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Conformational Basis of Energy Transduction in Membrane Systems
VIII. Configurational Changes of Mitochondria
In Situ and *In Vitro*

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Abstract

A method has been devised for the study of configurational changes in mitochondria *in situ* during the transition from nonenergized to energized conditions. The method depends upon the following component features: (a) subdivision of the tissue into finely diced sections; (b) the use of a modified Krebs-Ringer phosphate solution as the suspending medium; (c) aerobic conditions as the tactic for imposing the energized state; (d) anaerobic conditions or the presence of uncoupler under aerobic conditions as the tactic for imposing the nonenergized state; and (e) rapid fixation of the diced sections by addition of a mixture of formaldehyde and glutaraldehyde at a controlled temperature. Regardless of the tissue of source (heart, liver, skeletal muscle, retina, kidney) or the species (beef, rat, canary), all mitochondria show unambiguous configurational changes during the transition from nonenergized to energized conditions. The present study has revealed various optional features of the configurational states. Thus, there are two nonenergized configurations of the crista—orthodox and aggregated. The osmotic pressure of the suspending medium determines which nonenergized configuration will be observed. There are at least two variant forms of the energized-twisted configuration—tubular and zigzag. Again the osmotic pressure of the medium is an important factor in determining the form of the crista in the energized-twisted configuration. Mitochondria, such as those of heart muscle with relatively little matrix protein, show the clearest and most regular configurational changes, whereas mitochondria, such as those of liver with an abundance of matrix protein, show a more complex and less regular pattern of configurational change. From this comparative study of mitochondria *in situ*, it can be concluded that no exceptions have been found to the generalization that changes in configurational state of the cristae accompany changes in the energy state; this exact correlation provides additional support for the hypothesis of the conformational basis of energy transduction in the mitochondrion.

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Introduction

In previous communications of this series, evidence was presented that configurational changes in beef heart mitochondria correlate with changes in the energy state.[1–4] These correlative studies provided the experimental foundation for the hypothesis of the conformational basis of energy transduction in the mitochondrion. It follows from this hypothesis, that all mitochondria, regardless of source, and whether *in situ* or *in vitro*, should show the same correlation between configurational change and the change in energy state. The present communication is addressed primarily to presenting the evidence that mitochondria *in situ* undergo configurational changes that parallel the energy states. By *in situ* we do not imply the whole animal, but rather the intact cell in fine minces of excised tissue. Thus, we are comparing configurational changes in mitochondria separated from the cell and other organelles, *in vitro*, with configurational changes in mitochondria in their normal milieu and relationship to other organelles within intact cells.

The present study has turned out to be more than a necessary check of theory. Some unexpected features of the configurational changes which mitochondria *in situ* undergo have come to light; this new information has helped to clarify several puzzling aspects of configurational changes in mitochondria.

It was not only necessary to demonstrate configurational changes *in situ*[5] but it was also necessary to relate the configurational states observed in mitochondria *in situ* to those observed in mitochondria *in vitro*. Our studies[1–4] with isolated beef heart mitochondria have shown that there are three configurational states, nonenergized, energized, and energized-twisted. Electron transfer or hydrolysis of adenosine triphosphate (ATP)* can initiate the transition from the nonenergized to the energized configuration.[3] The addition of inorganic phosphate to energized mitochondria initiates the transition from energized to the energized-twisted.[3] The binding of phosphate by the cristal membrane is an integral part of the transition from the energized to the energized-twisted configuration.[6]

We have become aware, from our studies with isolated beef heart mitochondria (suspended in 0·25 M sucrose), that there are two alternative forms of the nonenergized configuration.[30] The orthodox configuration can be induced by relatively high concentrations of an uncoupler such as *m*-ClCCP[3] or of endotoxin of *Bordetella bronchiseptica*,[30] whereas the aggregated configuration is the normal configuration in the sucrose medium. The orthodox configuration of the crista is nonenergized and is characterized by a maximally contracted intracristal space or lumen and a maximally expanded matrix space. Since this configuration is almost invariably the one observed in mitochondria when the tissues are fixed by standard methods, the term orthodox is generally used for its description. In fact, to some electron microscopists, the orthodox configuration is an assurance that the tissue has been properly fixed.[7–9] The aggregated configuration of isolated mitochondria is also nonenergized and is characterized by a maximally expanded intracristal space or lumen of the crista and a maximally contracted matrix space. The orthodox to aggregated transition in isolated mitochondria is independent of the energizing cycle and is related to the osmotic pressure exerted by sucrose in the suspending medium.[20]

*Abbreviations used: DNP, 2,4-dinitrophenol; KRP, Krebs-Ringer phosphate solution. ATP, adenosine triphosphate; *m*-ClCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

From our studies on configurational changes in isolated rat heart mitochondria we have become aware of two alternative expressions of the energized-twisted configuration—the zigzag mode and the tubular mode.[3] In isolated rat heart mitochondria both modes are found in the same mitochondrion—an indication of an equilibrium between the two modes. In isolated beef heart mitochondria only the tubular mode of the energized-twisted configuration was found.[1–3]

