt-Induced Swelling.—An invariant correlation has been established experimentally between mitochondrial swelling in presence of salt[20] and the binding of the cations of the salt in question to the structured sectors of the mitochondrial inner membrane. The amount of cation binding[21] achieved at the point of maximal swelling differs from one salt to another (see Table 1), but for a given salt the extent of swelling is proportional to the extent of binding.[22] The following two lines of evidence provide the justification for postulating a causal relationship between swelling and the ion-binding capability of the inner mitochondrial membrane.

(*a*) Large-amplitude swelling of mitochondria (non-energy-linked) induced by gramicidin, valinomycin, Ca^{++}, etc. will not occur in aqueous media containing nonionic solutes such as sucrose but will occur in aqueous media containing decimolar concentrations of alkali metal salts (see Table 2). Under the latter conditions, swelling agents will induce a two- to tenfold increase in the amount of cation *bound* by the mitochondrion. In general, it can be stated that in decimolar salt, whenever it is possible by proper selection of conditions to observe both a contracted and a swollen state, the swollen state will always be characterized by a significant increment of cation binding.

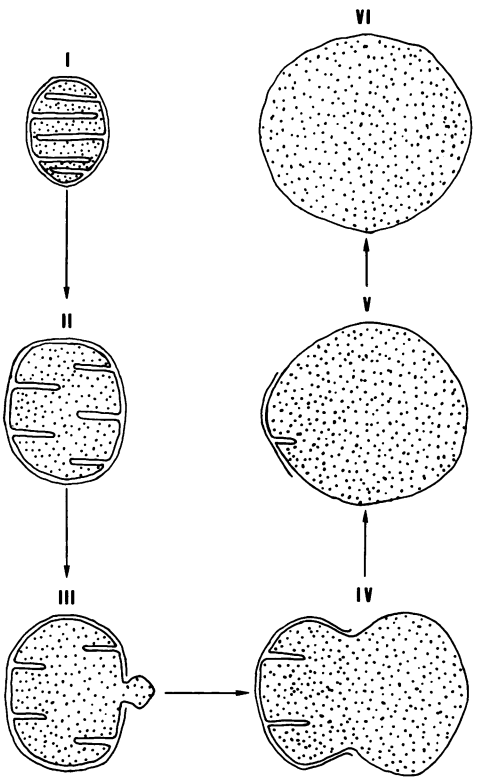

FIG. 1.—Diagrammatic representation of the morphology of the mitochondrion during various stages of large-amplitude swelling in decimolar salt media.

TABLE 1

CORRELATION OF MITOCHONDRIAL CHANGES IN VOLUME WITH THE BINDING OF ALKALI METAL CATIONS

Salt in medium	Increase in H_2O (mg per mg protein)	Cation bound (mμmoles per mg protein)	Oxygen consumed (mμatoms per mg protein per min)
Sodium acetate	8.67	187	46.4
Sodium acetate + cyanide	6.50	123	0
Potassium acetate	6.13	392	31.0
Potassium acetate + cyanide	1.37	95	0
Rubidium acetate	7.39	1520	25.8
Rubidium acetate + cyanide	1.37	498	6.9

The incubations were carried out at 25° for 20 min in 0.15 *M* alkali metal acetate which was also 0.01 *M* in Tris-acetate (pH 7.4), and, where noted, 5×10^{-4} *M* in KCN. The incubations were begun by the addition of freshly prepared heavy beef heart mitochondria to a final concentration of 2.5 mg of protein per ml (total volume, 5.0 ml), and terminated by sedimentation of the mitochondria (2.0-ml aliquot) for 1.0 min at 20,000 rpm. The volumes of the pellets were determined from measurements of wet weight versus dry weight, and are here recorded as increments with respect to a control experiment carried out in 0.25 *M* sucrose. The bound cations refer to the amounts present in the pellet in excess of that present on the assumption of complete equilibration of the intramitochondrial aqueous phases with the alkali metal salt media. The values reported were obtained by isotopic analysis with the use of Na^{22}, K^{42}, and Rb^{86}.

