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A Unified Model of Mitochondrial Morphology

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I. *Introduction*

In recent years there has been a growing awareness that mitochondria can exist in a bewildering variety of morphological states and that this multiplicity of states cannot be dismissed as a consequence of artifacts of fixation. The electron micrographs shown in Fig. 1 A–E illustrate the range of morphological variation that can be consistently reproduced in beef heart mitochondria isolated in 0·25 M sucrose. At first glance, one might incorrectly conclude that these are micrographs either of different kinds of mitochondria or even of entirely different organelles. Thus far, no single unified model has been offered which can describe the morphologies of mitochondrial membranes and relate the morphologies to the processes which give rise to the bewildering diversity that we see. In the present communication, we shall attempt to present such a unified model. In order to achieve this unification we have found it necessary to introduce a set of guiding principles and to correlate both morphological and biochemical data.

Despite their great morphological variability, mitochondrial inner membranes nevertheless have one property of their morphologies which is *common* to them all and which is *constant*. From the study of vast numbers of electron micrographs of mitochondria from a wide variety of tissue sources under a variety of biochemical states, it has been deduced that, *as surfaces*, all mitochondrial inner membranes are equivalent to a hollow sphere, and never lose that equivalence. A simple example of such a surface is an inflated rubber balloon. In just the same way that the surface of an inflated rubber balloon can be deformed from its original shape by distortions, so too can the surface of any mitochondrial inner membrane be deformed by distortions. The distortions of the surface of the mitochondrial inner membrane constitute the various morphologies we see in electron micrographs. As long as no holes are punctured through the surface during the distortions, both the rubber balloon and mitochondrial inner membranes remain unchanged as surface continua. This can be easily proved for the rubber balloon by the fact that its surface can revert back to its original shape. We have deduced the same to be true for the inner membrane of all mitochondria.

The very simple ideas sketched above for dealing with mitochondrial inner membranes as surfaces constitutes the essence of Membrane Topological Analysis. Topology is that branch of mathematics which deals with those properties of geometric figures (and their surfaces) which are *unaffected by deformations* other than tearing and joining. Therefore, in terms of topology, we have deduced that all mitochondrial inner membranes are topologically identical to a hollow sphere, and that they do not change that topology despite

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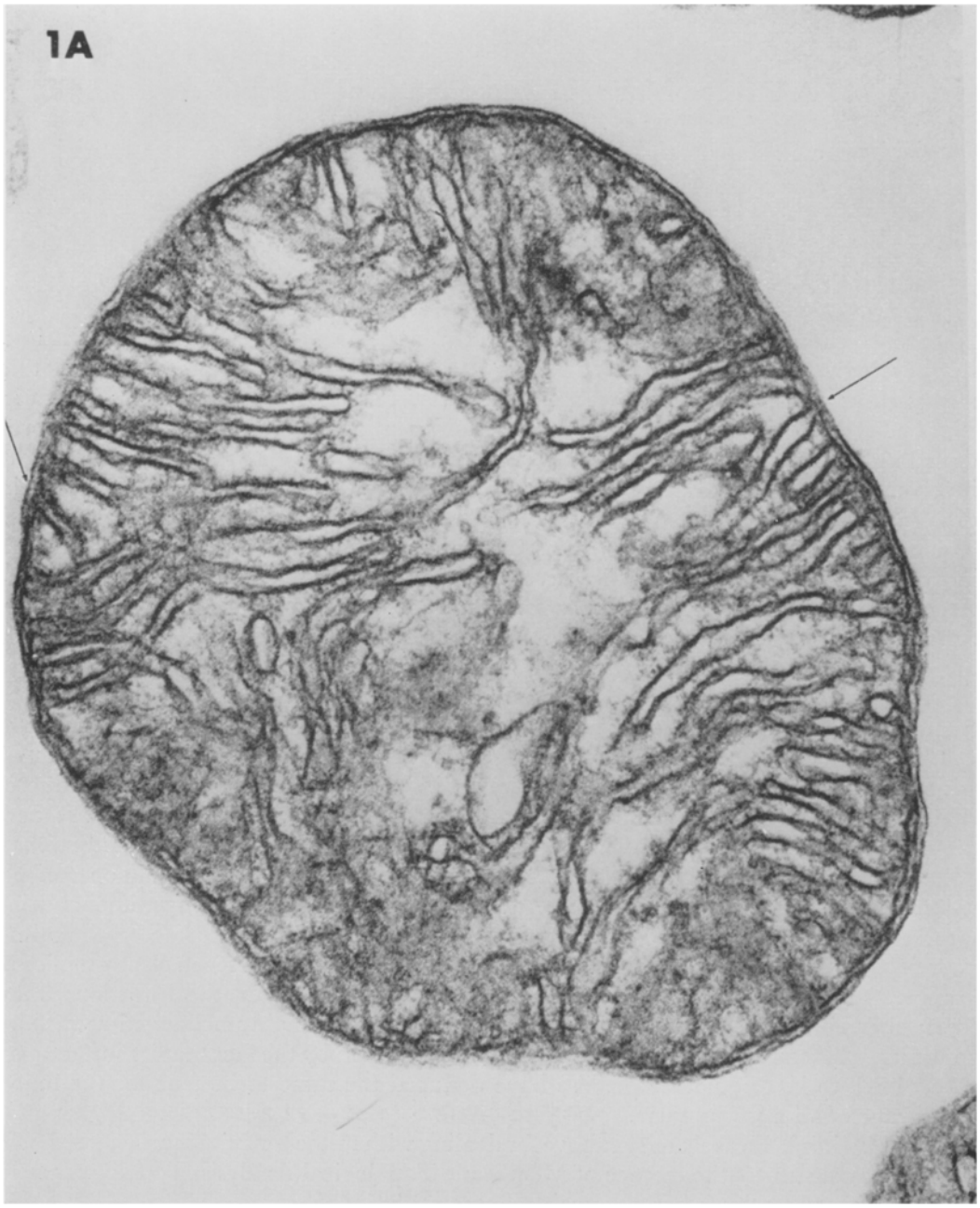


Figure 1A–E. Electron micrographs of isolated heavy beef heart mitochondria (HBHM) in all the known discrete configurational states.

Figure 1A. An electron micrograph of HBHM in the nonenergized (orthodox) configuration, NE (ortho). (86223-C-1). Arrows point to passageways connecting the two regions of the intracristal space.

Figure 1B. An electron micrograph of an HBHM in the nonenergized (aggregated) configuration, NE (Agg). (86219-C-1)

Figure 1C. An electron micrograph of an HBHM in the energized configuration, E. (86218-C-1)