Since, with very few exceptions, electron microscopists were not concerned with a correlative study, they processed the excised tissues in such a way that anaerobiosis was inevitable by the time the fixative was added to the specimen. This accounts for the fact that the vast majority of electron micrographs of mitochondria *in situ* show the cristae in the nonenergized, orthodox configuration. However, a few investigators, notably Fernandez-Moran,[10] Revel *et al.*,[11] Luft *et al.*,[12] Stenger and Spiro,[13] Slautterback,[14] Fawcett,[15] and Fawcett and McNutt,[16] took precautions with respect to the speed of processing the excised tissues or to the speed of fixation or to both. All these investigators recognized unusual configurational patterns of the cristal membrane. While they were unable to provide a causal explanation for these patterns, nonetheless, they were convinced that these patterns could not be dismissed as artefacts of fixation. Revel *et al.*[11] were particularly insistent on this point. The fact that the cristae of the mitochondria in the skeletal muscle of a hypermetabolic patient were consistently in the zigzag configuration led Luft *et al.*[12] to postulate some correlation between the disease state and the unusual configurational pattern of the mitochondrial cristae. The experiments of Slautterback[14] on the configurational patterns of canary heart mitochondria (rapidly fixed by injection of an osmium tetroxide solution into the beating heart) established the principle of multiple domains within a single mitochondrion—each domain representing sets of cristae in the identical configurational state be it zigzag or vesicular or orthodox. The fact of domains within a single mitochondrion provided the most elegant proof that the variation in configurational pattern could not be attributed to artefacts of fixation. Why would the artefactual pattern be observed in one domain and not in another adjacent to it?

Materials and Methods

Preparation of Mitochondria

Rat and canary heart mitochondria were prepared by the procedure described by Green *et al.*[3] Rat liver mitochondria were prepared by the method of Schneider.[17]

Preparation of Tissues

The heart, liver, and kidney of the rat (and also the heart and flight muscle of the canary) were rapidly removed from an animal anesthetized by cervical dislocation. The excised tissues were placed in ice-cold Krebs-Ringer phosphate (KRP) solution. The KRP solution was Ca^{2+}-free, 0·123 M in NaCl, 5 mM in KCl, 1 mM in $MgSO_4$, and 16 mM in sodium phosphate (pH 7·4). Rat heart ventricular tissue was chopped into very small pieces with a razor blade on a block of dental wax. Rat liver tissue was selected from the area near the edge of the lobe. Rat kidney tissue was selected from the cortical region. Only the ventricles of the canary heart were used. Canary flight muscle was excised from near the sternum bone. Each tissue was finely chopped, washed

with ice-cold KRP, and then transferred to the incubation flask. There was a time lapse of 1–2 min between removal of a specific tissue and the introduction of the minced, washed tissue into the flasks in which the experiment was carried out. One sample of each batch of chopped tissue was fixed with ice-cold glutaraldehyde–formaldehyde fixative without any further treatment. This zero-time sample served as a reference point for the treated samples.

Retinal tissue was dissected from bovine eyes obtained at an abattoir. The eyes were collected immediately after the death of the animal, wrapped in aluminum foil, placed on ice, and rapidly transported to our laboratory. There was a time lapse of 35–40 min between collection of the eyes and initiation of the experiments. A KRP solution fortified with 20 mM sucrose and 1 mM ascorbic acid was used as the basic medium for the experiments with retinal tissue.

Incubation of Tissues

All tissue suspensions were incubated at 20° for 3 min with 100% oxygen bubbling rapidly through the medium. The smallest pieces of chopped tissue (less than 1 mm in any dimension) were selected for the final embedding procedure. No apparent differences were noted in the appearance of mitochondria near the edge of the tissue block or deep inside the block.

Rat and Canary Tissues

The basic incubation medium was the KRP solution (6 ml) to which was added rotenone (17 μg/ml), rutamycin (67 μg/ml), and sodium iodoacetate (1 mM). The tissues were divided into three experimental groups—the first was fixed immediately without any incubation, the second was incubated with 10 mM sodium succinate as substrate, and the third was incubated with 10 mM sodium succinate as substrate in presence of 0·4 mM 2,4-dinitrophenol.

Bovine Retina

The incubation medium for bovine retina was the KRP solution fortified with 20 mM sucrose (to increase osmolarity) and 1 mM ascorbic acid (normal constituent of vitreous humor). Rotenone (17 μg/ml) was added as an inhibitor of DPNH-linked substrates. When substrate (sodium succinate) was added, the concentration was 10 mM; and when uncoupler (dinitrophenol) was added, the concentration was 0·4 mM.

Fixation of Tissue Samples

All the tissues were fixed by pouring 6 ml of ice-cold fixative into the incubation flask; aeration of the suspension with oxygen gas was continued for several minutes after the fixative was added. The fixed samples were then placed on ice for 1 h. The rat and canary tissues were fixed with KRP solution supplemented with 2% by weight of glutaraldehyde and formaldehyde. The bovine retinal tissue was fixed with KRP solution supplemented as above with glutaraldehyde and formaldehyde, and fortified with 20 mM sucrose and 1 mM ascorbic acid.

Preparation of Tissues for Electron Microscopy

All the tissues were washed with the Ca^{2+}-free KRP solution overnight in the refrigerator. They were stained with 2% osmic acid in KRP for 1 h at 3°. The tissues were

rinsed twice with KRP solution after the osmic acid staining. They were then stained with 1% uranyl acetate in 25% ethanol for 90 min at 3°. The uranyl acetate solution was removed and the tissues were processed slowly through a graded series of ethanol solutions and then with dry propylene oxide before embedding in Epon. The embedded tissues were sectioned with a glass or diamond knife on a Porter Blum ultramicrotome model MT-2. The sections were mounted on carbon-covered grids, stained with lead citrate, and examined in a Hitachi HU 11B electron microscope at 75 KV. We have examined 1250 electron micrographs from forty *in situ* experiments and all of these are consistent with the particular electron micrographs selected for demonstration purposes.

