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Studies on the Transition of the Cristal Membrane from the Orthodox to the Aggregated Configuration. I: Topology of Bovine Adrenal Cortex Mitochondria in the Orthodox Configuration

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Abstract

Bovine adrenal cortex mitochondria† examined by electron microscopy *in situ* or *in vitro* in 0·25 M sucrose have an unusual cristal membrane structure. The cristae usually appear as unconnected vesicles within a double membrane system. A few of the vesicles appear to be attached to the inner boundary membrane or to one or more other vesicles. The configuration of such mitochondria will be defined as the orthodox configuration. In this communication we will provide evidence that the inner membrane is not composed of multiple vesicles, but is one continuous membrane with tubular invaginations, and that these invaginations alternately are ballooned out and squeezed down. A mechanism has been proposed to account for the differentiated structure of the cristae of adrenal cortex mitochondria.

Introduction

This communication is the first of a series that will be concerned with the structure and function of bovine adrenal cortex mitochondria. Bovine adrenal cortex mitochondria *in situ* or *in vitro* can exist in two distinct and different configurations.[1–3] Adrenal cortex mitochondria in the aggregated configuration have essentially the same morphology as all mitochondria, regardless of tissue origin (Fig. 1)[4–8] However, the morphology of mitochondria in the orthodox configuration seems to vary depending on the tissue or origin. Mitochondria from such sources as heart, liver, and kidney[4–8] appear to have linear cristal invaginations which are continuous with the inner boundary membrane. On the other hand, mitochondria from the *zona fasciculata* of adrenal cortex[1–3, 9, 10, 11] *corpus luteum*,[12] and testes[13, 14] have a different morphology when they are in the orthodox configuration compared to mitochondria from heart, liver, and kidney (Fig. 2). In adrenal cortex mitochondria the cristae appear as "vesicles", vesicular cristae (VC), that seem principally to be unattached to any other membrane, i.e., they appear to be free-floating entities in the matrix space.[10] The question which then arises is, are the adrenal cortex mitochondria unique and different from most other mitochondria? Are we dealing with two basic types of mitochondria, one in which the cristae are clearly invaginations of the

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† In this paper the term adrenal cortex mitochondria always refers to mitochondria of the *zona fasciculata*.

inner membrane and another in which the cristae are clearly separated from any other membrane? Electron microscopic evidence will be presented to support the former thesis. The topology of the orthodox configuration of the adrenal cortex mitochondria is no different from that of all other mitochondria, i.e., it is isomorphic with a hollow sphere. What appear to be "unconnected vesicles" are, in fact, the result of sectioning

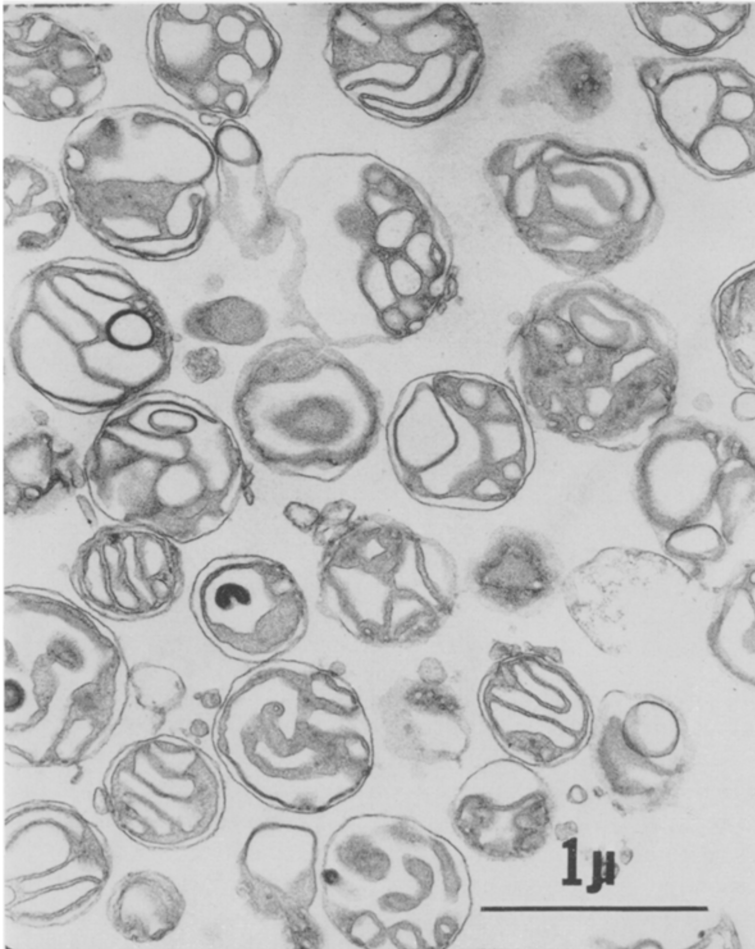


Figure 1. Electron micrograph of adrenal cortex mitochondria isolated in the STE medium. (38549)

tubular invaginations of the inner membrane in which the invaginations are alternately ballooned out and squeezed down.

Methods

Isolation of Mitochondria from the Cortex of Beef Adrenals

The excised adrenals of ten animals which had just been slaughtered were collected, placed in crushed ice and brought to the laboratory within 1 h of the death of the animal.