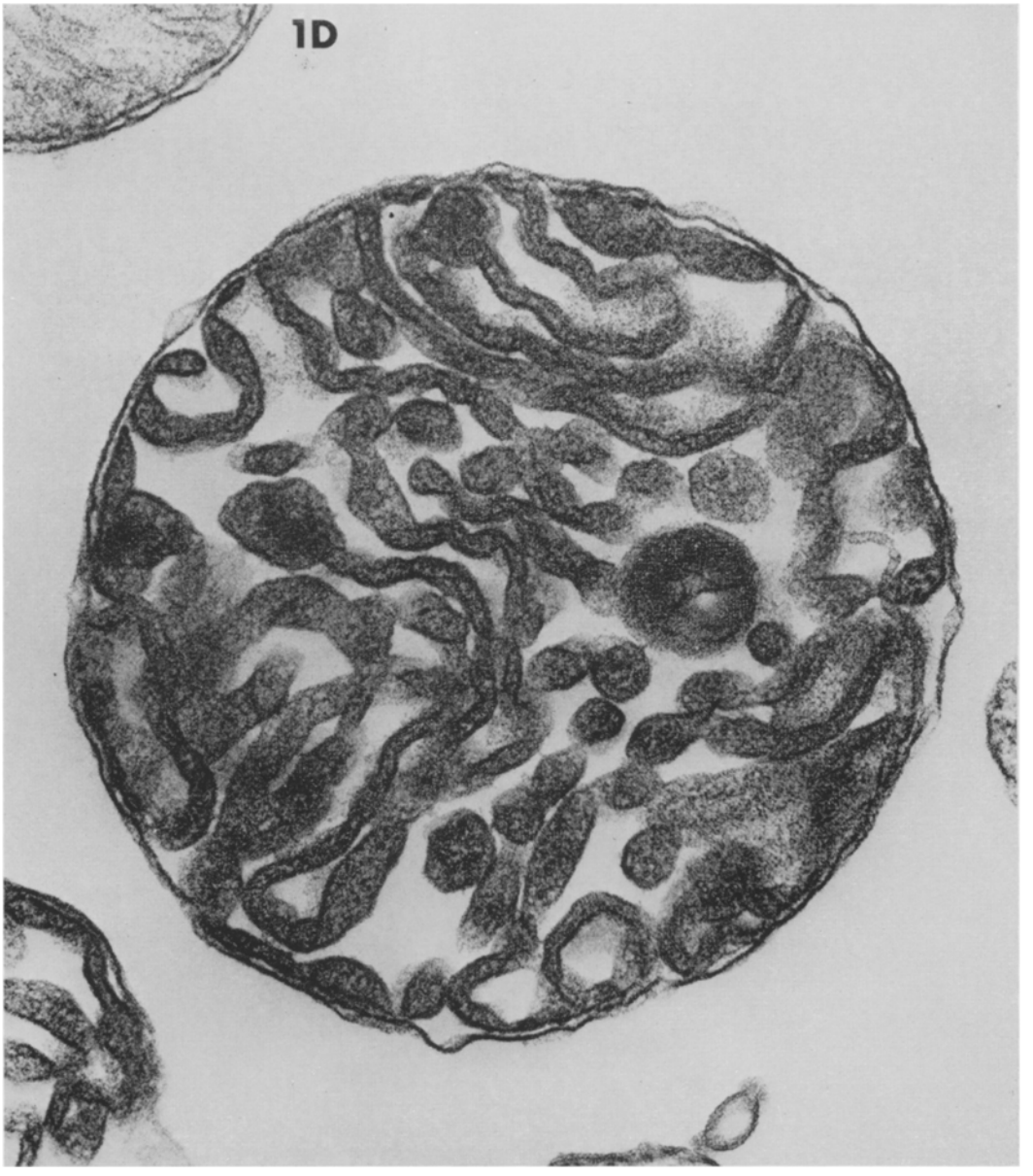


Figure 1D. An electron micrograph of an HBHM in the energized (twisted) configuration, E (T). (86220-C-1)

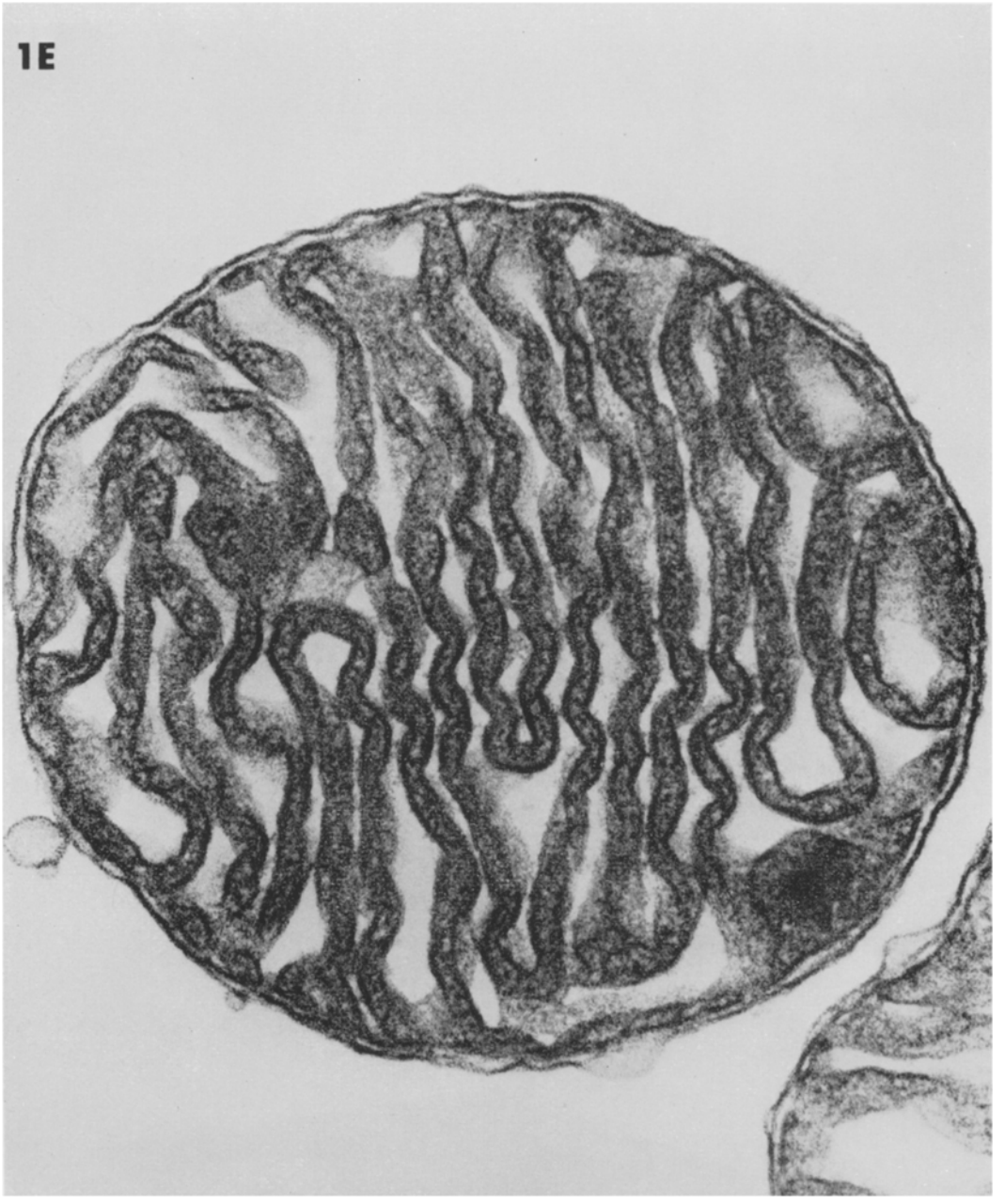


Figure 1E. An electron micrograph of an HBHM in the energized (zigzag) configuration, E (zigzag). (86222-C-1)

vastly different morphological appearances. The complete details of Membrane Topological Analysis will be given in this communication. This new analytical tool has made the enormous and confusing literature on mitochondrial morphology comprehensible.

II. *Mitochondrial Nomenclature*

For clarity of meaning, we shall adopt a carefully defined nomenclature when referring to the various structures within mitochondria. The heavy beef heart mitochondrion (HBHM), will serve as our model for this purpose, since it has been very intensely studied and it very clearly illustrates the structures of interest. The heavy beef heart mitochondrion, like all mitochondria, is a two-membrane system, with one membrane enclosed within another. Figure 2A is a highly stylized diagrammatic representation of this double-membrane system. We see a representation of a two-dimensional cut or slice across a typical heavy beef heart mitochondrion. The morphological state depicted is only one of the several morphological states that have been observed in electron microscopy. We shall arbitrarily use this morphological state as our starting point for the description of the mitochondrion and for developing the nomenclature to be used in this description, because this state, usually referred to as the orthodox state, is the most convenient for illustrative purposes.

As can be clearly seen in Fig. 2A, the two-membrane system of the mitochondrion is composed of an enclosing membrane and an enclosed membrane. The enclosing membrane will be called the outer membrane (OM). The outer membrane is a hollow, closed (but not necessarily empty), more or less spherical* surface. The morphology of the outer membrane is essentially invariant; that is, the membrane usually retains its size and essentially spherical shape unchanged except under drastic conditions. The membrane enclosed by the outer membrane will be called the inner membrane (IM). Unlike the outer membrane, the inner membrane is quite variable in its morphology. The various morphologies of the mitochondrion are, strictly speaking, the different morphologies of the mitochondrial inner membrane. In the orthodox state depicted diagrammatically in Fig. 2A, the inner membrane arranges itself in such a way that it can be thought of as being composed of two sets of surface elements. One set of elements lies adjacent and "parallel" to the outer membrane, and will be called the inner boundary membrane (IBM). The second set of surface elements consists of invaginations of the inner membrane surface. Each individual invagination will be called a crista (C). The cristae are known to be built up of repeating units,[1-3] very much like a wall which is built up of bricks. Unlike a brick wall, however, the cristal membrane is flexible. Each repeating unit of the crista is tripartite in character[1,4,5] and is known to be the site of electron transfer[6-14] and ATP synthesis.[15] The three parts of the tripartite repeating unit are: (1) a quasi-cuboidal basepiece, which, together with other quasi-cuboidal basepieces, comprises the membrane continuum where each basepiece carries out one part of the overall electron transfer process; (2) a spherical headpiece which carries out ATP synthesis; and (3) a cylindrical stalk which physically links headpiece to basepiece and may act to couple the processes of electron transfer to the synthesis of ATP. The repeating units of the inner boundary membrane are probably also tripartite, but their basepieces, unlike those of the

* Isolated mitochondria generally have a spherical or ellipsoidal shape. *In situ*, the exact shape varies dependent upon such considerations as close packing of the mitochondria within other structures.

repeating units of the cristae, possibly do not show electron transfer activity.[16] The question as to the exact function of these inner boundary membrane repeating units is not critical to the thesis of this communication.