TABLE 2

EFFECT OF GRAMICIDIN ON MITOCHONDRIAL SWELLING AND ALKALI METAL BINDING

Medium	Total water (mg per mg protein)	Na^+ bound (mμmoles per mg protein)
0.25 *M* sucrose	3.42	13.6
0.25 *M* sucrose + gramicidin	2.98	3.1
0.15 *M* NaCl	3.43	13.7
0.15 *M* NaCl + gramicidin	7.73	108

Conditions were those described in the legend of Table 1 except for the presence (where indicated) of gramicidin at a final concentration of 10^{-7} *M* and the substitution of Tris-Cl (pH 7.4) for Tris-Ac. Sodium was determined by extraction of the mitochondrial pellet in 0.3 *M* perchloric acid and subsequent analysis by atomic absorption spectrometry. The values for the experiments in sucrose represent total (endogenous) sodium whereas those for the experiments in NaCl represent net sodium content after correction for equilibration with the medium as described in the legend of Table 1.

(*b*) The energized uptake of alkali metal ions by mitochondria suspended in low-salt media usually leads to large-amplitude swelling.[7-9, 14] Although the present data do not permit a distinction to be made between an uptake of cationic species (in low-salt media) involving a form of ion binding and an uptake in which the cationic species is still osmotically active, it is clear that energized processes can be coupled to the binding of alkali metal cations (Table 1) with consequent swelling. It is reasonable to presume, therefore, that a significant portion of the actively transported alkali metal cations from centimolar salt media are bound by mitochondria and that this binding is intrinsic to the mechanism of salt-induced swelling. Further support for this hypothesis derives from the finding that the ratio of the amount of ions bound (in low-salt media) to the increase in mitochondrial volume is a function of the nature of the cation. Table 3 shows that this ratio is close to 2.5 times as high for Rb^+ as for Na^+, which is qualitatively in agreement with the results obtained in media decimolar in alkali metal salt and in the absence of coupling to energy-linked functions.

Our experimental studies have been restricted largely to salts of univalent cations (Li^+, Na^+, K^+, Rb^+, Cs^+, and NH_4^+) and to certain divalent cations (Mg^{++}, Ca^{++}, Sr^{++}, and Mn^{++}) but the salts of a large variety of anions (fluoride, chloride, bromide, iodide, sulfate, sulfite, phosphate, arsenate, formate, acetate, propionate, chloroacetates, etc.) have been examined in relation to the swelling phenomenon. At present we have no evidence which would speak against the thesis that swelling of intact mitochondria can be achieved by the cationic species of any salt. The important point to be made is that swelling is a function of the uptake of cations, and that the salts of all mono- and divalent cations tested can induce swelling under appropriate conditions.[22]

In relation to this hypothesis we have considered the possibility that, in fact, the reverse might be true; i.e., that swelling is the cause of increased ion binding in con-

TABLE 3

ENERGIZED ION UPTAKE IN MEDIA CENTIMOLAR IN ALKALI METAL SALT

Salt in medium	Total mitochondrial water (mg per mg protein)	M^+ bound (mμmoles per mg protein)	Ratio mμmoles M^+ bound per mg H_2O
Sodium acetate	11.07	487	44
Potassium acetate	13.26	963	72.6
Rubidium acetate	14.00	1428	102

The incubations were carried out at 25° in a total volume of 15 ml containing heavy beef heart mitochondria at a final concentration of 2.5 mg of protein per ml. The media were 0.1 *M* in mannitol; 0.04 *M* in Tris-acetate, pH 7.4; 0.0015 *M* in Tris-ATP; and 0.004 *M* in labeled (Na^{22}, K^{42}, Rb^{86}) alkali metal acetate. The reaction was begun by the addition of gramicidin to a final concentration of 10^{-8} *M*. Samples were withdrawn at the point of maximal ion uptake (Beckman cationic-sensitive electrode) and centrifuged at 20,000 rpm for 1 min. Determinations of volume and radioactive elements were accomplished as described in the legend of Table 1.