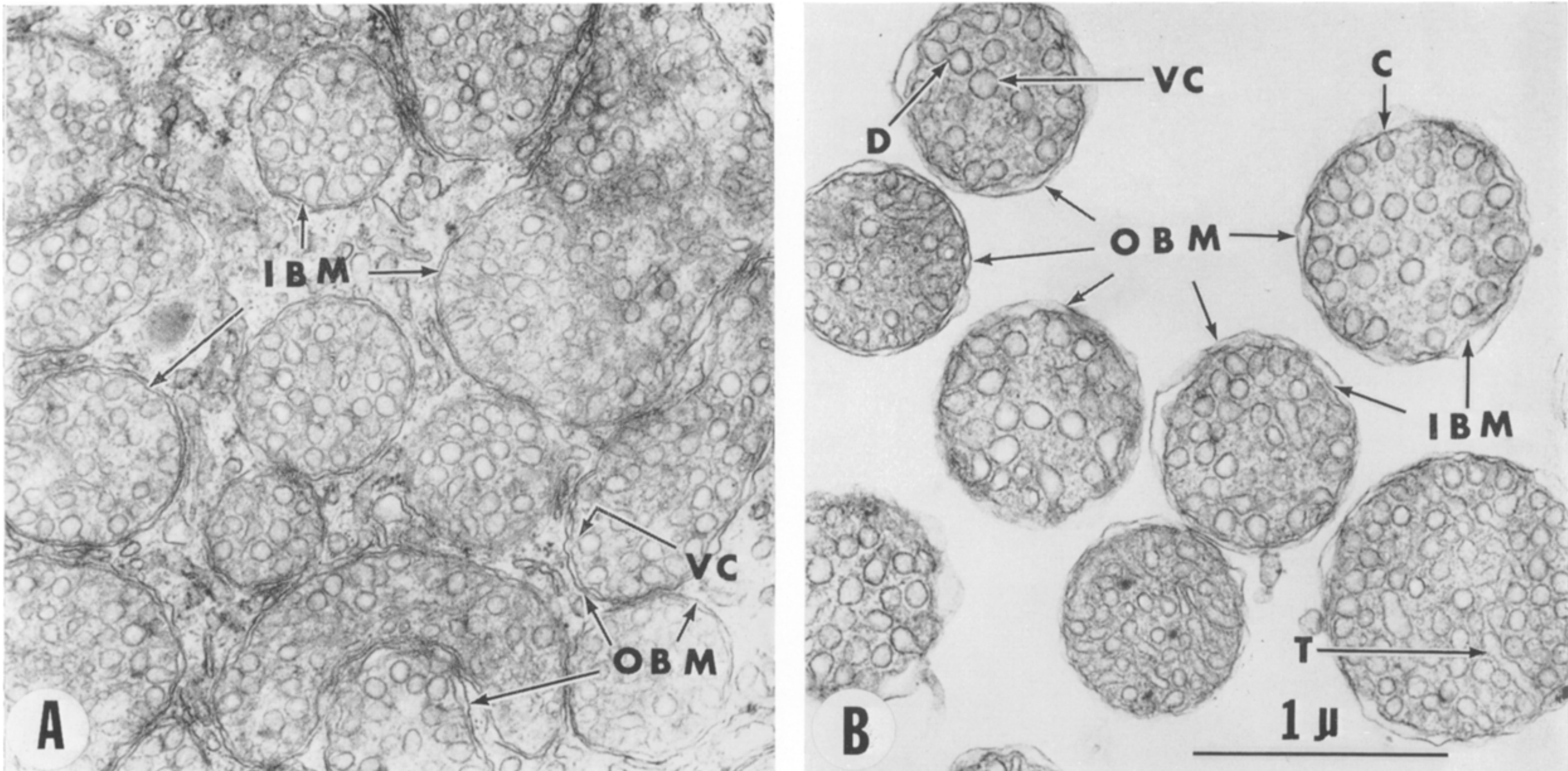


Figure 2. Electron micrographs of adrenal cortex mitochondria. A: a section containing mitochondria of the *zona fasciculata* of beef adrenal cortex *in situ*. (24299). B: a section of adrenal cortex mitochondria isolated in a medium 0·25 M in sucrose and 10 mM in TrisCl, pH 7·8. (19780). The arrows in both micrographs point to the three types of membranes: inner boundary membrane (IBM), outer boundary membrane (OBM), and "vesicular" crista (VC). The arrow D refers to cristal structures that look like "dumb-bells"; the arrow T refers to cristal structures that look like tubes with undulating walls; and arrow C refers to cristal structures that look like "dead-end" invaginations (cul-de-sacs).

The glands were trimmed to remove fat and cut in half longitudinally. The central medulla and a portion of the adjacent *zona reticularis* were scraped away and discarded. The next layer (mostly *zona fasciculata*) was obtained by scraping and immersed into the appropriate suspending medium cooled to 0°. Most of the *zona glomerulosa* adhered to the capsule and was discarded. The yield of *zona fasciculata* of the cortex was usually about 140 g from twenty glands. The chopped cortex was rinsed once with the fluid of the suspending medium and then resuspended in fresh medium (about 8 ml/g wet weight of chopped cortex). The suspension was homogenized first in a loose-fitting, glass-teflon homogenizer and then later in a tight-fitting homogenizer unit.

The isolation of the mitochondria was accomplished in one of two media: (a) a solution which was 0·25 M in sucrose, and 10 mM in TrisCl, pH 7·8 (ST), or (b) a solution which was 0·25 M in sucrose, 10 mM in TrisCl, pH 7·8, and 0·1 mM in NaK EDTA (STE). The abbreviations list all the ingredients of the medium. Medium (a) was used to prepare mitochondria in the orthodox configuration and medium (b) was used to prepare mitochondria in the aggregated configuration.

The homogenate obtained by the procedure described above was centrifuged for 10 min at 600 $\times$ *g* in a Spinco No. 30 rotor and the residue was discarded. The supernatant fluid was then centrifuged for 10 min at 10,000 $\times$ *g* in the same rotor. The fluffy layer was discarded and the heavy mitochondrial pellet was suspended in about 140 ml of medium; the suspension was centrifuged for 10 min at 10,000 $\times$ *g* in a Spinco rotor No. 40. The heavy layer was suspended in about 70 ml of medium and centrifuged for 10 min at 10,000 $\times$ *g* in the same rotor. The heavy layer was suspended in 50 ml of medium, and the suspension was centrifuged for 10 min at 15,000 $\times$ *g* in the same rotor. The heavy layer of the residue was suspended in 30 ml of medium; the suspension was centrifuged again for 10 min at 15,000 $\times$ *g*. The residue was suspended with sufficient medium so that the final protein content was 30–50 mg/ml. The protein concentration was determined by the biuret method of Gornall *et al.*[15] in the presence of deoxycholate.

Preparation of Calcium-Free Sucrose

Sucrose obtained from commercial sources usually contains calcium in significant amounts.* We found about 20 mμmoles of calcium/ml of 0·25 M sucrose. The calcium content of the medium can be reduced by more than 95% by mixing Amberlite MB-3 mixed bed resin (50 g) with 1 l of M sucrose for 1 h or longer. The resin was removed by filtration. The removal of Ca^{2+} was monitored by addition of $^{45}CaCl_2$ to the sucrose solution. The mixture was continuously stirred until the filtrate contained less than 5% of the radioactivity of the original ^{45}Ca. The ratio of resin to solution was set at the point at which the radioactivity of the filtrate could be reduced to $<$5% of the original. The concentration of Ca^{2+} in the sucrose medium before treatment with Amberlite was determined by atomic absorption (calcium standards were prepared in calcium-free sucrose of the same molarity as that of the sucrose in the experimental solutions).

