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Paired Moving Charge Model of Energy Coupling. III. Intrinsic Ionophores in Energy Coupling Systems

(ionophoroproteins/transport ionophores/catalytic ionophores/coupling modes)

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ABSTRACT **The experimental basis for the postulated role of intrinsic ionophores in mitochondrial ion transport and energy coupling is summarized. Intrinsic ionophores appear to be linked to, or contained within, specific ionophoroproteins localized in the inner membrane, and the isolation of these ionophores requires their release from the ionophoroproteins. At least ten different species of ionophores have been isolated from the mitochondrion, five of which have been wholly or in part chemically identified. Intrinsic ionophores have been implicated in the activation of inorganic phosphate in ATP synthesis and hydrolysis, and in the control of the coupling modes. The presence of ionophores in soluble proteins such as troponin and in ATP-energized kinases has been demonstrated.**

Ionophores occupy a central position in energy coupling systems by virtue of their unique capability for charge separation (1, 2). The present communication has two objectives—first, to summarize the rapidly growing body of experimental evidence relevant to the intrinsic ionophores of the mitochondrion, and second, to provide a framework and perspective for the new field of experimental inquiry revolving about these intrinsic ionophores.

Historical. The use of antibiotic ionophores in the study of energy coupling in mitochondria has a long history dating back to the discovery by Dubos and Hotchkiss (3) that the polypeptide antibiotics produced by *Bacillus brevis*, collectively referred to as gramicidin, were uncouplers of mitochondrial oxidative phosphorylation (4, 5) and facilitated the movement of K^+ across bacterial membranes (6). Lardy (7, 8) and Pressman (9, 10) have been the pioneers in examining the modulating role of antibiotic ionophores on mitochondrial energy coupling and in introducing the use of antibiotic ionophores for the study of energy coupling. Eisenman (11, 12) and Eigen (13) have developed the framework of principles which underlie the transport of ions across membranes by antibiotic ionophores. In recent years, ionophoretic activity has been demonstrated in a wide variety of molecular structures (14); moreover, the synthetic chemist has shown great aptitude in designing molecular structures that exhibit ionophoretic capability (14).

The curious point is that, although these experimental and theoretical developments established beyond peradventure that ionophores were active at incredibly high dilutions in facilitating ion transport, and provided the perfect models for the natural mediators of active transport and ion movements generally, nonetheless there was great reluctance, nay strong opposition, to taking the next obvious step of identifying the natural mediators of ion movements as ionophores. What was the basis for this resistance to the next obvious step? There were three main reasons. First, since extrinsic ionophores were antibiotics and potentially lethal reagents, the presence of natural counterparts of these ionophores in coupling systems was deemed to be highly improbable. Second, the concept of fixed channels in membranes had become so entrenched, particularly in the physiological literature (15), that it was treated as the ultimate reality even though there was no direct evidence to support the concept. Third, the preoccupation of biochemists with membrane transport proteins (15) effectively closed the door to any alternative approach. In our own laboratory, we indeed took the next obvious step 5 years ago and in time discovered the cellular tactics which effectively eliminate the possibility of what we may describe as the antibiotic catastrophe.

Elsewhere Blondin has related the chronology of steps by which he came to recognize that the intrinsic ionophores of the mitochondrion were not freely dispersed in the lipid phase of the inner membrane (16). They were found to be contained within proteins, and by virtue of this containment, unregulated participation of these ionophores in energy coupling was effectively excluded. The isolation and characterization of the intrinsic mitochondrial ionophores depend, therefore, upon their release from the proteins with which they are associated. The nature of the molecular strategy of containment is, accordingly, a paramount consideration for the systematic study of intrinsic ionophores.

We may formulate a set of key questions about intrinsic ionophores in mitochondria: how can they be released? how many are present, and in what concentrations? where are they localized, in the membrane or in the soluble fraction? what is their function? which are the proteins with which these ionophores are associated? what is the nature of the containment principle? The present communication is intended to provide the available answers to some of these questions.