The term cristal membrane as used in the literature refers to the entire inner membrane. This is probably because no distinction has been made in the past between the two

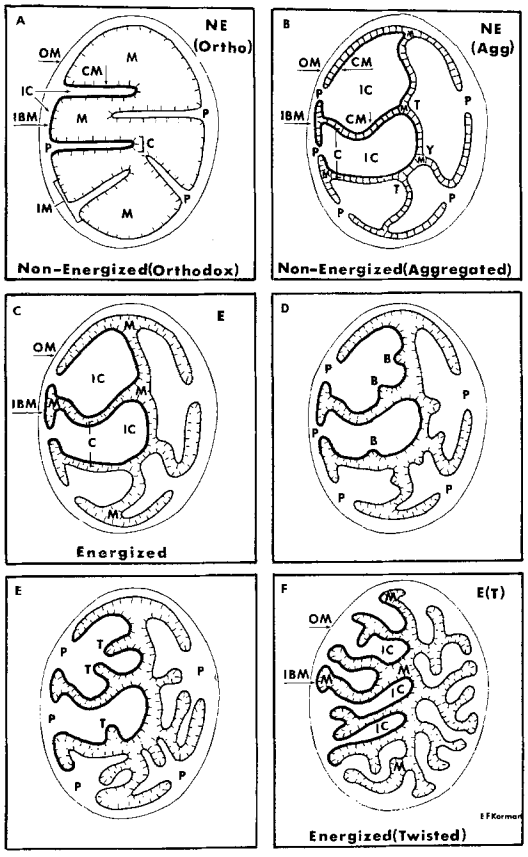


Figure 2. A diagrammatic two-dimensional representation of HBHM in all the known discrete configurational states, plus a representation of the transition of the E to the E (T) configuration. (86228-C-1)

A. A diagram of a cross section of an HBHM in the NE (Ortho) configuration. OM = outer membrane; IBM = inner boundary membrane; CM = cristal membrane; C (with brackets) = one crista; P = passageway; IM = inner membrane; IM = IBM + CM; IC = intracristal space; M = matrix space. The short lines drawn perpendicular to the CM surface represent projecting headpiece-stalk sectors. The IC space is at a volume minimum; the M space is at a volume maximum; cristae are maximally spatially separated. CM apposition is basepiece-to-basepiece. Two adjacent cristae and a portion of IBM between the two cristae are drawn with a heavy line.

B. A diagram of a cross-section of an HBHM in the NE (Agg) configuration. The IC space is at a volume maximum; the M space is at a volume minimum; the cristae are greatly expanded, with CM's from two neighboring cristae in close headpiece-to-headpiece apposition. Note the T-shaped and Y-shaped "fork-in-the-road" structures, labelled T and Y, respectively.

C. A diagram of a cross section of an HBHM in the E configuration. The general form of the E configuration is the same as the NE (Agg) configuration, except for the greater distance measured across the pairs of apposed sheet-like CM's.

D. A diagram of a cross section of an HBHM in the E configuration undergoing a transition in configuration to the E (T) configuration, upon the addition of inorganic phosphate. Note the bud-like reverse invaginations, B, of the CM's. The buds protrude perpendicular to the CM's and into the expanded IC space.

E. A diagram of a cross section of an HBHM in the E configuration undergoing a transition in configuration to the E (T) configuration, upon the addition of inorganic phosphate. Note the elongation of the bud-like reverse invaginations to the bulbous-ended, tube-like reverse invaginations, T. Also, note the enlargement of the passageways, P, connecting the two regions of the IC space.

F. A diagram of a cross section of an HBHM in the E (T) configuration. Note the complete tubularization of the IM, and the apparent loss of long stretches of the IBM, due to the widening of the passageways. The two regions of the IC space seen in other configurations have essentially lost their distinction. Also, note that certain regions of the IBM retain a constant spatial apposition to the OM, making the rapid discharge of the E (T) configuration back to the NE configuration possible.

surface elements of which the mitochondrial inner membrane is composed.[17] In addition, the term has been used when referring to the invaginations of the inner membrane, i.e., to the cristae *per se*. To avoid confusion, we shall restrict the use of the term cristal membrane (CM) to this latter sense. The term inner membrane, then, will be used to designate the entire enclosed membrane containing both cristal membrane and inner boundary membrane.

The two-membrane system of the mitochondrion divides the mitochondrion into two compartments or spaces. The space enclosed within the inner membrane is called the matrix space (M). The space between the inner and outer membranes is itself divided into two regions: (1) the region between the outer and the inner boundary membrane, and (2) the region enclosed within the cristal invaginations. These two regions are completely continuous, i.e., they are really parts of one interconnecting compartment. The regions enclosed within the cristal invaginations are collectively called the intracristal space (IC). Since the space between the inner boundary membrane and the outer membrane is actually part of the same compartment as the intracristal space, the name intracristal space is used to refer to both regions.

III. *Configuration and Conformation*

In order to have a clear understanding of the discussions which follow, the terms configuration and conformation, and the distinctions between them, must be clearly understood. By the term configuration we mean the morphology of a whole membrane or of a membrane element, such as a crista. A particular configuration can be seen in electron micrographs. By the term conformation we mean the shape and three-dimensional orientation of a single repeating unit. Thus, a particular configuration is a morphological phenomenon encompassing many repeating units, while a particular conformation is an intra-repeating unit phenomenon. A particular conformation of a repeating unit is, of course, determined not only by the summations of the three-dimensional foldings of the polypeptide backbones of the component proteins within the repeating unit, but also by the orientation and distribution of the chemically functional side-chains of the constituent amino acid residues of the proteins. In addition, conformation involves the three-dimensional orientation of the phospholipid molecules associated with the proteins. The intimate details of these three-dimensional aspects of conformation are as yet unknown, and we can merely detect *changes* in conformation. Such changes can be detected only by indirect means, such as by the release or uptake of protons.[18]

The scope of the present communication will be restricted to configurational phenomena, i.e., morphological patterns which can be visualized by electron microscopy. While we believe that the morphologies of mitochondrial inner membranes, which we call configurational states, have an underlying conformational basis, we will not deal with that relationship here except in an indirect way.

IV. *The Energy Cycle and the Configurational States*

Our laboratory has accumulated a body of evidence from studies with mitochondria from a variety of tissue sources, that profound and rapid morphological changes, which are easily visualized in electron microscopy, take place in the mitochondrial inner membrane during the energy cycle.[19–22] By the energy cycle is meant that sequence of events which occurs when mitochondria in the nonenergized state (a state in which the mitochondria are not capable of performing work) are first energized by the oxidation of oxidizable substrates or by the hydrolysis of ATP to an energized state (a state in which mitochondria are capable of performing work) and then are discharged back to the nonenergized state by the performance of work. Typical work performances which lead to

discharge of the energized state are ATP synthesis and ion translocation. The discharge of the energized state can also be achieved by the addition of uncouplers such as meta chloro-cyano carbonyl phenyl hydrazone (m-Cl-CCP) or dinitrophenol. Under such conditions the energy is dissipated in the form of heat.

The various states occurring during the energy cycle have been correlated with distinct morphologies of the mitochondrial inner membrane. These distinct morphologies have been called configurations or configurational states. The term configuration is defined above in Section III. The configurations are quite different and easily distinguishable from one another, and, when clearly seen,* each configuration corresponds in a one-to-one fashion to one of the states of the energy cycle. In general, the configurations are common to all mitochondria regardless of tissue source. A full description of the various configurations as they are related to the energy cycle will be given in Section VII below.

V. *The Topology of the Mitochondrial Inner Membrane*

The different configurational states of the mitochondrial inner membrane and the transformations from one configuration to another during the energy cycle can be understood only within carefully defined topological boundary conditions. The parameters which define these topological boundary conditions have been deduced from a study of a vast number of electron micrographs of mitochondria from a wide variety of tissue sources and in a variety of energy states. These topological boundary conditions are outlined below:

1. *Topological Isomorphism*

All mitochondrial inner membranes, regardless of the tissue source of the mitochondria, are identical topologically. This is equivalent to saying that all mitochondrial inner membranes belong to the same topological genus.