Procedure for Electron Microscopy

A sample of a given mitochondrial suspension which contained at least 10 mg of protein was treated with a glutaraldehyde solution to a final concentration of 10 mM. The inter-

* We are grateful to Drs. Leena Mela and Ronald Estabrook for pointing out that some commercial sources of sucrose are contaminated with Ca^{2+}. The sample of sucrose routinely used in these studies contained trace amounts of Ca^{2+}.

action of the mitochondrial suspension with glutaraldehyde was carried out at 25° for at least 10 min; then the sedimented pellet was resuspended and washed in Ca^{2+}-free ST for at least 60 min. The washed, fixed samples were then exposed to osmium tetroxide, uranyl acetate, and ethanol in the manner previously described.[16] The samples were dehydrated three times in 100% ethanol and later exposed twice to absolute propylene oxide for 10 min. The dehydrated samples were then exposed first to a mixture of equal parts by volume of Epon and propylene oxide over a period of 20 min, and finally exposed to 100% Epon (Luft's 1–3)[17] for 4–5 h. The samples in fresh Epon were placed into capsules and hardened at 60° for about 48 h. Fixation of the specimens with 1% glutaraldehyde and 1% acrolein[5,16] gave results identical with those obtaining when 10 mM glutaraldehyde was used as the fixative. Glutaraldehyde alone was generally used as fixative.

Samples of adrenal cortex tissue (1 mm cubes) were fixed immediately upon arrival from the slaughterhouse in either of two solutions: (A) a solution which was 0·25 M in sucrose, 50 mM in cacodylate, pH 7·5, 1% in glutaraldehyde, and 1% in acrolein, or (B) in Krebs-Ringer-Phosphate containing 1% glutaraldehyde and 1% formaldehyde (4). The fixed samples were washed with the fixative-free medium and treated with uranyl acetate, osmium, etc., as described above for the mitochondrial suspensions. The two fixatives gave identical results.

The specimens were sectioned with a diamond knife, post-stained with lead citrate[18] and examined in a Hitachi HU-11B electron microscope operated at 75 kV.

Beef adrenal glands were generously supplied by Oscar Mayer and Co., Madison, Wisconsin.

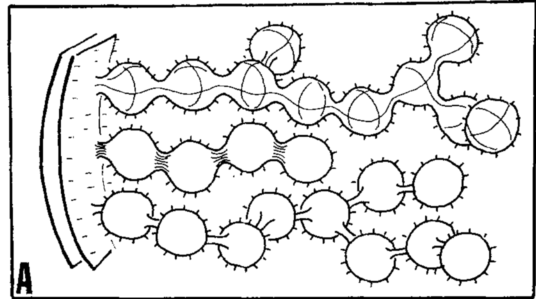
Results

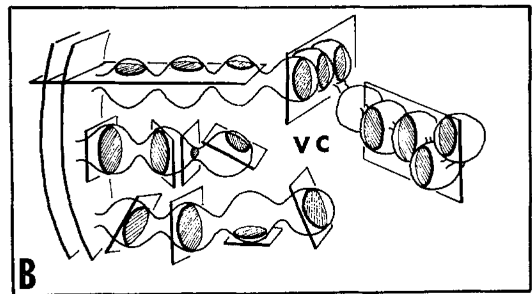
Adrenal cortex mitochondria *in situ* (see Fig. 2A) or isolated (see Fig. 2B), which are in the orthodox configuration, show a very characteristic ultrastructural pattern. The mitochondria have an outer boundary membrane (OBM), an inner boundary membrane (IBM), and internal structures which appear as "unconnected vesicular" structures (VC).

The schematic diagram in Fig. 3 shows the relationship of the outer boundary membrane, inner boundary membrane, and cristal membranes (see also refs. 16 and 19 for further discussion of this membrane system). Thus, these mitochondria give the appearance of having three independent and separate membrane systems. Each vesicular crista appears to be either "unconnected" to any other structure (arrow VC), connected to one or more other vesicular structures, or connected to the inner boundary membrane. Except for those vesicular structures which are clearly connected to the inner boundary membrane, the cristae do not appear to be invaginations of the inner boundary membrane. In attempting to visualize the ultrastructure of adrenal cortex mitochondria in the orthodox configuration, we were then faced with the problem of whether the cristae of these mitochondria have an entirely different type of cristal membrane morphology from that of the cristae of the mitochondria of heart, liver, kidney, etc. In these latter-listed mitochondria the inner boundary membrane is continuous with the cristae.[15] In topological terms, such mitochondria have an inner membrane (i.e., inner boundary membrane plus cristal membrane) which is isomorphic with a single hollow sphere, because it retains its topological identity no matter how many invaginations or what form these

invaginations take. However, if the cristal membrane of orthodox adrenal cortex mitochondria is composed of multiple unconnected, i.e., free-floating, vesicles, the topology of the inner membrane is no longer isomorphic with that of the mitochondria of heart, liver, kidney, etc.

On close examination, however, we are able to rationalize the topology of the cristal membrane of orthodox adrenal cortex mitochondria as isomorphic with that of other mitochondria. That is to say that the vesicular cristae are, in fact, modified tubular invaginations of the inner boundary membrane. This latter statement is rationalized and supported by the diagrams and the electron micrographs shown in Figs. 3 4, and 5. Some of the structures and patterns in the drawings in Fig. 3 have been lettered, and these lettered structures and patterns should be compared with the corresponding lettered structures seen in actual electron micrographs in Figs. 4 and 5. Figure 3A represents typical cristae drawn in different three-dimensional representations. All of these representations are equivalent. As indicated in these drawings we visualize the cristae as tubular invaginations of the inner membrane. The tubes are represented as variable in diameter, i.e., the diameter along the length of each tube alternately "balloons out" and "squeezes down". This results in a tube which alternates between almost "spherical" regions and narrow tubular connecting parts. The diameter of the tubular connecting part can vary between a minimum of 0·1 of the diameter of the spherical regions, up to a maximum equalling the diameter of the spherical regions. We shall refer to the structure of the crista as a "scalloped tube". The headpiece-stalk sectors represented by the short straight lines project into the matrix space. The drawings given in Figs. 3B and 3C illustrate how various patterns seen in actual electron micrographs could arise as a result of sectioning the scalloped tubular cristae at a variety of angles. The shaded areas designate what the various resulting patterns are. In Fig. 3B we see that no matter at which angle the