Rationale of the Incubation Conditions

The KRP medium in which the various tissue minces were suspended approximates fairly closely the composition and osmotic pressure of the extracellular fluid as judged by two criteria—the absence of mitochondrial swelling; and the preservation of membrane ultrastructure. The design of the standard experiments was to determine electron microscopically the configuration of the mitochondria (a) immediately before the incubation was started; (b) after aerobic incubation in presence of substrate; and (c) after aerobic incubation in presence of substrate and uncoupler. When carbon monoxide gas was bubbled through the suspension (this led to anaerobiosis and inhibition of electron transfer) the configuration of the mitochondria was orthodox; i.e., identical with that in the mitochondria of samples incubated aerobically with substrate in the presence of uncoupler. The nonenergized configuration (orthodox) can be achieved either by imposing anaerobic conditions or by adding uncoupler under aerobic conditions.

Various inhibitors were added to suppress processes that could complicate the results. Since succinate was the standard electron donor, the presence of rotenone in the medium ensured the suppression of electron transfer via endogenous DPNH-linked substrates. Rutamycin blocked coupled ATP synthesis and energizing of the mitochondrial membrane by ATP hydrolysis; its presence in the medium was yet another way of increasing the stability of the energized state once generated. Iodoacetate was added to the medium to suppress glycolysis. This reagent prevented the glycolytic synthesis of ATP and it eliminated the possibility of ATP acting as an energy source for energizing the mitochondrial membrane. In general, the presence of these reagents increased the uniformity of the mitochondrial population with respect to the energized configurations, but any one or all of them could be left out of the incubation medium without any gross effect in a particular experiment as long as the tissue was incubated aerobically.

The incubation was carried out for 3 min at 20°. The temperature of 20° was chosen because the rate of turnover of the energized state of the mitochondria was slower, and the speed of fixation was apparently faster than the speed with which the energized configuration could be spontaneously discharged. At higher incubation temperature (e.g., at 37°) and with warm fixative (e.g., at 25°), the discharge of the energized state seemed to be faster than the speed of fixation. The net result was always the orthodox configuration. For this reason we lowered the incubation temperature to 20° and used an ice-cold fixative solution.

We have used a combination of glutaraldehyde and formaldehyde for purposes of fixation. This combination, under our conditions, appears to be eminently suited for preserving the configurational states present at the moment of fixation.