2. *The Topological Genus Zero*†

All mitochondrial inner membranes belong to the topological genus zero, a grouping which includes three-dimensional surfaces isomorphic with a hollow sphere. Distortions of the spherical surface, technically referred to as transformations, such as folds, wrinkles, invaginations, and evaginations of the surface, do not change the genus of that surface. Thus, a spherical surface can be transformed to a hollow ellipsoid, to a hollow cube, or to a hollow, irregularly shaped surface such as that of an amoeba, and still remain a member of genus zero. The mitochondrial inner membrane, which has an essentially spherical surface with numerous cristal invaginations, where the invaginations can vary greatly in size and shape, is thus a member of genus zero.

* The one-to-one correspondence between the energy cycle and the configurational states is demonstrable only under non-steady state conditions. Only when the mitochondrial membrane has a sufficiently large number of repeating units in one energy state does the membrane assume one discrete configuration.

† The genus of a three-dimensional surface is defined by the number of nonintersecting closed cuts that can be made on the surface without cutting the surface into two pieces. Thus, there are zero, i.e., no closed nonintersecting cuts, which can be made on a spherical surface without cutting that surface into two pieces. A doughnut, i.e., a torus, on the other hand, can be cut with one closed nonintersecting cut and not be cut into two pieces. A torus is therefore genus one. See Fig. 3 illustrating these principles.

3. *Topological Invariance*

All mitochondrial inner membranes, regardless of the tissue source of the mitochondria, retain their topology, i.e., remain members of genus zero, during normal metabolic operation, as well as under experimental conditions other than drastic conditions which lead to membrane dissolution or rupture. In other words, the topology of the mitochondrial inner membrane is a constant.

The topological boundary conditions outlined above are few in number and very simple in principle, but a strict adherence to them is completely indispensable for a full comprehension of the relationships among the welter of configurational states and their variations seen in mitochondrial inner membranes. In the past, the lack of awareness that such topological boundary conditions are operative led us to incorrect interpretations of the structures of the various configurational states (see Section VII, 1, below).

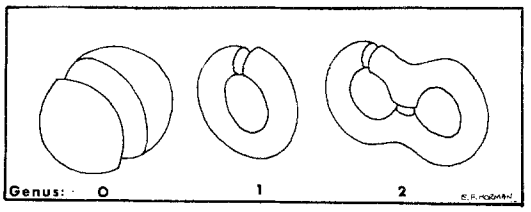


Figure 3. A diagrammatic representation of several topological surfaces cut by closed, non-intersecting curves. A hollow sphere cut by even a single closed curve will be divided into two unconnected pieces. It is genus zero. A doughnut (i.e., a torus) can be cut by, at most, one closed curve and still remain one continuous piece. It is genus one. Two cuts through a torus will divide the surface into two unconnected pieces. A double doughnut can be cut by, at most, two closed curves and still remain one continuous piece. It is genus two. (86225-C-1)

VI. *Morphological Variability in the Form of Mitochondrial Cristae*

As was pointed out in Section V, the mitochondrial inner membrane has been deduced to be a member of topological genus zero regardless of the cristal invaginations. Therefore, all cristae share this common characteristic and have identical topologies. Although all cristae have identical topologies, they nevertheless have important differences in structural detail. The exact shape and size of such cristal invaginations are of very great interest to us. This is so because, as was pointed out above, the cristae are built up of the tripartite repeating units which are the molecular instruments for coupling electron transfer to the synthesis of ATP. It is thus the cristal membrane in which the energy cycle actually takes place. Therefore, it is also the cristal membrane which most directly reflects the events occurring during the energy cycle, and which imposes the forces upon the entire mitochondrial inner membrane which ultimately lead to the configurations seen in electron micrographs.

In the nonenergized state, the cristae of mitochondria from heart,[19–22] liver,[23–24] and kidney[24–25] generally have the shape of hollow, flattened pillow-cases or hot water bottles, which are arranged within the mitochondria in parallel sets. Figure 4A is a diagrammatic representation of the three-dimensional arrangement of such cristae in a mitochondrial inner membrane in the nonenergized state. We see that each crista is connected to and is continuous with the inner boundary membrane by a narrow "neck". The cristae are very much like pockets in a garment, except that unlike most ordinary pockets, they have very narrow passageways leading into the interiors of the pockets. It must be carefully noted that topologically the insides of pockets are really on the outside of a garment. That is why the term passageway rather than the term hole is used when referring to the connection of the crista to the inner boundary membrane. The term hole would imply a puncture of the membrane continuum. Thus, by analogy with a garment,

the interiors of the cristae, i.e., the intracristal space, is actually on the outside of the inner membrane.

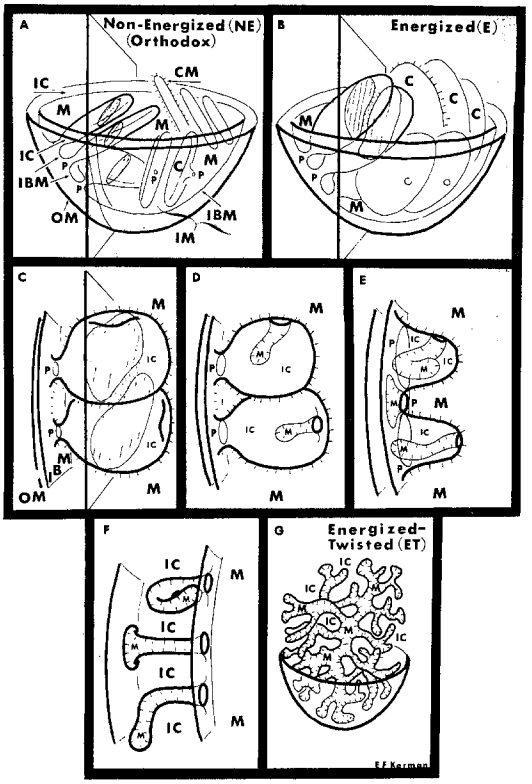


Figure 4. A diagrammatic three-dimensional representation of an HBHM in the NE (Ortho), E, and E (T) configurations plus a representation of the transition from E to E (T) configuration. (86216-C-1)

A. A diagrammatic three-dimensional representation of an HBHM in the NE (Ortho) configuration. The diagram represents a mitochondrion cut across its diameter with the top half removed. The bowl-like outer hemisphere is the outer membrane, OM. A second bowl-like invaginated hemisphere enclosed inside the OM is the inner membrane, IM. The portion of the IM forming the hemispherical part of the surface ("parallel" to the OM) is the inner boundary membrane, IBM. The invaginations of the inner membrane, in the shape of hot water bottles or hollow pillow-cases, are the cristae, C, whose surfaces are called the cristal membrane, CM. The CM is built up of tripartite repeating units. The short lines drawn perpendicular to the CM surface represent the headpiece-stalk sectors. The cristae are arranged in parallel sets, like stacks of pennies. The narrow "necks" or passageways, P, connect the CM surface with the IBM surface. The space enclosed within each crista, the intracristal space, IC, is continuous with the space between the hemispherical OM and IBM. The space enclosed within the IM is the matrix space, M. A vertical plane is represented which cuts through the mitochondrion. It cuts the OM and IBM to generate hemispherical lines, which are seen in the electron micrographs of whole mitochondria as closed lines. The plane also cuts across cristae to generate long tube-like patterns (shaded for identification) which are closed at both ends enclosing IC space. A plane sometimes cuts across a passageway and CM surface to generate a line in electron micrographs showing the IBM continuous with the CM.

B. A diagrammatic three-dimensional representation of HBHM in the E configuration. The IC space has expanded to a maximum, the M space has reduced to a minimum, and neighboring cristae have their CM in close headpiece-headpiece apposition. A vertical plane cuts through the mitochondrion generating a network with T-, Y-, and X-shaped structures. This network is the electron dense M space filled with headpiece-stalk sectors. The shaded areas represent the electron transparent IC spaces.

C. A diagrammatic three-dimensional representation of two expanded neighboring cristae in the E configuration. The "dimples" indicated on the CM surfaces are the beginnings of reverse invaginations.

D. A diagrammatic three-dimensional representation of tubular reverse invaginations of the CM surfaces feeding into the IC space. The tubes enclose M space, and are lined with headpiece-stalk sectors, and have bulbous ends. Note that the passageways are slightly larger in diameter.

E. A diagrammatic three-dimensional representation of the reverse invagination process carried further. The passageways have increased in diameter to the point that the tubular reverse invaginations can pass through them. A portion of the IBM retains its apposition to the OM with formation of a tube.

F. A diagrammatic three-dimensional representation of the reverse invagination tubularization process which has gone to completion. The two regions of IC space have now become indistinguishable.

G. A diagrammatic three-dimensional representation of the E (T) configuration. The entire IM is shown. The IM has assumed a form of a highly ramified labyrinth or network of interconnecting tubes which are lined with headpiece-stalk sectors. The structure is similar to "worm coral". Regions of the IBM retain their apposition with the OM.