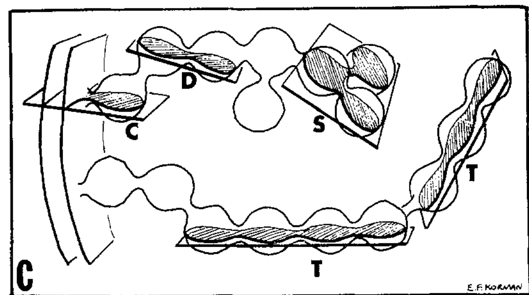


Figure 3. A series of schematic drawings illustrating our interpretation of the tubular cristal membranes of beef adrenal cortex mitochondria in the orthodox configuration. A represents typical cristae drawn in different three-dimensional representations. B and C illustrate how various patterns seen in actual electron micrographs could arise as a result of sectioning the scalloped tubular cristae at a variety of angles. The shaded areas designate what the various resulting patterns are. The various patterns are lettered, and the letters refer to actual examples seen in Figs. 4 and 5.

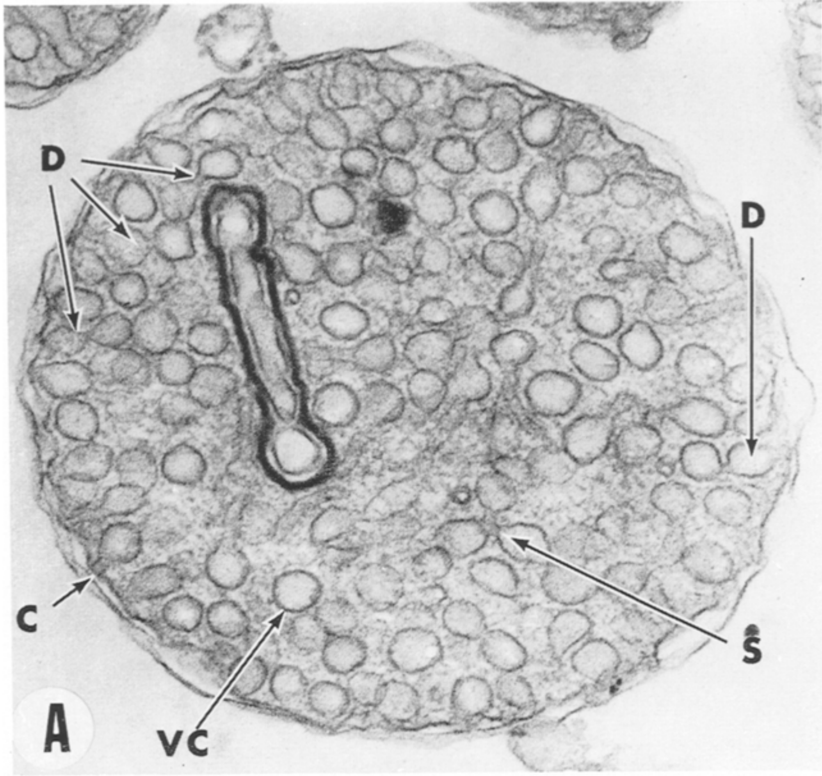
cristae are sectioned across their diameters, the *predominant* pattern to be expected is that of "unconnected vesicles". "Unconnected vesicles" also result from sectioning down the length of a scalloped tube, as long as the section does not pass through a connecting tube. If the plane of sectioning bisects the tubular connections then structures depicted in Fig. 3C will be seen. Among them is a "dumbbell" pattern, D, and a branched "shamrock" pattern, S, as well as closed tubes with undulating walls, T. In addition, a frequent pattern seen near the inner boundary membrane is what looks like a short, single scalloped "dead-end" invagination (cul-de-sac, C). The explanation for this single scalloped invagination (see Fig. 4, arrow C) protruding from the inner boundary membrane into the matrix is illustrated in Fig. 3C, arrow C. It should be noted that contrary to the first impression, this pattern results not from sectioning a single scalloped invagination but rather from sectioning a typical crista in a particular plane. To completely understand this observation it must be realized that spherical regions are not always linked absolutely linearly. Such a non-linear arrangement of spherical regions along the length of a single tube is seen in the electron micrographs in Fig. 5. If this non-linear arrangement had been sectioned in a plane indicated by the dark line in Fig. 5B, the resulting pattern would have been a single scalloped invagination of the inner membrane (see Fig. 3C, arrow C, and Fig. 4, arrow C). If it had been sectioned in a plane indicated by the dark line in Fig. 5C, the resulting pattern would have appeared to be a "dumbbell" invaginating from the inner boundary membrane, as illustrated in the drawing in Fig. 5C. An actual example of such a "dumbbell" invagination is seen in Fig. 4 (see arrow D). If it had been sectioned in a plane indicated by the dark line in Fig. 5D, the resulting pattern also would have appeared to be a "dumbbell", as illustrated in Fig. 4 (see arrow T).

Evidence for a linear or branched array of alternating tubular and spherical structures can be verified by examining areas T in Fig. 4, where we see three or four such connecting arrays. In some cases (Fig. 4D, arrow T) we have seen up to seven to nine connecting spheres. With these examples, we thus can easily see that any given crista which has linear, non-linear, and branched segments and which has a fixed orientation in space, when sectioned at a variety of different planes, can give rise to *all* the patterns seen in the electron micrographs.

The configurational state of adrenal cortex mitochondria discussed thus far has been designated orthodox. This designation has been assigned by analogy with the orthodox state of heart and liver mitochondria.[4–8] In adrenal cortex mitochondria, as well as liver and heart mitochondria, the orthodox state is characterized by: (a) maximal separation of individual cristae, (b) relative volume minimum of the intracristal space, and (c) the relative volume maximum of the matrix space.