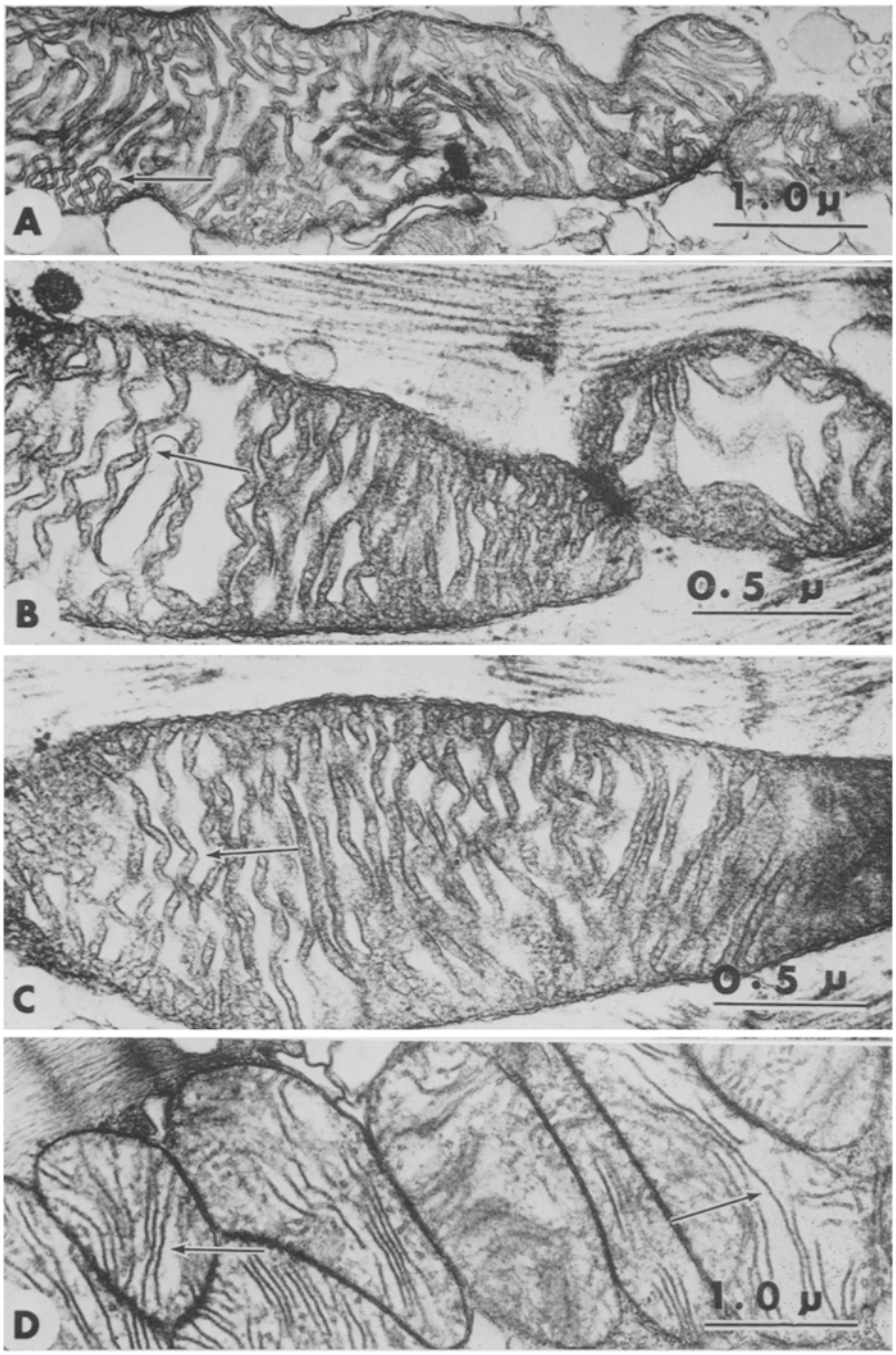


Figure 1. Configurational states of canary heart muscle mitochondria *in situ*. The mitochondria shown *in situ* in electron micrographs A, B, and C were incubated in KRP with substrate plus oxygen. The arrows point to particularly good examples of the cristal membrane in the energized-twisted configuration. The mitochondria shown *in situ* in electron micrograph D were incubated in KRP with substrate, oxygen, and the uncoupler, DNP. The arrows of electron micrograph D point to cristal membranes in the orthodox configuration.

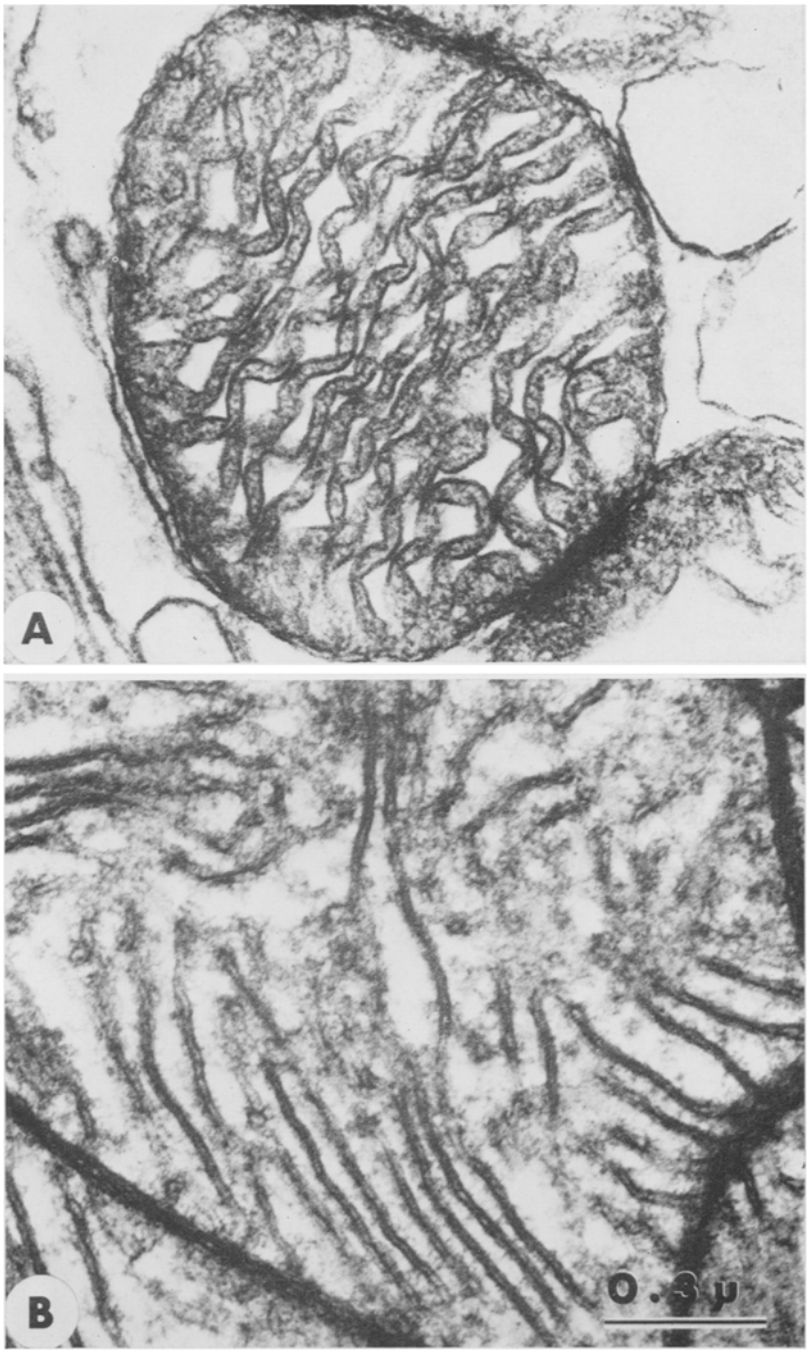
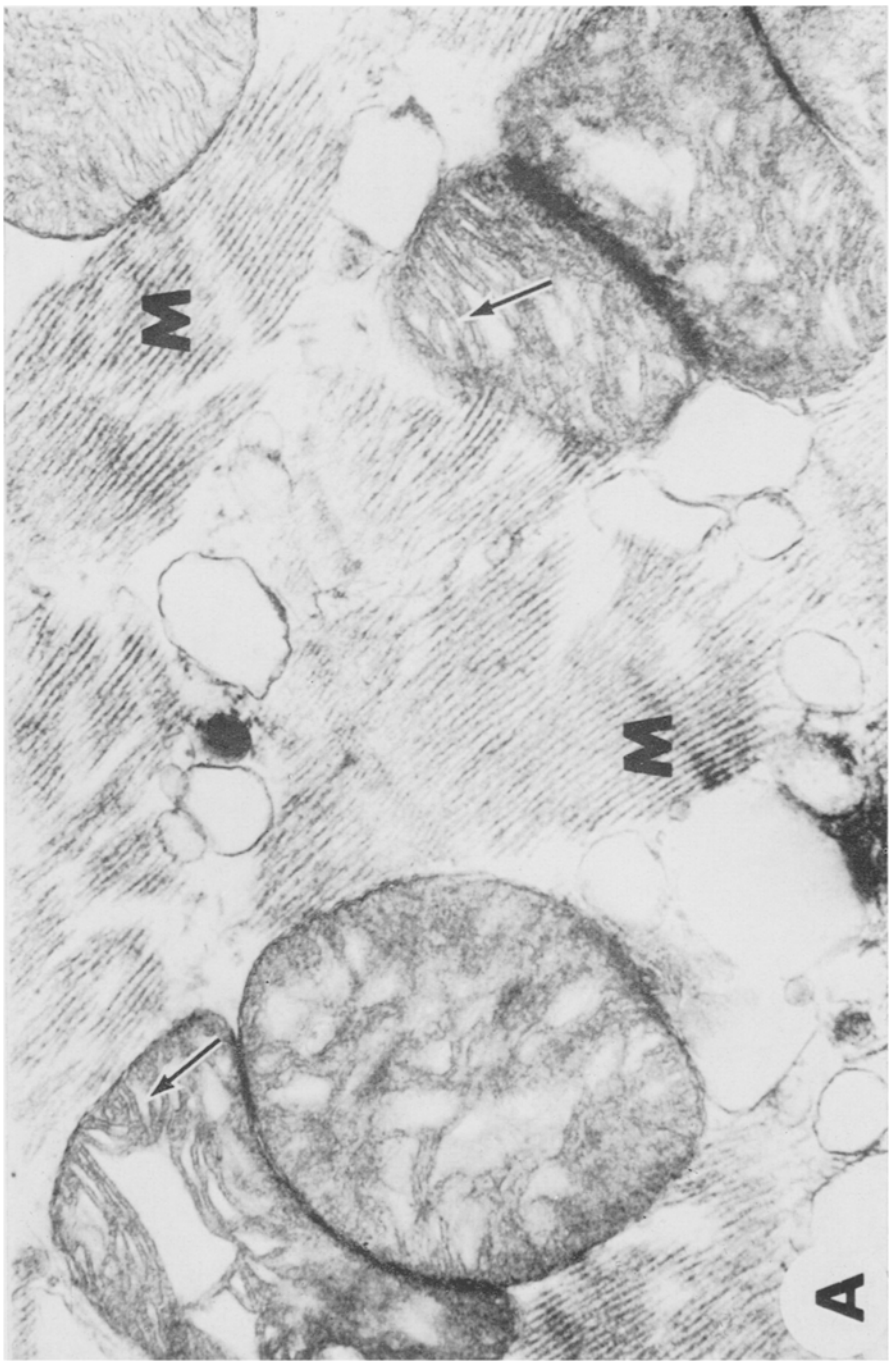