The cristae of mitochondria from other tissues have forms different from the pillow-case or hot water bottle type. The cristae of mitochondria from the adrenal cortex,[26] skeletal muscle,[26] and other tissues are long tubular invaginations. The tubes either wander irregularly back and forth, as they do in the adrenal cortex mitochondria, or they lie principally along the longitudinal axis of the mitochondrion. Despite these

differences, the mitochondrial inner membranes with tubular cristae are all topologically isomorphic with mitochondrial inner membranes with the pillow-case or hot water bottle type of cristae. Although greatly elongated and snake-like, tubular cristae, nevertheless, are only invaginations of the mitochondrial inner membrane just as are the pillow-case type of cristae. They, too, are elements of a surface which is isomorphic with a hollow sphere.

There are further important variations in detail which are found among the class of tubular cristae. The tubes can be either uniform or non-uniform in diameter down their length. For example, in guinea pig liver mitochondria, the tubular cristae are uniform in diameter, while in the adrenal cortex mitochondria, the cristae vary in diameter along the length of the tube.[28] The form of the adrenal cortex mitochondrial cristal membrane is illustrated diagrammatically in Fig. 5. The cristal tubes seem to be alternately ballooned out in diameter and then squeezed down in diameter, to give rise to "scalloped" cristae. Here again, despite important differences in detail, the topology of the mitochondrial inner membranes with scalloped cristae is isomorphic with the mitochondrial inner membrane having uniform tubular cristae, as well as with the mitochondrial inner membranes having the pillow-case type of cristae.

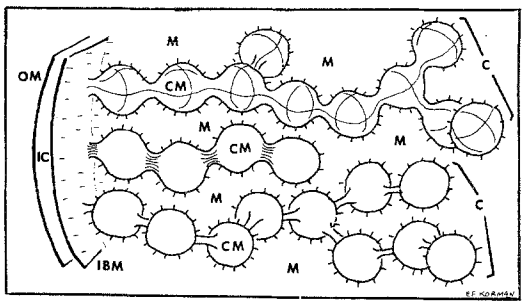


Figure 5. A diagrammatic three-dimensional representation of adrenal cortex mitochondrial cristae. The cristae are tubular, with a diameter which varies in dimension along the length of the tube. The alternate ballooning out and squeezing down of the diameter results in "scalloped" cristae. The cristae represented here are all equivalent. The diagram shows the scalloped nature of the tubes drawn in three different pictorial renditions. The short lines represent the headpiece-stalk sectors. (86217-C-1)

With the idea of topological isomorphism of all mitochondrial inner membranes firmly in mind, and with the recognition that within the limits of topological isomorphism that there exist important differences in cristal form, we are ready to discuss the second topological boundary conditions, namely, the topological invariance of the mitochondrial inner membrane regardless of energy state. We maintain that despite vast and easily detectable changes in the morphology, i.e., in the configurational state of the mitochondrial inner membrane during the energy cycle, there is never any change in the topology of that membrane. A change in topology would result from the tearing or the shredding of the mitochondrial inner membrane or the puncturing of the mitochondrial inner membrane with true holes. This boundary condition will be examined carefully in Section VII below.

VII. *The Configurational States Correlated With the Energy Cycle*

The normal configurations of the mitochondrial inner membrane seen during the energy cycle will be discussed in detail below.

1. *The Pillow-Case Type of Cristae*

Extensive electron micrographic studies in beef, rat, and canary heart mitochondria, both *in situ*[29] and in isolated mitochondria,[19–22] have revealed the following sequence of

configurational states of the mitochondrial inner membrane as related to the energy cycle:

(*a*) *The nonenergized (orthodox) configuration.* Electron micrographic examination of HBHM isolated in 0·25 M sucrose has shown that under nonenergizing conditions (i.e., in the absence of substrate or ATP and in the presence of inhibitors of both electron transfer and ATP hydrolysis, in which case the mitochondrion is incapable of performing work), the mitochondria occur principally in two morphological forms. The less frequent of these two forms is shown in Fig. 1A. Although infrequent, we shall begin our discussion with this form because it is the most convenient one for our analysis. The other more frequent form will be discussed next in order.

The intracristal space enclosed within the walls of any one particular pillow-case-shaped crista is at a volume minimum, the matrix space is at a volume maximum, and individual cristae are spatially maximally separated from one another, as illustrated diagrammatically in Fig. 2A. It should be noted that each cristal invagination has close basepiece-to-basepiece apposition. This configuration is called the nonenergized (orthodox) configuration, NE (Ortho).

The nonenergized (orthodox) configuration shown in Fig. 1A occurs rather infrequently in HBHM mitochondria isolated in 0·25 M sucrose. However, when rat heart or canary heart mitochondria are studied *in situ*[29] under anaerobiosis or in the presence of uncoupler in Krebs-Ringer-Phosphate (KRP) medium, both of which are nonenergizing conditions, essentially *all* the mitochondria are in the nonenergized (orthodox) configuration. The rat heart and canary heart mitochondria can be easily studied *in situ*, because it is possible to fix rapidly the fresh tissue shortly after sacrificing the animal, something which is impossible to do with HBHM. The nonenergized (orthodox) configuration can also be induced in fresh rat and canary heart mitochondria by a short pre-incubation before fixing, in which case endogenous ATP and oxygen are depleted. The appearance of the nonenergized (orthodox) configuration in both rat and canary heart mitochondria *in situ* is essentially identical with that of the HBHM in Fig. 1A.

The cristae in the nonenergized (orthodox) configuration seen in the electron micrograph in Fig. 1A look like long, small-diameter tubes with only a few connections to the inner boundary membrane. It has been deduced, however, that the cristae cannot be in the form of small-diameter tubes because, if they were tubes, we would expect to see a statistically calculable number of circular and elliptical cross-sections of the tubes in the electron micrograph. We actually see no such circular or elliptical cross-sections. Therefore, the structures which look like tubes are really two flat membrane surfaces or "sheets", in close apposition, which have been sectioned. In Fig. 4A these structures are indicated by shading, and it can be seen how a tube-like structure, closed at both ends, could result from sectioning at a random angle across a flat pillow-case or hot water bottle-shaped crista. The shaded areas also indicate how structures unconnected to the inner boundary membrane in the electron micrograph could arise from sectioning a hot water bottle-shaped crista, despite the fact that all cristae are thought to be continuous with the inner boundary membrane. The fact that the cristae are continuous with the inner boundary membrane is proved by the existence of at least a few passageway openings which are seen in electron microscopy and which are indicated by the arrows in Fig. 1A. We see so few passageways into the interiors of the cristae because the neck-like connections by which the pillow-case-shaped cristae are attached to the inner boundary mem-

brane are so small in diameter that we infrequently cut across them. For the purpose of simplicity of presentation, the diagram of the nonenergized (orthodox) configuration given in Fig. 2A shows every crista continuous with the inner boundary membrane.

(*b*) *The nonenergized (aggregated) configuration.* Most of the HBHM isolated in 0·25 M sucrose and examined electron microscopically in the nonenergized state, have an inner membrane which is not in the nonenergized (orthodox) configuration seen *in situ* in rat and canary heart mitochondria, but which is rather in a quite different configuration, shown in Fig. 1B. In this electron micrograph what we see enclosed by the outer membrane are large electron transparent spaces plus a very dark, almost black, network-like system of what must be the mitochondrial inner membrane. There are curious T-shaped and Y-shaped "fork-in-the-road" structures, and X-shaped "cross-road" structures seen in this network. See letters T, Y, and X in Fig. 1B.

This nonenergized configuration of the mitochondrial inner membrane of isolated mitochondria is so different from the nonenergized configuration of mitochondria *in situ* that the two cannot possibly be mistaken. In fact the two configurational states are so different in appearance that it is quite difficult to see how the two states can be related to one another and how the one can arise from the other. However, by invoking the boundary conditions of topological invariance, the two configurational states can be rationalized with each other and it can be easily seen how either configuration can arise from the other.

Figure 2B illustrates diagrammatically the "aggregation" of the mitochondrial inner membrane into this new configurational form. This new configuration is called the nonenergized (aggregated) configuration, NE (Agg). The nonenergized (aggregated) configuration results from a redistribution of the intramitochondrial water between the two mitochondrial compartments. This redistribution occurs during the isolation of mitochondria because sucrose is used in the isolation medium. Sucrose can penetrate into the intracristal space, but not into the matrix space.[30] The matrix is a sucrose-impenetrable space. This sucrose distribution compels the movement of water from the matrix space into the intracristal space, and a "ballooning out" of the cristae. The cristae expand to the point at which the intracristal space achieves a volume maximum, the matrix space achieves a volume minimum, and the membranes of neighboring cristae are forced into close apposition. The cristal membranes now have very close headpiece-to-headpiece apposition. In the nonenergized (aggregated) configuration, each one of the paired apposed membranes is derived from two different cristae. By comparison, the pair of apposed membranes in the nonenergized (orthodox) derive from one and the same crista.