In situ the cristae of bovine adrenal cortex mitochondria are arranged in orderly arrays (orthodox) as shown in Fig. 6A. When the mitochondria *in situ* are exposed to a medium 0·25 M in sucrose for 20 min prior to the addition of the fixative as shown in the electron micrographs of Fig. 6B, the intracristal space undergoes swelling to the point that neighboring cristae now touch (engage)* and the headpiece-stalks from the repeating units of one crista may interdigitate with the corresponding structures of the apposed crista. The swelling of the crista has an asymmetric character; the squeezed-down connecting regions between ballooned-out vesicular structures swell more than the vesicular

* The term "engagement" in this context refers to the touching or near touching of headpiece-stalk sectors of apposing cristae.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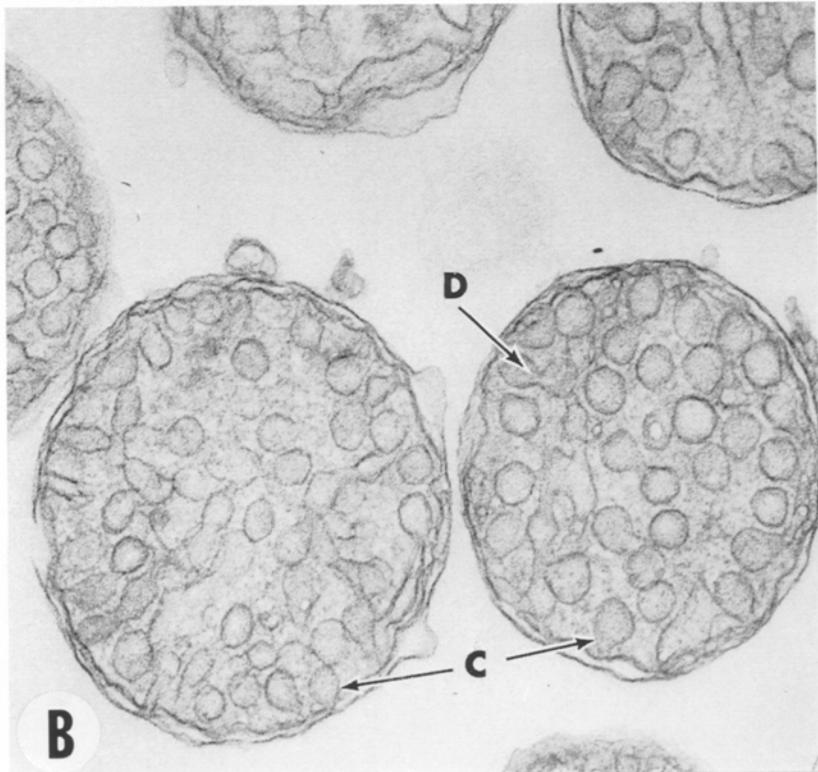
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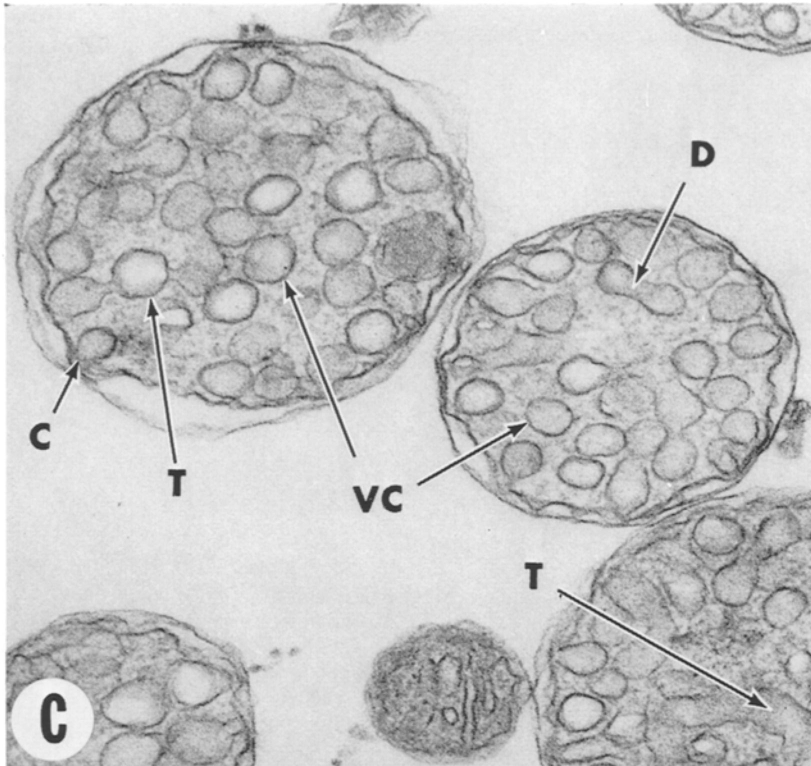