Figure 2. Configurational states of canary heart muscle mitochondria *in situ* at high magnification. The mitochondrion shown in electron micrograph A was incubated in KRP with substrate plus oxygen. The energized-twisted configuration of the cristal membrane is clearly shown in this specimen. The mitochondria shown in electron micrograph B were incubated in KRP with substrate, oxygen, and the uncoupler, DNP. The orthodox configuration of the cristal membrane is evident.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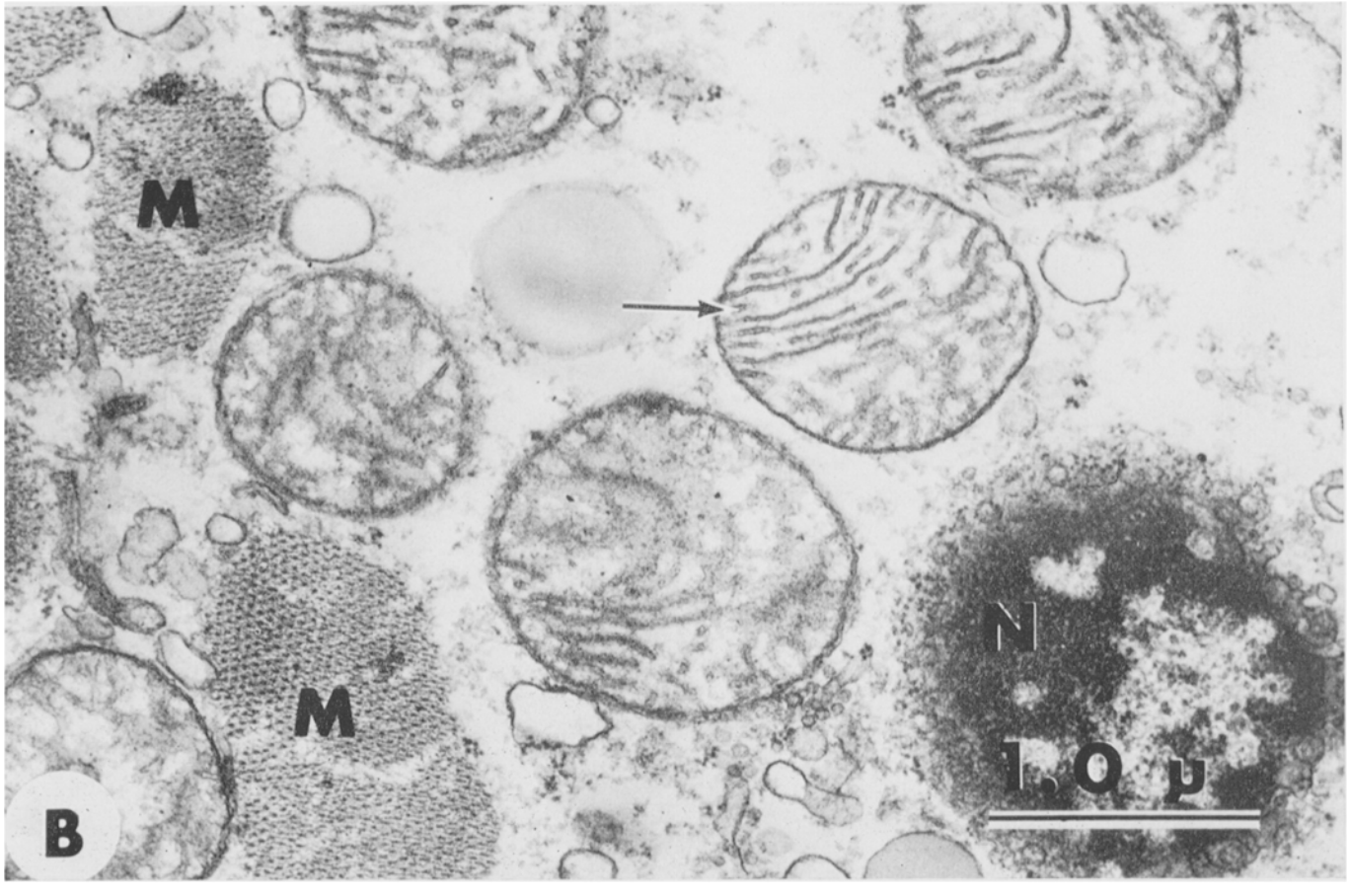