Divalent Cation Ionophores of Mitochondria. The combination of tryptic digestion of mitochondria extracted with 90% acetone, followed by exposure of the aqueous digest to *sec*-butanol and acetic acid, has been found by Blondin to be highly effective in releasing the divalent metal ionophores (17). The released ionophores are quantitatively extractable into chloroform/*sec*-butanol in the quaternary phase system, chloroform/*sec*-butanol/acetic acid/water. The extract is subfractionated by alkali extraction and chromatography on Sephadex into an acidic polar fraction, an acidic nonpolar fraction, a neutral fraction, and a K_2CO_3-insoluble acidic fraction (18). Each subfraction is chromatographed separately on

silicic acid and LH-20 columns and resolved into its component ionophore fractions. In this resolution, phospholipids were eliminated and free fatty acids were accountable in specific fractions outside the range of ionophoretic components. This is not to say that the ionophoric capabilities of phospholipids and free fatty acids can be ignored or dismissed, but a distinction is made between ionophore species which are present in catalytic amounts and ionophore species such as cardiolipin or oleic acid which are present in amounts two or three orders of magnitude greater.

Blondin has demonstrated multiple major ionophoric species by the above extraction procedure, seven in chemically pure state (18). The different species are distinguishable by infrared and ultraviolet light absorption, by molecular weight determinations, by nuclear magnetic resonance spectroscopy, and by chromatographic properties (column and thin-layer). Five species have been identified chemically.* Two acidic species have been identified as 9-hydroxy- and 13-hydroxy-octadecadienoic acid, respectively, and one neutral species has been identified as the methyl ester of the 9-hydroxy octadecadienoic acid.† A fourth species has been identified as 9- or 13-keto-octadecadienoic or a mixture thereof; a fifth species as the methyl ester of the ketoacid. The positions and configurations of the two double bonds in each of the five species have been determined.† In addition, two of the seven species are definitely not of the octadecadienoate family.

Ionophoretic capability is initially assessed and routinely followed by the assay for the induction of mitochondrial swelling in Ca^{++}-, Mg^{++}-, or K^+-containing media (17). The ultimate and absolute test, which is applied only after the achievement of chromatographic homogeneity, is ionophoretic activity in the Pressman cell (19). This assay depends upon the transfer of cation between two aqueous phases separated by an organic phase. The ionophore is localized in the organic phase. Only ionophores will give a positive response in the Pressman cell.

There are two other criteria by which the presence of an ionophore can be detected. Ionophores will augment the metal-dependent fluorescence of anilinonapthalene sulfonic acid either in water (high concentrations of metal ions) or in chloroform [equilibrated with an aqueous metal-salt-containing medium (20)]. Acidic ionophores, particularly, will induce the transfer of metal ion from an aqueous to an organic phase such as chloroform (21). With labeled metal ions, this test can be an exceedingly sensitive and diagnostic test for the presence of ionophore.

The molecular weights of the divalent metal ionophores isolated by Blondin cover the range from about 300 to 500.* Thus, these are all relatively small molecules not unlike, in size range, the antibiotic ionophores. The present indications are that the different species cover a spectrum of molecular structures and some surprises are to be anticipated as these structures are elucidated.

Monovalent Cation and Anionic Ionophores of Mitochondria. The first introduction to the existence of intrinsic ionophores was provided by the isolation from mitochondria of a peptide ionophore active on monovalent cations (equally active with both Na^+ and K^+) (22). This isolation could be duplicated only in about one in 50 trials. In fact, it was this string of failures that finally alerted Blondin to the fact that the intrinsic ionophores of mitochondria were not free species. In rare instances, some enzyme or factor, as yet unidentified, induced the release of the ionophore and rendered it extractable with the lipid. As yet, no consistently reliable method has been found for the quantitative release of the monovalent cation ionophores. Among the reagents which are known to induce partial release are trypsin, fluorescein mercuric acetate, dodecyl sulfate* and cetyl triethylammonium bromide* (16, 23). When the monovalent cation ionophore was isolatable by virtue of spontaneous release, the yield per g dry weight of mitochondria corresponded to about one molecule of ionophore per molecule of cytochrome c_1. By virtue of this unpredictability of the success of the isolation, it has not been possible to complete the chemical characterization of the monovalent cation ionophore. It appears to be a peptide of molecular weight of about 1000 with a specific activity not unlike that of valinomycin (22).