The details of the headpiece-stalk structures in the nonenergized (aggregated) configuration are completely obscured. It has been calculated[27] from the thickness of the two cristal membranes which are in close apposition in the nonenergized (aggregated) configuration, that the matrix space has been so reduced in volume between the two membranes that the 85–90 Å spherical headpieces attached to the 50 Å-long cyclindrical stalks cannot possibly be extended to their full length into the matrix space. Apparently, the headpiece-stalk sectors are "collapsed", with the headpieces distorted in shape in order to just barely fit into the space available between the two apposed membranes.

The complete inversion or reversal of the relative volumes of the two mitochondrial

compartments which is seen in heavy beef heart mitochondria isolated in 0·25 M sucrose as compared with the beef heart mitochondria *in situ*[29] in the absence of sucrose was not understood in this laboratory for quite a long time after the electron micrographs of these two configurational states were first observed. This made the explanation of the configuration of the mitochondrial inner membrane in isolated mitochondria extremely difficult. There was the need to explain the curious T-shaped, Y-shaped, and X-shaped structures. The only way to explain such structures, if one assumes that the electron transparent regions are the matrix space in isolated mitochondria in the same way that the electron transparent spaces are matrix *in situ*, was to invoke a breaking or dissolution of the mitochondrial inner membrane, followed by a process of resealing or anastomosis, which could, if it were carried out in just the right way, give rise to these strange structures. That explanation, of course, violates our currently held view on the topological boundary condition of topological invariance. In addition, it was hard to understand how such a mechanism of membrane dissolution and resealing would allow for the known impenetrability of the mitochondrial inner membrane to sucrose. These difficulties become irrelevant in light of the mechanism now held to be correct. The T-, Y-, and X-shaped structures can now all be completely explained by the process of volume inversion of the two mitochondrial compartments. Thus, T-, Y-, and X-shaped structures have become diagnostic of this volume inversion resulting from the migration of water from the matrix space into the intracristal space.

(*c*) *The energized configuration.* The addition of substrate and oxygen to either the nonenergized (orthodox) configuration *in situ*[29] or the nonenergized (aggregated) configuration in isolated mitochondria[21] leads to a common energized configuration, E. Figure 1C is an electron micrograph of HBHM isolated in 0·25 M sucrose in the energized configuration, and Figs. 2C and 4B are diagrammatic representations of this configuration. This energized configuration is characterized by the presence of T-, Y-, and X-shaped structures which, as was indicated above for the nonenergized (aggregated) configuration, are diagnostic of expanded or ballooned out intracristal space.

Although the general *form* of the energized configuration is similar to the nonenergized (aggregated) configuration, the detailed appearance of the apposed cristal membranes is quite different. The average distance measured across the two apposed cristal membranes has increased in the energized configuration as compared with the nonenergized (aggregated) configuration, and the membranes which appeared intensely dark in the nonenergized (aggregated) configuration now appear less darkly stained and contain considerable internal structure. We see between the membranes what can be interpreted as headpiece-stalk structures. From the distance measured across the apposed cristal membranes at their positions of closest approach it has been deduced that the headpiece-stalk sectors from one cristal membrane must be interleaving or interdigitating with the headpiece-stalk sectors of the apposed cristal membrane. Figure 6 is a diagrammatic representation of such interleaving headpiece-stalk sectors of two apposed cristal membranes.

The energized configuration has essentially the same relative volume distribution within the two mitochondrial compartments as does the nonenergized (aggregated) configuration. Thus, the energizing of mitochondria *in situ*,[29] in which case the mitochondria are initially in the nonenergized (orthodox) configuration, causes a movement of water out of the matrix space. Mitochondria isolated in 0·25 M sucrose, which are

in the nonenergized (aggregated) configuration, already have a relative volume distribution almost the same as that in the energized configuration.

(*d*) *The energized (twisted) configuration.* When inorganic phosphate is added to HBHM isolated in 0·25 M sucrose in the energized configuration, another profound change in configuration rapidly takes place in the mitochondrial inner membrane, as seen in the electron micrograph in Fig. 1D.[21] We consider this configuration to be a variant of the energized configuration and essentially equivalent to it in energy level. The relatively few phosphate ions resulting from the hydrolysis of ATP during the energizing of the mitochondrial inner membrane are apparently insufficient to cause this rapid configurational change,[21] and additional relatively massive amounts of phosphate must be added to achieve the new configuration. There is the appearance of what look like long, twisted, snake-like tubes with bulbous ends. These structures are true tubes, since we see the statistically calculable number of circular and elliptical cross-sections which are expected when tubes are sectioned across their diameters. These tubes appear to wind their way throughout the entire volume of the mitochondrion. In addition, the inner boundary membrane, which in all other configurations of the mitochondrial inner membrane appears to retain its position of close apposition parallel to the outer membrane and to have relatively few small openings or passageways linking the two regions of the intracristal space, now has numerous wide openings. The snake-like tubes are relatively darkly stained against a background which is essentially transparent to the electron beam. The densely stained tubes have internal structure, which can be accounted for in terms of extended headpiece-stalk sectors. This snake-like configuration has been called the energized (twisted) configuration, E (T).

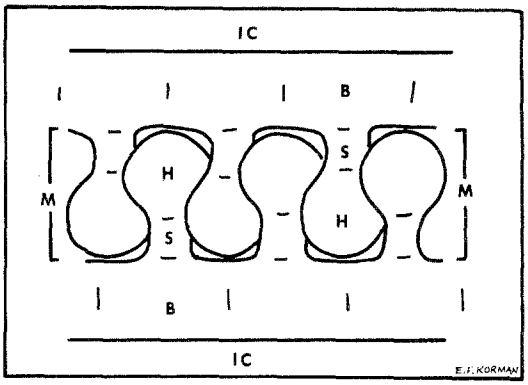


Figure 6. A diagrammatic representation of the interleaving or interdigitating of headpiece-stalk sectors of two apposed cristal membranes. H = headpiece; S = stalk; B = basepiece. The diagram is representative of the interleaving only. Precise details are still uncertain. The dimension across the pairs of cristal membranes in electron micrographs indicate that the headpieces are probably not spherical in shape, as drawn here. The topological aspects are, however, independent of these details. (86226-C-1)

We have interpreted the energized (twisted) configuration to be the result of a "reverse invagination" of the energized cristal membrane, which is in the form of apposed sheets, leading to twisted, helical, or corkscrew-shaped tubes. The tubes are envisioned as being "fed" into the expanded intracristal space with a concomitant enormous expansion of the passageways linking the two regions of the intracristal space, as illustrated diagrammatically in the sequence of drawings in Figs. 2 C–F and 4 C–G. Electron micrographic evidence for the process of reverse invagination has been obtained. In Fig. 7 we see an electron micrograph of a mitochondrion which is "caught in the act" of changing from the energized configuration to the energized (twisted) configuration. We see a bud-like projection, B, emanating perpendicular to a pair of flat, apposed, sheet-like cristal membranes in the energized configuration. The bud, which is projecting into the electron transparent intracristal space, has a bulbous end similar to those seen at the ends of the