sequence of the exposure of new binding groups as suggested by Bartley and Enser.[23] However, our recent evidence suggests that increase in the binding capability as a result of swelling cannot be the major determinant of the order of events. Thus, mitochondria in a medium which is 0.15 *M* in NaAc and 0.25 *M* in sucrose do not swell (in absence of energizing conditions); yet the mitochondria will bind almost as much sodium as they do in the absence of sucrose when swelling occurs. This evidence suggests that the process of swelling does not make available previously unavailable binding sites in amounts sufficient to account for the increased alkali metal cation binding observed under swelling conditions.

Facilitation of Ion Binding.—Mitochondrial swelling in media decimolar or higher in salt proceeds spontaneously, i.e., in the absence of energy-coupled processes; however, facilitation by other reagents in addition to the salt may be required. Such facilitation of mitochondrial swelling in solutions 0.15 *M* in sucrose and centimolar in salt is almost always required, and is accomplished by reagents such as gramicidin and valinomycin, or by reagents which lead to the energized state (substrate plus oxygen, or adenosine triphosphate (ATP). Both categories of reagents are usually needed for swelling in presence of centimolar salt.[7-9, 13, 14] The former type of facilitation relates to the availability of the binding groups in the membrane to the action of salt. Gramicidin, valinomycin, monactin, and other similar-acting reagents so modify the structure of the repeating units as to facilitate the entry or permeation of salts. The electrostatic barrier imposed by the layer of phospholipid enveloping the repeating units is modified by these reagents[24, 25] and this modification permits the more ready penetration of salts into the interior of the membrane itself.

The latter type of facilitation, i.e., the generation of the energized state, induces a structural alteration in the interior of the repeating units such that potential binding groups become exposed, and thus are made readily available for interaction with penetrating salts. Whatever the nature of this alteration, it is different in kind from that induced by antibiotic polypeptides such as gramicidin. These polypeptide reagents probably influence the penetrability of the repeating units to salts, whereas generation of the energized state achieves primarily the release of groups that can react with salt.

High salt concentrations are capable of achieving the same end effect as the combined effects of polypeptide reagent, energizing conditions, and salt in centimolar concentration. That is to say, the repeating units are more permeable to certain salts when these are present in high concentration, and the groups that can react with salt are fully available. From the correspondence in the swelling behavior of the mitochondrion under these two different sets of conditions we may conclude that the state of the repeating units induced in media of high salt concentrations is equivalent to that of the energized state. If we accept the thesis that the repeating units can assume two conformations, the one characteristic of the nonenergized state (buried or unexposed groups that can react with salt) and the other characteristic of the energized state (exposed groups), then high salt induces the same conformational state as does substrate or ATP at lower salt concentrations.

Contribution of Phospholipid to the Ion Binding Capability of Mitochondria.—Phospholipid can be excluded as the major instrumentality for ion uptake because mitochondria which have been depleted of their lipid content according to the pro-

TABLE 4

BINDING OF ALKALI METAL CATIONS BY LIPID DEPLETED MITOCHONDRIA

Salt in medium	Cation Bound (Mμmoles per Mg Protein)	
	M^+ acetate	M^+ chloride
Sodium (0.15 *M* acetate or chloride)	81.4	83
Rubidium (0.15 *M* acetate or chloride)	1261	1185

Experimental conditions were as described in the legend of Table 1. The mitochondria were rendered lipid-deficient according to the procedure of Fleischer *et al.*[26]

cedure of Fleischer *et al.*[26] show an abundant capability for binding alkali metal ions (Table 4). However, in view to the well-known ability of phospholipid micelles to retain mono- and divalent cations,[27, 28] it is probable, as well as consistent with the data in Tables 1 and 4, that a portion of the binding observed in intact mitochondria is referable to the charged groups of phospholipid. The exact extent of the interaction between phospholipid and alkali metal ions in intact mitochondria is at this stage not clear; however, on the basis of data of the kind shown in Tables 1 and 4, it is probable that these interactions contribute only to a form of ion binding which does not lead to swelling.[29]