The Theoretical Number of Isolatable Ionophores. If we assume a 1:1 correspondence between the number of cation transport capabilities in mitochondria and the existence of separate ionophores, there would be five different ionophores [one for K^+/Na^+ (22), two for divalent metals (24), one for chloride (25), and a nigericin-type ionophore specific for Na^+ (26)]. Assuming a similar correspondence between passive transport capabilities and ionophores, there would be at least four different ionophores [one each for P_i, ADP, monocarboxylic acids, and dicarboxylic acids (27)]. Finally, assuming a 1:1 correspondence between ionophores and kinases (2) and between ionophores and divalent-metal-requiring systems, we would anticipate five ionophores derivable from kinases (ATP–succinate, ATP–acetate, ATP–long chain fatty acids, monophospho-, and diphosphokinase), two from carboxylases (pyruvate and α-ketoglutarate dehydrogenase complexes), and two for activation of P_i and ADP in ATP synthesis. Theory would predict a minimum of 18 different ionophore species (assuming that no two ionophores are identical). Thus far, nine divalent cation ionophoric species and one monovalent cation ionophore have been isolated. The point to be emphasized is that the number of isolated ionophores is not a source of embarrassment. If anything, the number has to be larger. The discrepancy could in part be a reflection of the fact that some ionophoric species derivable from different enzyme systems are identical, and in part a reflection of the variable degree to which the different ionophoric species are releasable with the presently available methods.

The Molar Concentrations of Intrinsic Ionophores. Perhaps the most appropriate way of expressing the concentration of ionophores in the mitochondrion would be in terms of multiples of the molar concentration of cytochrome c_1. For each complete electron transfer chain, there would be one molecule of cytochrome c_1. Of the ten divalent cation ionophores isolated by Blondin, the actual yield of purified ionophore would correspond to at least one molecule per molecule of cytochrome c_1, if we assume no more than 50% recovery during the isolation.* The yields of the two hydroxy isomers of octadecadienoic acid would be significantly higher—about 4 molecules per molecule of cytochrome c_1.* For the ionophores concerned in transport and in ATP synthesis or hydrolysis, this correspondence of the molar concentration with that of

* G. A. Blondin, unpublished studies.
† Unpublished studies of G. A. Blondin and H. K. Schnoes.

the electron transfer chain or ATPase would be expected. For the ionophores associated with kinase systems, no such correspondence would be predictable.

Localization of the Intrinsic Ionophores. This aspect of the ionophore problem has yet to be fully explored, but a few qualitative observations are relevant. The lipid-free complexes of the electron transfer chain, as well as the lipid extractable by 90% acetone, appear to be devoid of ionophores other than those attributable to the phospholipids.* The ionophores appear to be concentrated, if not exclusively present, in the lipoprotein fraction soluble in chloroform–methanol 2:1.‡ This fraction is devoid of both the complexes of the electron transfer chain and of F_1. It contains predominantly the Criddle structural protein fraction (28) (molecular weight 29,000), and the Beechey protein (molecular weight 10,000)—the protein which reacts with dicyclohexylcarbodiimide (DCCD) (29). The protein in this fraction accounts for some 20–25% of the total protein of the inner membrane and it tightly binds some 20–21% of the lipid.‡

The mitochondrion can be resolved into water-soluble protein and the intrinsic membrane proteins (30). The localization of ionophores in the chloroform–methanol fraction applies only to the intrinsic membrane proteins. There are ionophores associated with the kinases of the soluble protein fraction, but these proteins are not soluble in chloroform–methanol 2:1.‡

The Beechey Protein—An Ionophoroprotein. The first direct demonstration that intrinsic ionophores are protein-bound was provided by the studies of Kessler *et al.* (31) on the Beechey protein (29). This protein, brought to the stage of chromatographic purity, can be shown to contain one molecule of ionophore per molecule of protein. The ionophore has been provisionally identified as the methyl ester of 9-hydroxyoctadecadienoic acid.§ The ionophore can be released from its protein in the presence of butanol/acetic acid/water (31). Under the conditions of isolation, the Beechey protein exists as a stable ionophoroprotein. Since the protein is soluble in chloroform, the ionophore is directly demonstrable by the metal-dependent augmentation in fluorescence of anilinonaphthalene sulfonic acid. After removal of the ionophore, by the butanol/acetic acid/water procedure, the apoprotein loses residual lipid, tends to polymerize, and is no longer soluble in chloroform.

The ionophoroprotein, but not the released ionophore, can bind inorganic phosphate in chloroform in 1:1 stoichiometry with Mg^{++} (31). This binding of inorganic phosphate is absolutely dependent upon the presence of a divalent metal in the ionophore. We equate this metal- and ionophore-dependent binding of inorganic phosphate with the activation of inorganic phosphate that underlies coupled synthesis of ATP from ADP and P_i. There is abundant evidence to support this interpretation.

The capacity of the Beechey protein to bind P_i is suppressed by three reagents which specifically suppress oxidative phosphorylation, namely, dicyclohexylcarbodiimide, oligomycin, and tripropyl tin (31). These three reagents, in some fashion as yet unclear, modify the Beechey protein so that the ionophore becomes dissociable. The Beechey protein that has been isolated from mitochondria exposed to any of these three reagents fails to show any evidence of ionophore or of the capacity to bind either divalent metal or P_i.