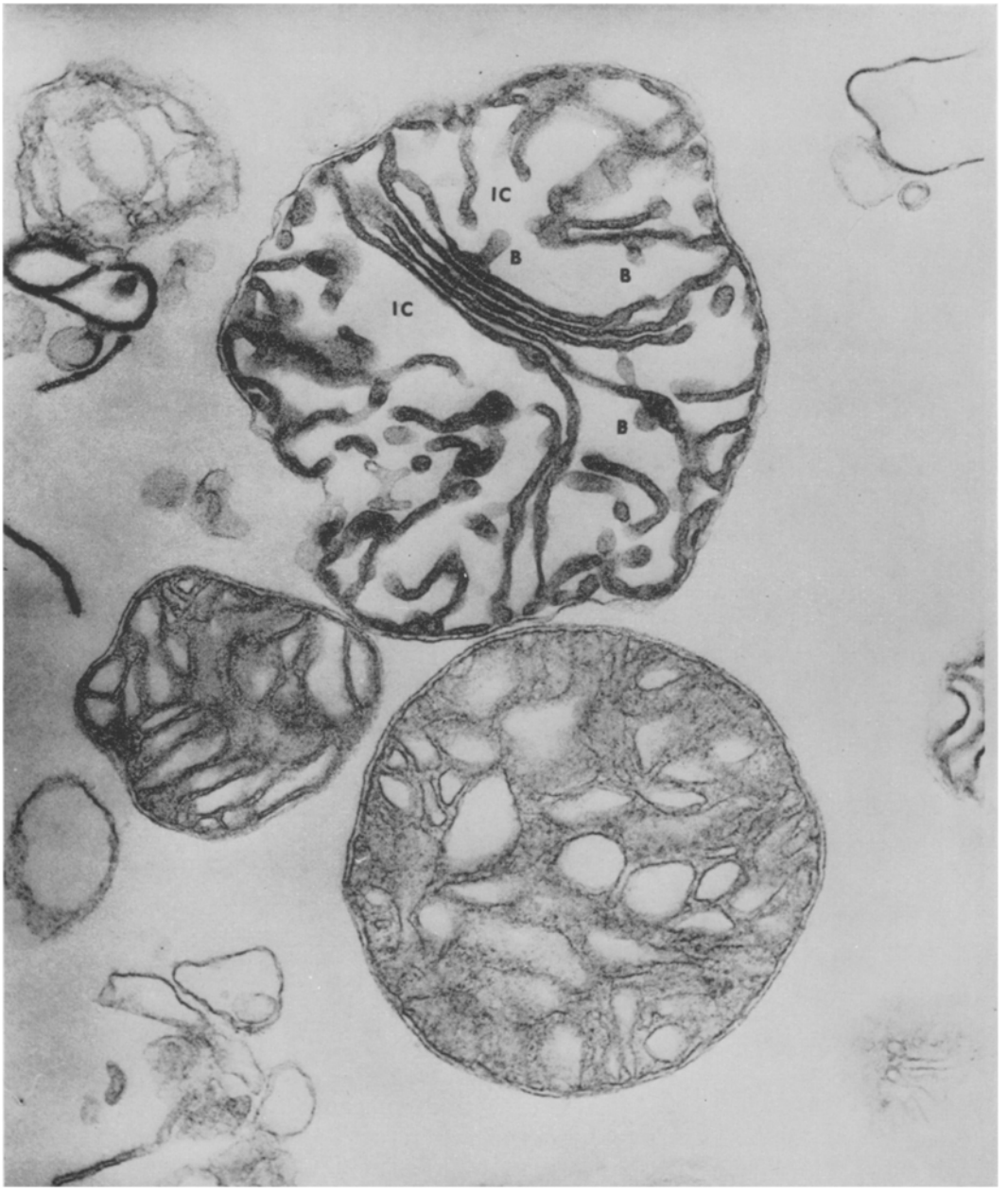


Figure 7. An electron micrograph of HBHM in the process of transition from the E configuration to the E (T) configuration. Regions of the mitochondrion are clearly in the twisted and tubular E (T) configuration, while other regions are in the E with flat sheets. At numerous points, bud-like reverse invaginations, B, are seen emanating perpendicular to the flat sheets, and thus extending into the electron transparent IC space. (86221-C-1)

snake-like tubes seen in the fully developed energized (twisted) configuration. The end result of this process of reverse invagination is the appearance of a large number of wide openings of the inner boundary membrane and the formation of a highly ramified labyrinth of interconnecting tubes, T, whose lumens are lined with headpiece-stalk sectors. It should be carefully noted that there has been no change in the topology of the inner membrane in the transformation outlined above.

In the formation of the energized (twisted) configuration, the process is represented diagrammatically as resulting from the formation first of a bud-like protuberance of the energized cristal membrane into the intracristal space, and then subsequent elongation of the bud into a twisted tube with a bulbous end. This process requires a perturbation of the flat, sheet-like cristal membrane continuum. The association of headpieces "sideways" with their nearest neighbors on the same cristal membrane continuum could easily account for that perturbation. In the flat, sheet-like configuration of the cristal membrane continuum in the energized configuration, the extended headpiece-stalk sectors are distributed on the membrane in such a way that there is a gap of approximately 25 Å in length between the headpieces. Upon the addition of inorganic phosphate the headpieces touch sideways in a way which is analogous to their having been drawn together by a purse-string. This headpiece-headpiece association is illustrated diagrammatically in Fig. 8. It will be noticed that in the drawing together of the headpieces, the flat membrane continuum has achieved a curvature which is consistent with the perturbation required for the formation of a bud-like reverse invagination. If the process continues further, a "zippering" together of the headpieces along the membrane can generate a tube with a bulbous end.

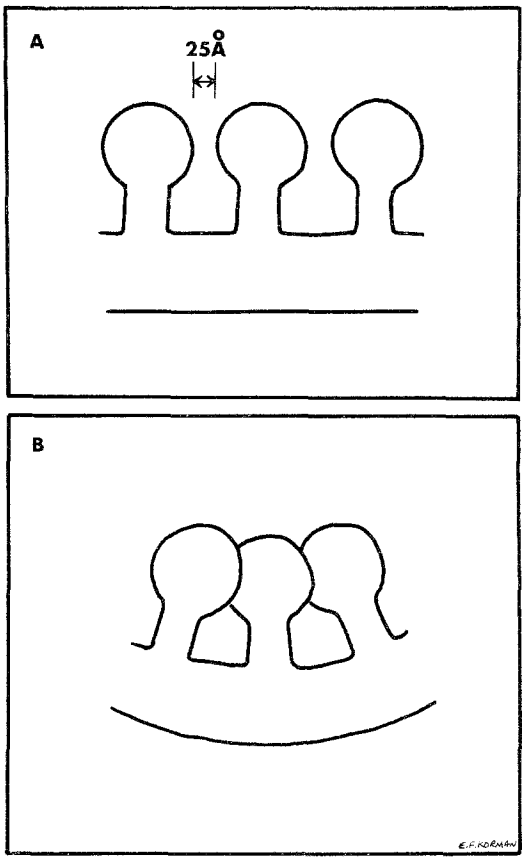


Figure 8. A diagrammatic representation of the "sidewise, purse-stringing" together of headpiece stalk sectors on the cristal membrane. (86224-C-1).

A. The CM surface is in its flat, sheet-like configuration seen in nonenergized and energized configurations. A 25 Å gap exists between headpieces.

B. The CM surface is curved with headpieces touching.

The tube formed in the model proposed above is pictured as twisted or corkscrew in form. The twisted nature of the tube has been partly rationalized by Penniston in our laboratory.[27] When hard glass spheres are stacked in hollow flexible plastic tubes [where the diameter of the glass spheres bears the same relation to the diameter of the plastic tubes that the 90 Å spherical headpieces bear to the diameter of the twisted tubes seen in the

electron micrographs of the energized (twisted) configuration], the glass spheres can stack only in helical arrays. The stacking of headpieces according to this helical model could lead to the corkscrew form of the tubes seen in electron microscopy.

In a previous communication[19] on the configurational cycle, we interpreted the transition of the mitochondrial inner membrane from the energized configuration to the energized (twisted) configuration as a tubularization associated with a "comminution" of the membrane into numerous separate snake-like twisted tubes. At that time, the idea of multiple tubes was proposed because we could not visualize the transformation other than by a shredding of the membrane. Such shredding is, of course, a violation of our currently held position of topological invariance. It also made the fact that the matrix is a sucrose-impenetrable space very difficult to understand, unless, as was originally done in a similar incorrect fashion for the explanation of the nonenergized (aggregated) configuration, the comminution and resealing of the membrane is carried out in a way which somehow does not allow sucrose to penetrate into the matrix space. Such a process seems very unlikely. Here again, the question becomes irrelevant once the currently held model is accepted, because the topology of the mitochondrial inner membrane is a constant with no tearing or shredding of that membrane during the transformation.

Discharge of the energized or energized (twisted) configuration of the mitochondrial inner membrane by a work function or by uncouplers results in the reappearance of the nonenergized configuration.[21] This discharge process leading to the nonenergized configuration is extremely rapid. Our current view of the topological invariance of the mitochondrial inner membrane during this discharge makes that rapid process more understandable than in the past. The concept of multiple tubes resealing to form flat, sheet-like membranes in the nonenergized configuration in the short period of time measured for the change presented formidable difficulties. We now realize that the membrane can revert to the nonenergized configuration almost instantaneously, because the membrane has not really changed its form except in terms of the kinds of folds present in its unbroken surface. This is another example of a problem which has been obviated by a proper interpretation which is imposed by a careful adherence to the topological boundary conditions.