Additional evidence which would argue in favor of at least two different cation binding modalities in mitochondria has been forthcoming from a study of proton movements. In recent years a considerable number of reports have been published dealing with the extrusion of protons from the mitochondria as other cations are taken up in an energy-dependent process.[3, 30–32] Our findings in centimolar alkali metal chloride media and in the absence of energy-coupled processes strongly suggest that the cation-for-proton exchange is related only to "nonspecific" binding, i.e., it is accompanied by alkali metal ion uptake which does not give rise to swelling. On the other hand, when facilitating agents such as valinomycin or gramicidin are introduced into the same medium, there is an additional uptake of cations which is accompanied by large-amplitude swelling and by an apparent proton accumulation. The significance of these proton movements is at this stage hard to assess, all the more so since salt-induced conformational changes in the membrane may drastically alter the pH of the incubation medium.

Salt-Induced Helix Formation in Poly-L-Lysine.—A study of the interaction of poly-L-lysine with alkali metal salts has afforded us considerable insight into the comparable interaction of membrane repeating units with salt. When the amino groups of poly-L-lysine are fully dissociated (pH about 10.5–11.0) the polypeptide can assume 100 per cent helical configuration.[33] The degree of helix formation of poly-L-lysine is influenced not only by the pH of the medium but also by the concentration of salt in the medium. For a given pH in the range 8.5 to 11.0, the degree of helix formation is increased by the presence of salt,[33] and under similar conditions considerable quantities of alkali metal cation have been found to be associated with the polymer (determined by equilibrium dialysis).[22] The degree of cationic association roughly parallels the helical content of the polymer and the order of association for alkali metal ions appears to be much the same for poly-L-lysine as for mitochondria and is the reverse of the order ($Na^+ > K^+ > Rb^+$) which has been observed for the electrostatic association of alkali metal cations with phospholipid micelles.[34–36] What is of particular interest is the fact that helix formation induced by salts leads to the uptake of salt by poly-L-lysine; and when the helical content of

poly-L-lysine is reduced by lowering the pH or by raising the temperature, the salt taken up during helix formation is released.

Localization of Groups in the Membrane Which React with Salt.—Exactly where the salt-reacting groups are localized in the repeating units of the membrane is still an open question, but all the available evidence points to the localization of these groups in the interior of the proteins of the repeating units and within an electrostatic barrier, probably provided by phospholipid. Not only is this postulated localization consistent with the evidence that penetration of alkali metal salts into repeating units often has to be mediated by reagents which modify the charge characteristic of phospholipid micelles, but it is also consistent with the absence of a barrier in lipiddepleted mitochondria to the interaction of alkali metal cations with groups in the membrane (see Table 4).

Two Modalities of Membrane Penetration.—There are two ways by which salts can penetrate the mitochondrion: (1) between repeating units into the mitochondrial spaces; and (2) directly into repeating units. The inability to distinguish experimentally between these two types of penetration in large measure accounts for the confused state of the field during the past decade. Repeating units fit (nest) together to form a two-dimensional membrane continuum;[37] the narrow spaces at the points of junction between the repeating units of the inner membrane provide a pathway for the penetration of the solvent (water plus small solute molecules) through the membrane into the spaces in the interior. The rate of this penetration of ions between repeating units appears to be dependent only upon the ionic dimensions of the diffusing species and appears not to be influenced by the state of the mitochondria, i.e., whether in the energized or nonenergized state.[22] Implicit in this postulate of penetration between repeating units is the existence of aqueous channels, probably fluctuating in position, as a permanent feature of the membrane. Such a view, long overlooked in the biochemistry of the mitochondrial inner membrane, is based on (*a*) the absence of a barrier to the diffusion of water through the inner membrane,[22, 23] and (*b*) the finding that the macroaqueous compartments of the mitochondrion are capable of almost instantaneous equilibration (<1.0 min) with media decimolar in sodium or potassium chloride.[22, 38] This phenomenon of penetration between repeating units is unrelated either to the binding of ions or to, mitochondrial swelling.