The Beechey protein is clearly implicated in oxidative phosphorylation and not active transport. It thus provides direct evidence that ionophores play a role in catalytic processes as well as in active transport. Note that the released ionophore of the Beechey protein is indistinguishable from classical ionophores in respect to transport properties and yet it is committed to a catalytic role.

The Two Coupling Modes of Mitochondria. Mitochondria can undergo a transition between two coupling states (32). In one state, the transducing units carry out one set of coupled functions, including oxidative phosphorylation; in the other state, the transducing units carry out another set of coupled functions, including active transport of K^+ and Mg^{++}. Mercurials and heavy metals can induce this transition in coupling mode. Southard and Green have analyzed the state change and presented evidence that it can be rationalized in terms of a transition from free to latent ionophores (32). In the oxidative phosphorylation mode, one set of ionophores (Ca^{++}, P_i) is free and another set is latent (Mg^{++}, K^+). In the active transport mode, the previously latent ionophores become free and the previously free ionophores become latent. The latency of the Mg^{++} and K^+ ionophores is interpreted in terms of these ionophores' being committed to energy coupling rather than active transport. This would mean that ionophores can switch from a role in active transport (in the free state) to a role in energy coupling (in the latent state). The essence of the control of mitochondrial coupling function would then involve this switch in ionophore function.

Only mitochondria in the oxidative phosphorylation mode can carry out antiport coupling as in reversed electron flow, energized transhydrogenation, and coupled ATP synthesis (32). In the active transport mode, the mitochondria are committed entirely to symport coupling. As we have discussed in a previous communication, antiport coupling is impossible without a linkage system of circulating positive charges which translates the overall antiport process into two component symport processes which proceed in opposite directions. The latent ionophores are presumed to generate the linkage system of circulating positive charges required for oxidative phosphorylation and other antiport coupled processes.

Fig. 1 shows diagrammatically how this latent → free transition of paired ionophores can be paralleled by a shift from antiport to symport coupling. Implicit in this diagram is the notion that the Mg^{++} and K^+ ionophoroproteins can exist in either of two states—fused or separated. When fused, these ionophoroproteins provide the linkage system required for antiport coupling and have latent active transport capability. When separated, these ionophoroproteins act exclusively in active transport. The Ca^{++}, P_i ionophoroproteins are fused when the Mg^{++}, K^+ ionophoroproteins are separated. This is the basis of the inverse relation in the change in coupling mode. Whereas the fusion of the Mg^{++}, K^+ ionophoroproteins leads to antiport coupling, the fusion of the Ca^{++}, P_i ionophoroproteins does not. The active transport capabilities of these two ionophoroproteins become latent during fusion, but the fused ionophoroproteins do not provide a linkage system. Hence, antiport coupling parallels exclusively the fusion of the Mg^{++}/K^+ pair of ionophoroproteins. As shown in the diagram, the headpiece–stalk unit is

‡ Unpublished studies of R. Kessler and C. Tyson.
§ Unpublished studies of G. A. Blondin and R. Kessler.

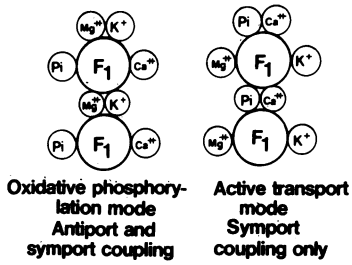

FIG. 1. Diagrammatic representation of the ionophore patterns in the two coupling modes. In the oxidative phosphorylation mode, the Mg^{++}/K^+ pair is fused and the P_i/Ca^{++} pair is separated. In the active transport mode, the P_i/Ca^{++} pair is fused and the Mg^{++}/K^+ pair is separated. The headpiece–stalk (F_1) is assumed to slide from one position to another in the transition from one mode to the other. The diagram represents a surface view of the membrane.

the moving unit in the transition from one coupling mode to the other. It either separates the Mg^{++}/K^+ ionophoroprotein pair or the Ca^{++}/P_i pair. How the headpiece–stalk slides from one position to the other is unknown.

Ionophores in Other Energy Coupling Organelles and in Kinases. Blondin and Greaser[¶] have identified in troponin a neutral divalent cation ionophore. Troponin is the Ca^{++}-binding protein of the actomyosin system and would be the logical protein from which an ionophore could be extracted. The ionophore was extracted with the usual butanol/acetic acid/water mixture that has been so successful in the isolation of divalent cation ionophores from mitochondria. Here again, we have evidence that ionophores can fulfill a role other than that of ion transport. We have suggested elsewhere that in muscle a moving charged filament would correspond to a moving ion in energy coupling (33).