Figure 3. Configurational states of canary flight muscle mitochondria *in situ*. The mitochondria in electron micrograph A were incubated in KRP with substrate plus oxygen. The arrows point out the energized cristal membranes. The mitochondria in electron micrograph B were incubated in KRP with substrate, oxygen and the uncoupler, DNP. The arrow points out the typically orthodox cristal membrane. Muscle fibers (M) and a nucleus (N) are present in the electron micrographs.

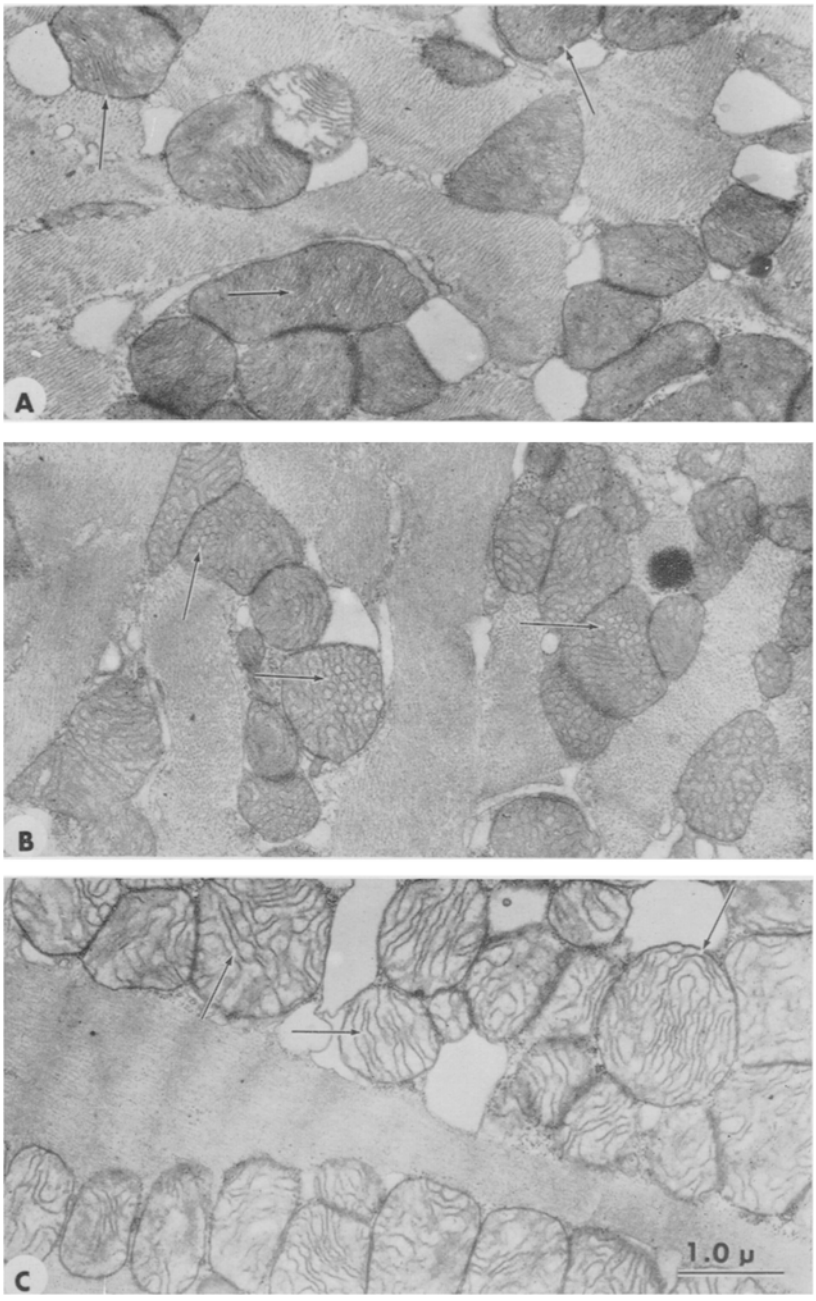


Figure 4. Configurational states of rat heart muscle mitochondria *in situ*. The mitochondria shown in electron micrograph A were fixed immediately without incubation. The arrows are pointing to the cristal membranes "trapped" in a non-orthodox configuration. The mitochondria in electron micrograph B were incubated in KRP with substrate plus oxygen. The arrows are pointing to the "honeycomb" structure of the cristal membrane. This appearance is the result of cutting the tubularized cristal membranes in cross section. The mitochondria in electron micrograph C were incubated in KRP with substrate, oxygen, and the uncoupler, DNP. The arrows are pointing to the typically orthodox cristal membranes.