However, from the evidence which has been presented, it is clear that mitochondria possess a potential for the uptake of ions which leads to swelling, and that this potential function also imposes a barrier capable of ionic discrimination. The principal characteristics of the latter barrier are (*a*) that the size of the anion is no longer a determinant of penetrability, i.e., the penetration of sodium salts is approximately the same whether the anion be formate, acetate, propionate, isobutyrate, trimethylacetate, or phenylacetate; (*b*) that the dissociation constant of the acid formed from the anion is a critical factor in the penetrability, i.e., the weakly dissociating anionic acids (acetic, phosphoric, sulfurous) penetrate more readily than do the strongly dissociating anionic acids (hydrochloric, hydrobromic, sulfuric); (*c*) that the barrier in question appears not to be concerned primarily with access to any of the macroaqueous compartments of the mitochondrion; and (*d*) that the penetration is influenced by both classes of facilitating agents. From the above considerations, it is reasonable to presume that the uptake of ions by mitochondria in-

volves not only surface interactions but also the entry of these ions into a structured segment of the mitochondrion, i.e., into the repeating units of the inner membrane.

A Unified Theory of Mitochondrial Swelling.—The basic and constant feature of mitochondrial swelling of all types is the rearrangement of the inner membrane from a contracted state with multiple invaginations (cristae) to an expanded state with few or no invaginations. This change in membrane form is a consequence of a change in geometry of the repeating unit brought about by helix formation. There are three ways of inducing helix in the mitochondrial repeating units: (1) decimolar salt; (2) a combination of centimolar salt and the energized state; and (3) a combination of no salt and distilled water.[39] Sucrose (0.1–0.25 *M*) suppresses helix formation by modes (1) and (3) but has no effect on helix formation by the second mode.

When mitochondria begin to swell under the three sets of conditions specified above which lead to helix formation, the inner membrane has a characteristic appearance. The pouchlike cristae break up into tubular sections and the space within the tubule disappears. The point at issue is that the change in geometry of the repeating units and of the membrane induced under these three different sets of conditions appears to be the same. It should be stressed that there are multiple regions for potential helix formation in the repeating units; each mode of helix formation probably involves alternative helical regions.

When mitochondria are carrying on coupled respiration, the repeating units are in the energized state. In this state, residue groups in the protein which can participate in helix formation are exposed and made available for interaction with salt. Given this interaction with salt, helix formation is induced. But the helix formed by interaction of salts with groups in the protein of energized repeating units is stable only as long as the repeating units remain in the energized state. When the repeating unit becomes de-energized, the salt-induced helix opens and the ions bound during helix formation are released. Thus, swelling induced by centimolar salt under energizing conditions can be reversed merely by suppressing electron transfer; the helix is then opened and the ions taken up are released.

There appears to be a totally unexpected way in which helix formation induced by salts can lead to the containment of salts within the domain of the helix. How this containment is achieved is unknown. However, the phenomenon has been verified in our laboratory for poly-L-lysine by three independent physical observations; namely, by techniques involving equilibrium dialysis, gel filtration, and the use of ion selective electrodes.[22]

Some Summarizing Comments.—Mitochondrial swelling has provided insight into the conditions required for inducing helix formation in repeating units and into the consequences of helix formation for the arrangement of the inner membrane. An entirely novel way of containing ions within a membrane (via helix formation) has been revealed by the study of mitochondrial swelling and this discovery may open the door to the molecular tactics for accumulating ions within membranes.

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† Postdoctoral trainee of the Institute for Enzyme Research.

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