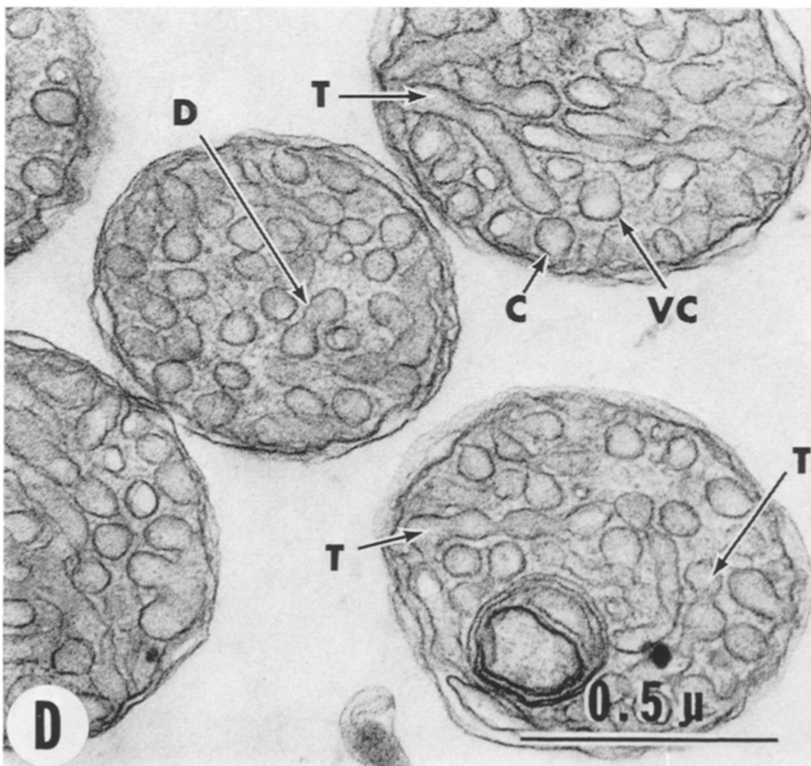
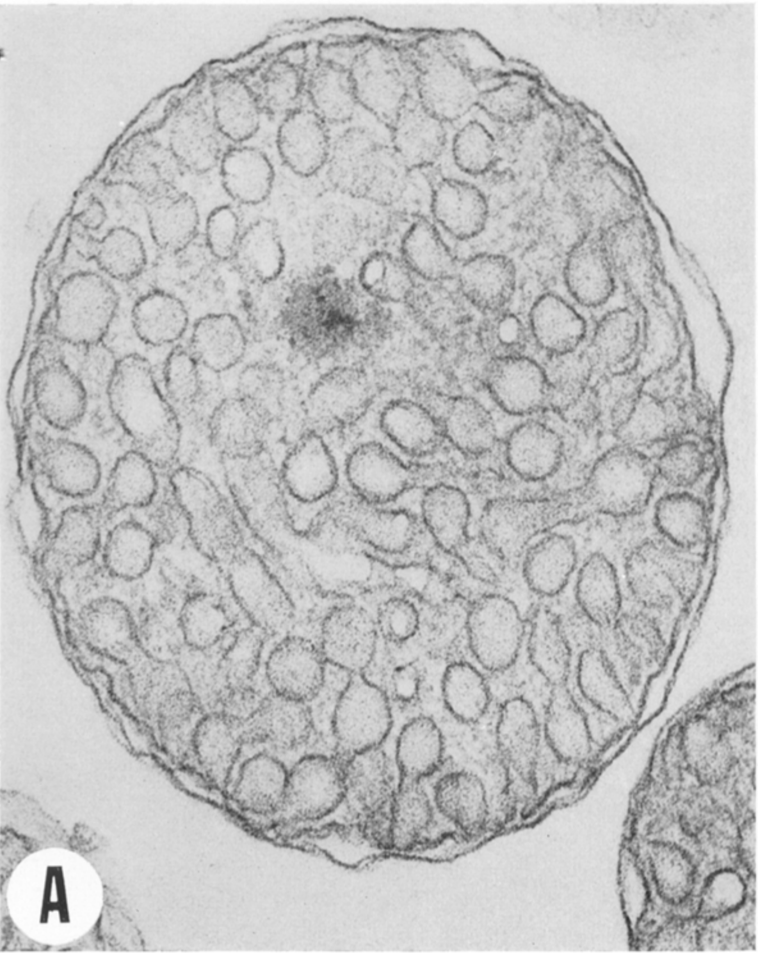