Results

I. *Transition of Cristae in Mitochondria In Situ from the Nonenergized to the Energized Configurations*

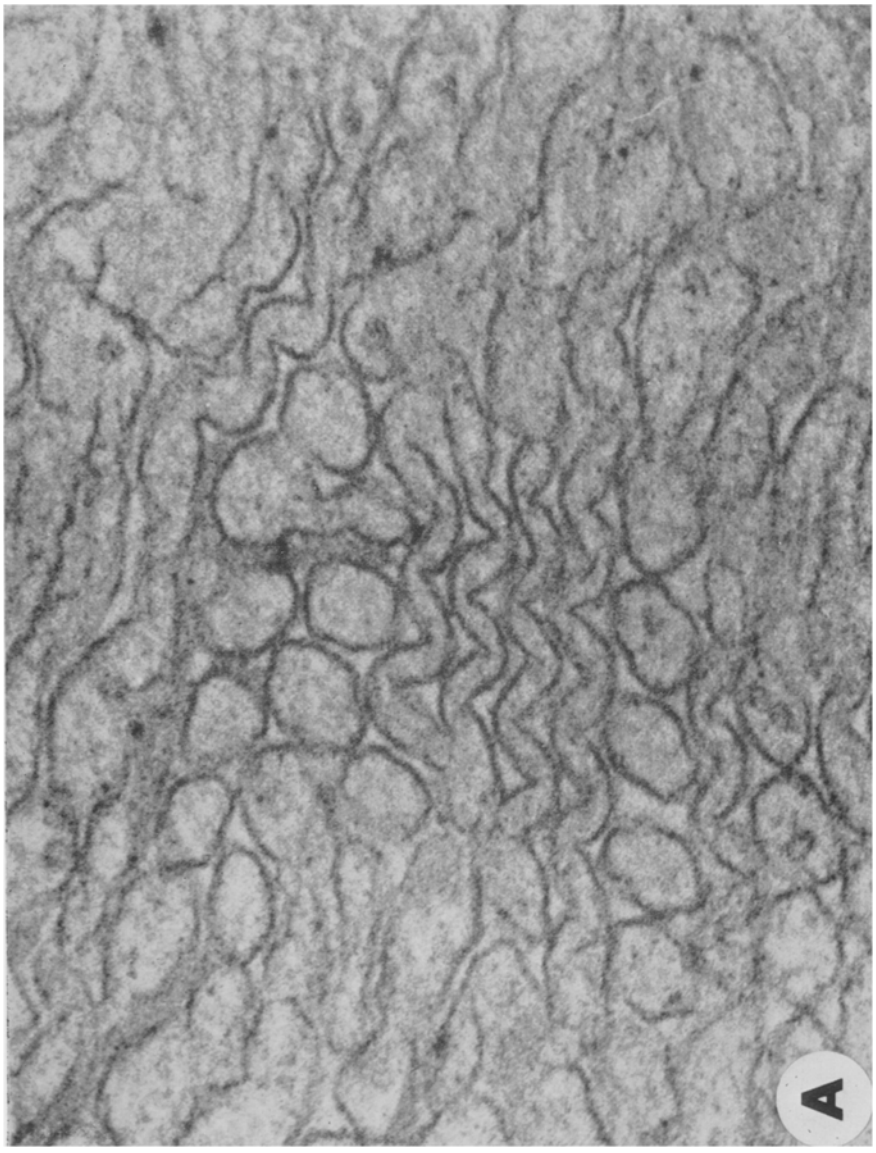
The configurational transitions for mitochondria *in situ* of canary heart muscle, rat heart muscle, rat kidney (convoluted tubular region), rat kidney (perinuclear region), rat liver, and bovine retinal cones are shown respectively in the electron micrographs of Figs. 1–12. Our immediate concern is with the comparison between the configurations in the energized state (aerobic plus uncoupler). In every case the nonenergized configuration of the cristae (uncoupled with DNP) is in the orthodox mode, Fig. 1(D); Fig. 2(B); Fig. 3(B); Fig. 4(C); Fig. 5(B); Fig. 6(C); Fig. 7(B); Fig. 8(B); Fig. 9(C); Fig. 10(B); Fig. 11(B); Fig. 12(B). The intracristal space or lumen of the crista in the orthodox configurational mode is maximally contracted, and the matrix space is maximally expanded. The intracristal space or lumen of the crista is barely visible in heart muscle mitochondria, Figs. 1–5, but is quite definite in rat kidney mitochondria, Fig. 7, and bovine retinal cone mitochondria, Fig. 12. Since the nonenergized configuration is by definition the configuration which was obtained in the presence of dinitrophenol as uncoupler, we may conclude that all of the mitochondria from the different tissues and from the two sources of the same tissue are in the orthodox configurational mode when in the nonenergized state.