(*e*) *The energized (zigzag) configuration.* When inorganic phosphate is added to HBHM isolated in 0·25 M sucrose in the energized configuration, in addition to the characteristic twisted tubular configuration, a certain small proportion of mitochondria is in a configuration quite different in appearance. In Fig. 1E we see an electron micrograph of this configuration, called the energized (zigzag) configuration. This configuration is similar to the energized configuration, except that the apposed cristal membranes have a zigzag character. There is no evidence of tubularization, since no circular or elliptical cross sections are seen.

The energized (zigzag) configuration shown in Fig. 1E occurs only infrequently in HBHM which have been isolated in 0·25 M sucrose and energized. However, when rat heart or canary heart mitochondria are studied *in situ* under energizing conditions, essentially *all* the mitochondria are in the energized (zigzag) configuration.[29] Here again, rat and canary heart mitochondria can be easily studied *in situ* because it is possible to fix rapidly the fresh tissue shortly after sacrificing the animal, something not feasible with beef heart muscle. The appearance of the energized (zigzag) configuration in both rat

and canary heart mitochondria *in situ* is essentially identical with that of HBHM in Fig. 1E.

The energized (zigzag) configuration seems at first to have very little in common with the energized (twisted) configuration, the configuration seen in mitochondria in the comparable energy state, but isolated in 0·25 M sucrose. However, they do have a very important characteristic in common. The energized (zigzag) configuration has regions on the cristal membrane continuum which have the same kind of curvature and purse-stringed headpiece-stalk sectors that were invoked in the explanation of the formation of the tubes in the energized (twisted) configuration. This similarity is illustrated diagrammatically in Fig. 9. From this we conclude that the zigzag sheet and the twisted tubes are variations upon the same structural perturbation of the energized configuration. In a sense, the formation of tubes is the farthest possible extreme to which the perturbation which gives rise to the zigzag sheet can be carried. Just why the perturbation goes to completion into tubes in mitochondria isolated in sucrose and only to the zigzag sheet in mitochondria *in situ* is as yet not understood.

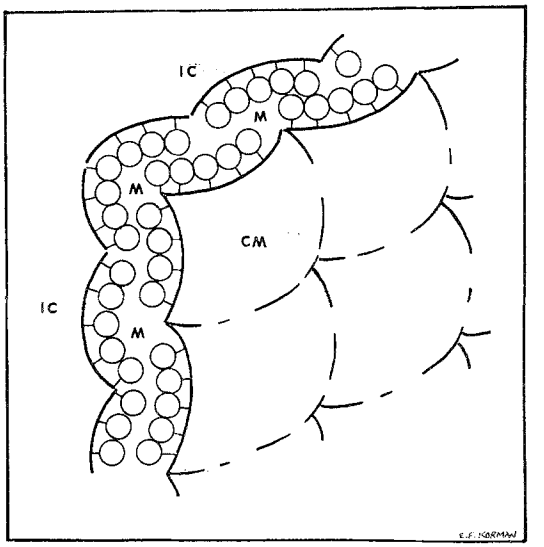


Figure 9. A diagrammatic three-dimensional representation of portions of two apposed cristal membranes in the E (Zigzag) configuration. Each CM is perturbed with numerous bud-like, reverse invaginations. The headpieces are purse-stringed together over the entire surface of each CM. The two membranes mesh so that when cut at any angle whatsoever, the zigzag configuration is generated in electron microscopy. (86227-C-1)

(*f*) *The configurations observed in mitochondria in situ in sucrose.* When canary and rat heart mitochondria are studied *in situ* in 0·25 M sucrose medium,[29] the configurational states which are seen in electron micrographs during the energy cycle are essentially the same as those observed with mitochondria isolated in 0·25 M sucrose.

(*g*) *Summary of configurations observed during the energy cycle.* The various configurational states observed during the energy cycle for heart mitochondria studied both *in*

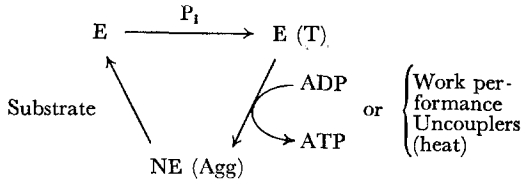
TABLE I. Configurations observed during the energy cycle

	Configurations		
Energy state	Mitochondria *in situ* (no sucrose)	Mitochondria isolated (sucrose)	Mitochondria *in situ* (sucrose)
Nonenergized	NE (Ortho)	NE (Agg)	NE (Agg)
Energized	E	E	E
Energized + P_i	E (Zigzag)	E (T)	E (T)
Discharged to:	NE (ortho)	NE (Agg)	NE (Agg)

situ and isolated are summarized in Table I. The configurations can also be summarized in cyclic diagrams (in which the vertical axes represent the energy level) which describe the operation of the energy cycle in non-steady state systems:

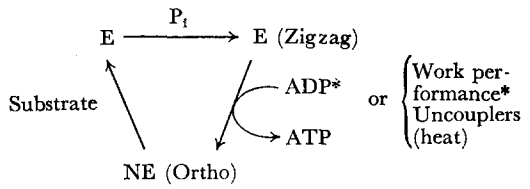
A similar cyclic diagram can be written for mitochondria *in situ* (0·25 M sucrose).

(1) *Mitochondria, isolated in 0·25 M sucrose in situ in 0·25 M sucrose*



A cyclic diagram can also be written for mitochondria *in situ* (KRP), parts of the cycle having been experimentally observed, and other parts of which have been deduced by analogy with the cycle in isolated mitochondria.

(2) *Mitochondria in situ (KRP) medium;*



2. *The Tubular Type of Cristae*

The detailed descriptions of the configurational states associated with the energy cycle outlined above for mitochondria possessing the pillow-case type of cristae have their essentially identical counterparts in mitochondria with tubular cristae. For example, in the study of isolated bovine adrenal cortex mitochondria the mitochondrial inner membrane exhibits easily identifiable configurational states related to the energy cycle in a one-to-one fashion and which, except for a few minor details due to the scalloped tubular nature of the cristal membrane, are identical with those seen in beef heart mitochondria. Allmann *et al.* in our laboratory have studied these configurational states extensively, and a complete discussion of these observations and their relationship to the topological boundary conditions are presented in this journal.[28]

VIII. *Discussion*

It was the stated purpose of this communication to examine the various morphological states of the mitochondrial inner membrane and to formulate a unified view of their three-dimensional form within the framework of certain topological parameters. We believe that we have succeeded in deducing a simple, self-consistent model which

* Processes deduced by analogy with those in diagram (1) above.

operates within the framework of reasonable topological boundary conditions, and which at the same time accounts for all the configurational phenomena observed in mitochondria.

The configurational states have been correlated with events in the energy cycle in a one-to-one fashion, and therefore we believe that the configurational states and the changes which occur in the transformation from one observed configurational state to another are significant observations which must be understood in relation to the biochemical processes in mitochondria which they accompany. For example, in the transition from the nonenergized (orthodox) *in situ* configuration to the energized configuration, there is an inversion or reversal of the relative volumes of the two mitochondrial compartments. This volume reversal can also be effected by a nonenergized process, namely by isolation of the mitochondria in 0·25 M sucrose. The osmotic gradient resulting from the inability of sucrose to penetrate the mitochondrial inner membrane into the matrix space causes a water movement which leads to the nonenergized (aggregated) state. The similarity of the morphological form of the nonenergized (aggregated) configuration and the energized configuration is striking. The question which immediately suggests itself is how the energizing process can lead to water movements which are similar to water movements observed in an osmotically imposed condition. The answer to this kind of question could prove to be crucial to our ultimate understanding of the energizing process, and could possibly clarify the relation of that process to oxidative phosphorylation.

The conversion of the nonenergized (aggregated) configuration to the energized configuration is of immense interest. This transformation, although subtle, is unmistakable. The general morphological form for the two configurations is almost the same, complete with T-, Y-, and X-structures, except for the dimension measured across the two mitochondrial inner membranes. The most striking difference between the two configurations is the appearance in the space between the apposed membranes. In the nonenergized (aggregated) configuration the space is uniformly electron dense, while in the energized configuration the structure within the space is recognizable as in the form of headpiece-stalk sectors. This difference results from a change in the *conformation* of the repeating units, i.e., a change in the relation of the headpiece-stalk sector of the repeating unit to the basepiece. We interpret the increase in the dimension across the pair of membranes and the appearance of visible structure between the membranes as resulting from the change in conformation of the headpiece-stalk sector from the collapsed to the extended conformation. This is direct visual evidence for the conformational basis of energy transduction.

The new questions which we can now raise, and the new interpretations we can now make, depend completely upon our current understanding of the various configurations of the mitochondrial inner membrane. Membrane Topological Analysis has proved to be indispensable for this understanding. This technique should provide further information about mitochondrial structure and function.

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