Figure 4. Electron micrographs of four typical fields of adrenal cortex mitochondria in the orthodox configuration. The lettered areas correspond to the same lettered areas seen in the schematic drawings in Fig. 3. (38962, 38911, 38955, 38961)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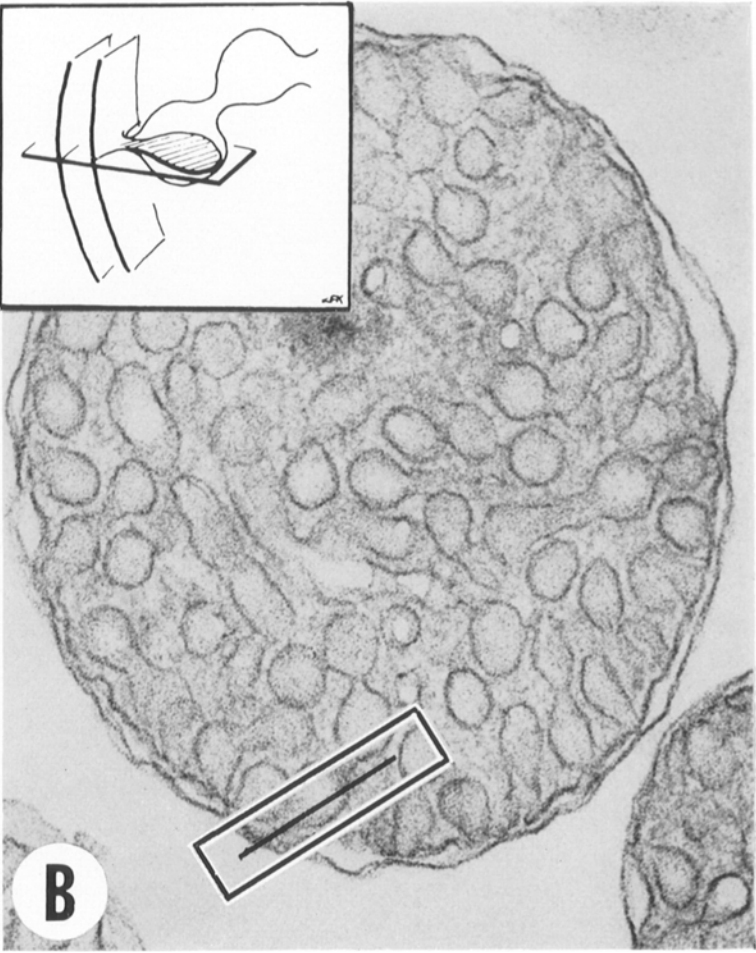
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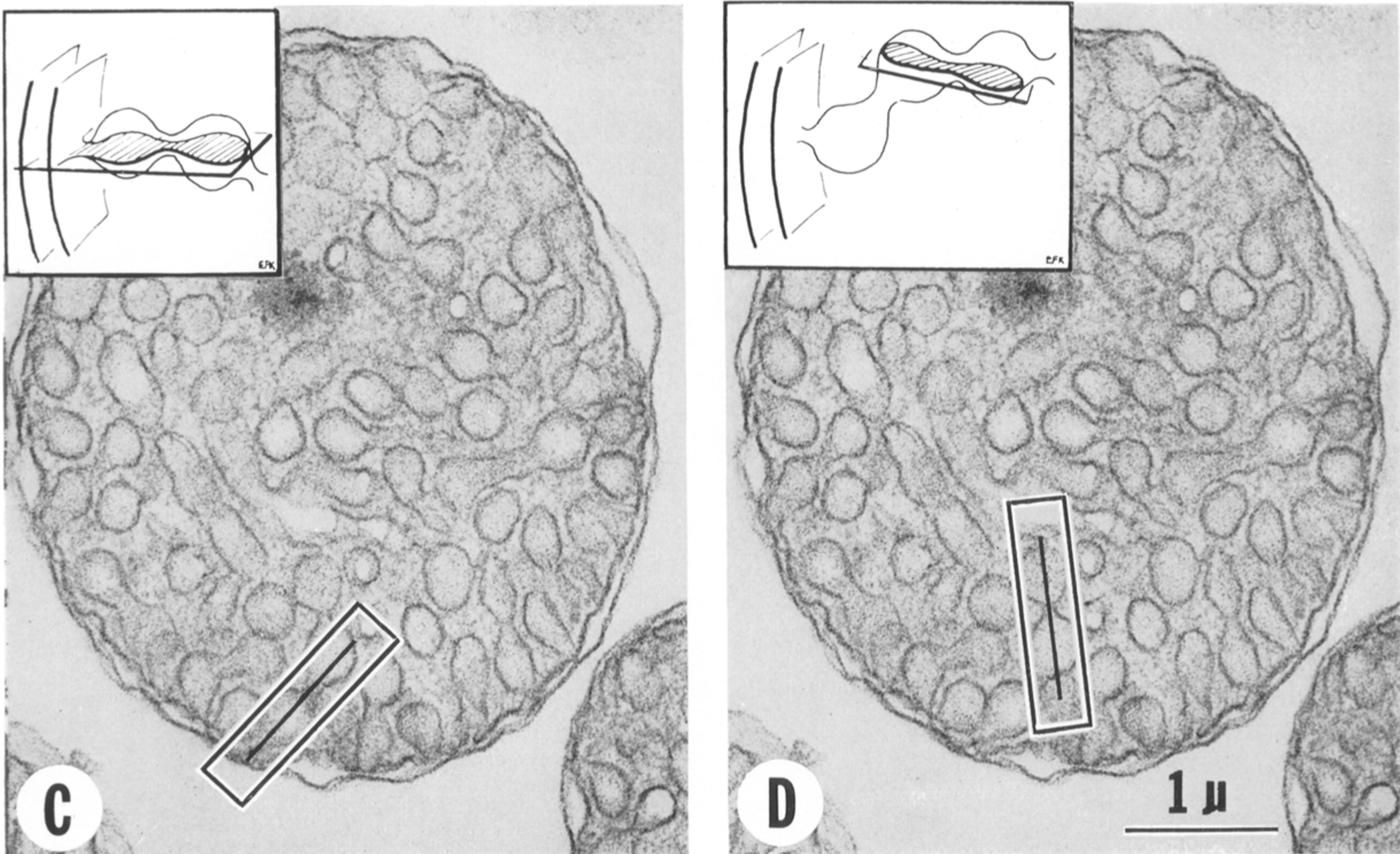
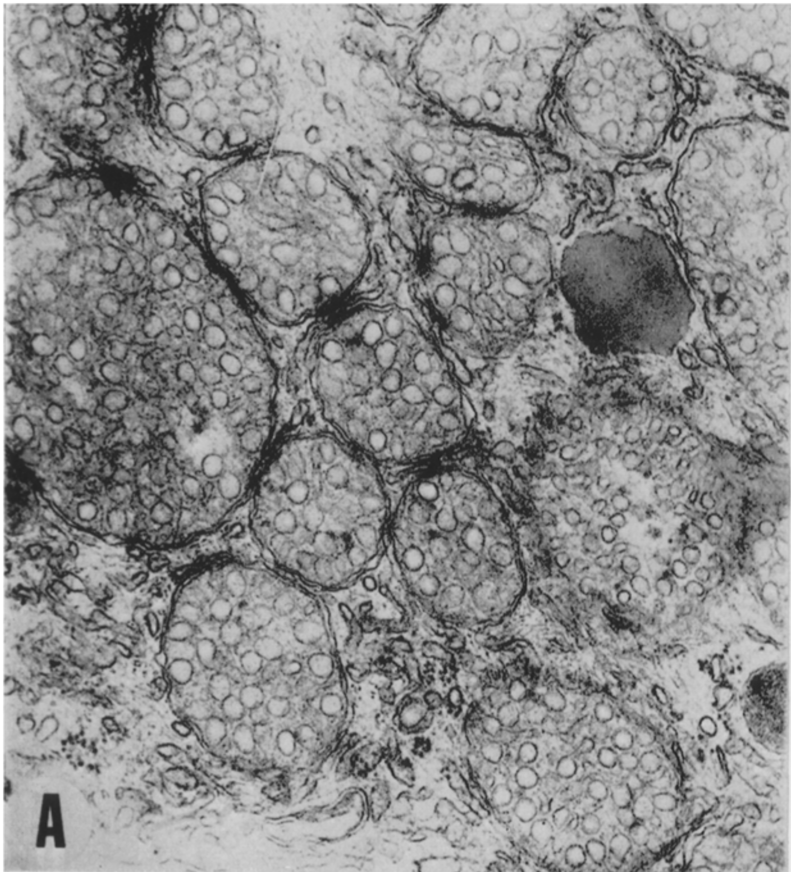


Figure 5. Electron micrographs of a single mitochondrion illustrating a non-linear arrangement of spherical regions along the length of a single tube. A shows crista as it was sectioned. (38965). B shows that if the plane of sectioning had been as indicated by the dark line the resulting pattern would have been a single scalloped invagination of the inner membrane (see also Fig. 3, arrow C, and Fig. 4, arrow C). C shows that if the plane of sectioning had been as indicated by the dark line the resulting pattern would have appeared to be a "dumbbell" invaginating from the inner boundary membrane as seen in Fig. 4, arrow D. D shows that if the plane of sectioning had been as indicated by the dark line the resulting pattern would have appeared as a "dumbbell" as seen in Fig. 4, arrow T.