The presence of divalent cation ionophores has also been detected in sarcoplasmic reticulum subjected first to tryptic digestion, and then to extraction with butanolacetic acid-water.[‖] These qualitative studies have yet to be followed by direct isolation and characterization of the ionophores, but at least it can be stated that isolatable ionophores are present in this organelle.

The association of ionophores with soluble proteins would be predictable on the basis of the postulated presence of ionophores in kinase systems. Troponin was the first instance in which an association with a purified soluble protein was directly demonstrated. In mitochondria, we have found qualitative evidence for the presence in the soluble protein fraction of extractable ionophores (after extraction with 90% acetone and exposure to butanol/acetic acid/water[**]). Similar evidence is now available for the presence of extractable ionophores in soluble kinases such as hexokinase.[**]

Shamoo and MacLennan have recently reported that proteins obtained by tryptic digestion of the sarcoplasmic reticulum increase the conductance of thin films by a factor of several hundred in the presence of Ca^{++} (34). It would appear that the products of fragmentation by trypsin are still ionophoroproteins in which the contained ionophore can shuttle ions across the lipid phase. By our own extraction procedure with butanol/acetic acid/water, the ionophores in the sarcoplasmic reticulum can be stripped from their proteins and assayed directly as the free ionophores.

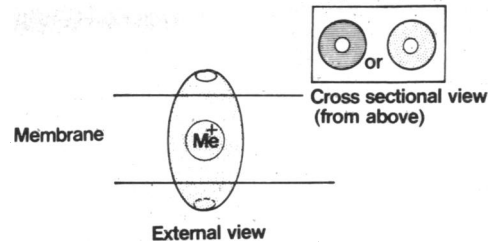

FIG. 2. Diagrammatic representation of the Criddle protein. The protein is oriented so as to span the membrane. The ionophore (Me^+) is contained within the fluid interior of the hollow protein. Assuming that the interior contains phospholipid, the orientation of the phospholipid molecules could be in the plane of the membrane or perpendicular to the plane.

The Structure of Ionophoroproteins. It is obvious that ionophoroproteins will have entirely different internal structures, depending on the role of the protein. If the protein and the associated ionophore fulfill a catalytic role, such as the Beechey ionophoroprotein, the ionophore in this instance will be concerned in phosphate activation and in the transfer of activated phosphate to an acceptor system. The ionophore with its composite ion, e.g. $(MgP_i)^-$, could be transferred from one protein to another of a transfer chain or the composite ion could be transferred from ionophore to acceptor protein. By contrast, in ionophoroproteins which are concerned with ion transport functions, the ionophore–metal ion combination must be capable of moving from one side of the membrane to the other. This would call for a container-type protein with a fluid interior in which the ionophore would have free translation (see Fig. 2). The protein would have to span the membrane with its long axis at right angles to the plane of the membrane. There are circumstantial grounds for postulating that the family of chloroform-soluble proteins of molecular weight 29,000 would correspond to this postulated genre of membrane-spanning transporting proteins. That the family of 29,000 molecular weight proteins spans the membrane has been demonstrated by suitable labeling techniques (35). Moreover, these contain so-called bound lipid (difficulty extractable) which fails to show the usual free rotation of bilayer lipid (36). It is reasonable to postulate that the bound lipid forms an internal core in which the ionophore is contained and in which the ionophore can undergo translation within the protein from one side of the membrane to the other. A very significant fact is that the bulk of the extractable ionophores are found to be present in the chloroform–methanol fraction of which the 29,000 molecular weight protein is the predominant species.

Ionophoroproteins and Heme Proteins. Both ionophores and cytochromes can serve as charge-separating devices. It should be noted that the prosthetic group of cytochromes is in effect an ionophore. Iron is by no means the only metal that can coordinate with porphyrin. In chloroplasts, the metal is Mg^{++}, not Fe^{++} (37). The point to be emphasized is that there is a continuity in structure between ionophores and porphyrins. It is not surprising that porphyrins show positive responses in systems that test ionophoric capability††.

¶ G. A. Blondin and M. Greaser, unpublished studies.
‖ Charles Tyson and G. A. Blondin, unpublished studies.
** E. Løvaas and D. E. Green, unpublished studies.
†† Charles Tyson and D. E. Green, unpublished studies.

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