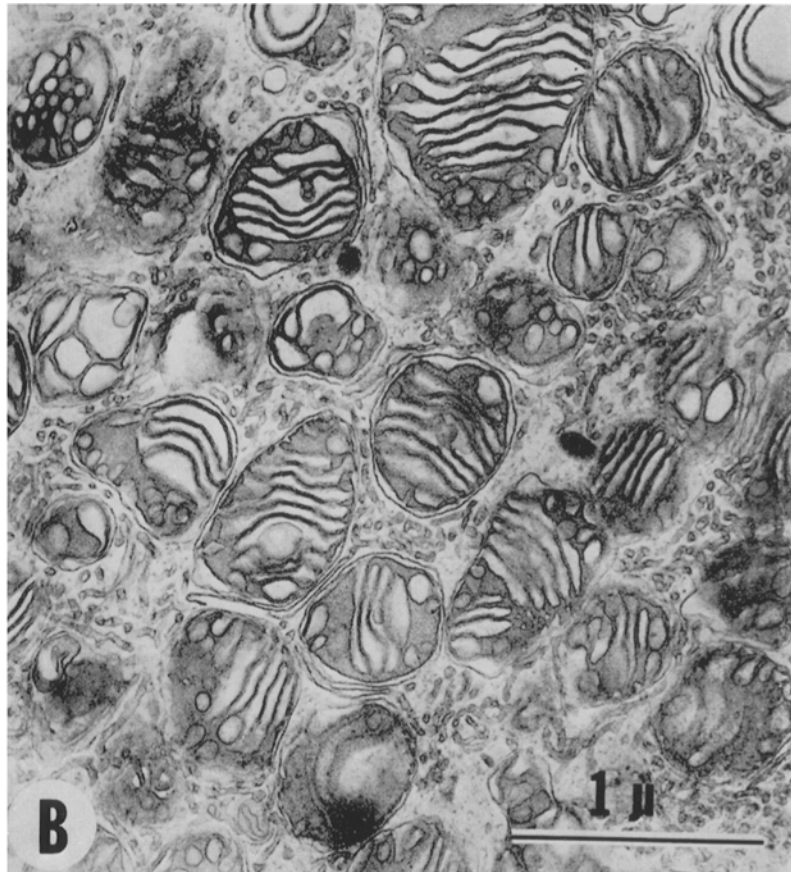


Figure 6. Electron micrographs of adrenal cortex mitochondria *in situ*. A: a section of a slice of adrenal cortex incubated for 20 min at room temperature in a Ca^{2+}-free Krebs-Ringer-Phosphate buffer prior to fixation. (24300). B: a section of a slice of adrenal cortex incubated for 20 min at room temperature in 0·25 M sucrose (Ca^{2+}-free) prior to fixation. (37149)

parts during this treatment. The net result is the obliteration of the scalloped nature of the crista and the expansion of the crista to the point of contact with neighboring cristae above and below. The configuration of the crista formed by this expansion of the connecting sections between vesicles is defined as the aggregated configuration. In this configuration the intracristal space is maximal and the matrix space is minimal.

We have deliberately considered the orthodox to aggregated transition, as illustrated first in mitochondria *in situ*, because in these mitochondria the original orderly alignment of the cristae in the orthodox configuration makes it possible to easily visualize the essential features of the ultrastructural change, because the transition occurs orderly. When adrenal cortex mitochondria are isolated in a sucrose medium (STE), however, the transition of the cristae from the orthodox to the aggregated configuration appears to result in a chaotic aggregated configuration (see Fig. 1). This is so because in the sucrose medium used in the isolation the cristae in the orthodox configuration no longer maintain their orderly alignment. Instead of one crista neatly engaging only its neighbors above and below, the cristae now engage indiscriminately with many cristae, resulting in what appears to be a featureless network. The formation of aggregated cristae in isolated adrenal cortex mitochondria as well as in isolated liver mitochondria leads to what Hackenbrock aptly described as the condensed state.[7] The important feature to notice is that the extensive electron-transparent spaces (unstained) which fill the interior of mitochondria in this aggregated or condensed state are the expanded lumens of the cristae. The darkly staining regions are the areas of engagement of the headpiece-stalk sectors extending from the repeating units of the apposed cristae.

Discussion

This new ultrastructural analysis of the orthodox configuration clearly establishes that the inner membrane of adrenal cortex mitochondria is not unique in having cristal membrane as free-floating vesicles, but is isomorphic with the inner membrane of mitochondria generally. The curious appearance of the orthodox configuration derives from the tubularity of the cristae and the bimodal (scalloped) nature of the tubular cristae. The scalloped tubular character of the cristal membranes of adrenal cortex mitochondria is unusual among mitochondria. What accounts for the unusual structural idiosyncracies of the cristae of adrenal cortex mitochondria? It is a reasonable presumption that the repeating units in the ballooned-out sections of the tubular cristae are *different* from the repeating units in the squeezed-down sections. Otherwise there would be no basis for the scalloped structure. It could be argued that the interior of the ballooned-out sections contains structured material that prevents close basepiece-to-basepiece apposition; however, the fact that the interior of these sections is electron transparent does not support such a postulate. Another possibility for the scalloped nature of the cristae is suggested by the enzymatic functions observed in adrenal cortex mitochondria. It has been well established that adrenal cortex mitochondria, as well as other mitochondria with steroidogenic activity, contain two kinds of electron transfer chains:[20-23] (a) the classical chain which couples electron transfer to synthesis of ATP, and (b) a steroidogenic electron transfer chain containing P_{450}, a cytochrome species with unique absorbing properties. We are suggesting that this enzymatic bifunctionality underlies the bimodal structure of the cristae of adrenal cortex mitochondria. The repeating units in the

ballooned-out sections of the cristae presumably contain the steroidogenic electron transfer chain containing P_{450}, while the repeating units in the collapsed sections of the cristae presumably contain the tripartite repeating units which implement oxidative phosphorylation. This assignment is reasonable, since in mitochondria with no steroidogenic activity the cristae have no ballooned-out regions, but are uniform along their length with diameters similar to the diameter of the squeezed-down sections. Since steroidogenesis is maximal in the orthodox configuration of the cristae while coupled phosphorylation is maximal in the aggregated configuration, the orthodox-aggregated transition would play a key role in controlling whether steroidogenesis or coupled phosphorylation will take place at a given time.[2,3] Further communications in this series will attempt to correlate in depth the structure-function relationships alluded to here.

Acknowledgements

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