Under energizing conditions, all the mitochondria we have examined are clearly in a different configurational state than that obtained in the presence of uncoupler, Fig. 1(A–C); Fig. 2(A); Fig. 3(A); Fig. 4(B); Fig. 5(A); Fig. 6(B); Fig. 7(A); Fig. 8(A); Fig. 9(B); Fig. 10(A); Fig. 11(A); Fig. 12(A). In canary heart and rat heart muscle and in rat kidney convoluted tubules, the energized mitochondria are clearly either in the energized or energized-twisted configuration.

The energized configuration is recognizable by the parallel double membranes that are closely apposed in a head-to-head fashion with what we interpret as densely staining headpiece-stalk sectors between the membranes. The energized-twisted configuration is the same set of parallel membranes with a zigzag character. Rat heart mitochondria show twisted-tubular structures both in longitudinal section and in cross-section Fig. 13(B). The twisted tubules arise by a reverse invagination of the cristal membrane—a process equivalent to forming a glove-shaped structure by poking fingers into an inflated balloon.[18] It should be noted that the two apposed membranes in the energized configurations are the membranes of opposite cristae (the space between is the matrix space) apposed in a head-to-head manner, whereas the two apposed membranes in the orthodox, nonenergized, configuration are the membranes of the same crista (the space between is the intracristal space) apposed in a back-to-back manner.

In rat liver the energized mitochondria are in the condensed configurational mode. It is very difficult to distinguish between the energized and energized-twisted configuration of *in situ* rat liver mitochondria [see Figs. 9(B) and 10(A)]. However, we can clearly see the energized-twisted tubules in isolated rat liver mitochondria and liver tissue exposed to 0·25 M sucrose [see Figs. 14(A) and 17]. The twisted tubules are much larger than those observed in other mitochondria, but this is probably due to the greater amount of matrix proteins contained in liver mitochondria.

Thus, we have two extremes, that of heart mitochondria or kidney convoluted tubule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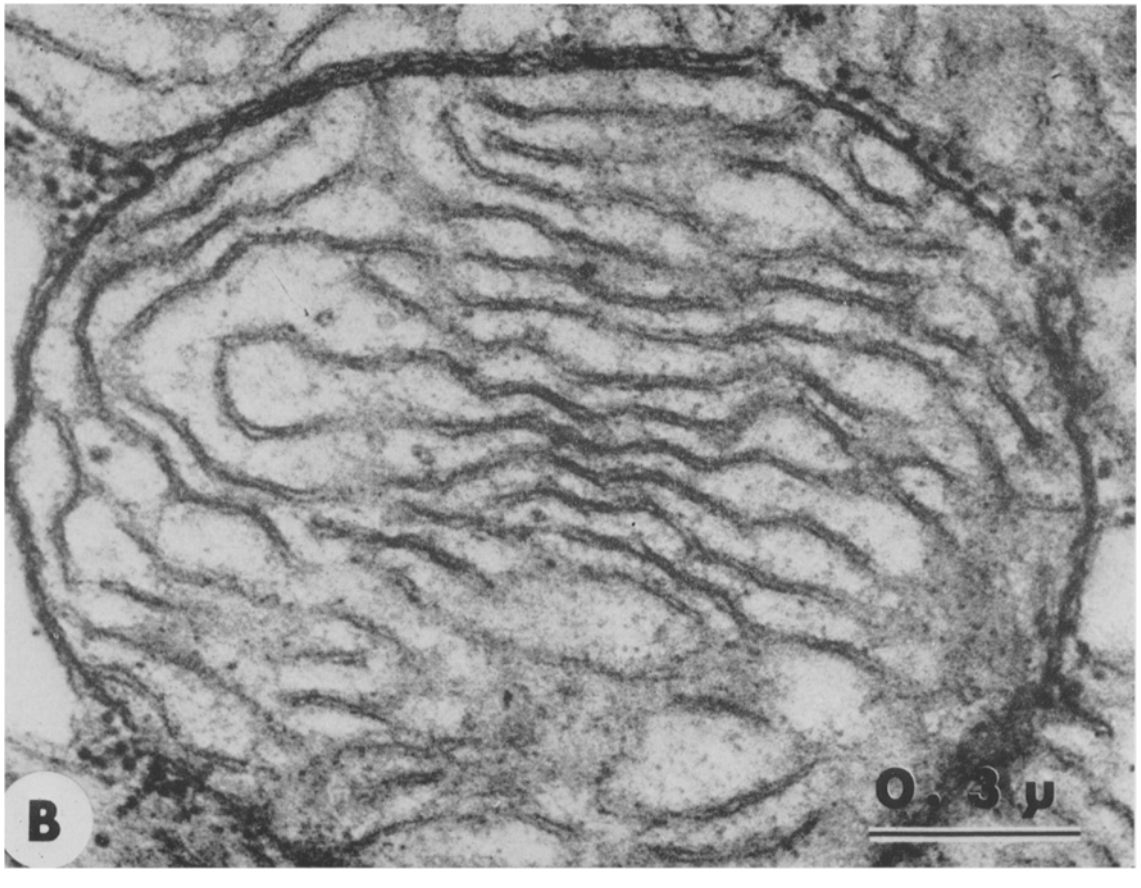


Figure 5. Configurational states of rat heart muscle mitochondria at high magnification. The mitochondrion in electron micrograph A was incubated in KRP with substrate plus oxygen. The energized-twisted tubules in cross-section and longitudinal section are predominant in the center of the micrograph. The mitochondrion in electron micrograph B was incubated in KRP with substrate, oxygen, and the uncoupler, DNP. Orthodox cristae are the dominant feature in this uncoupled mitochondrion.

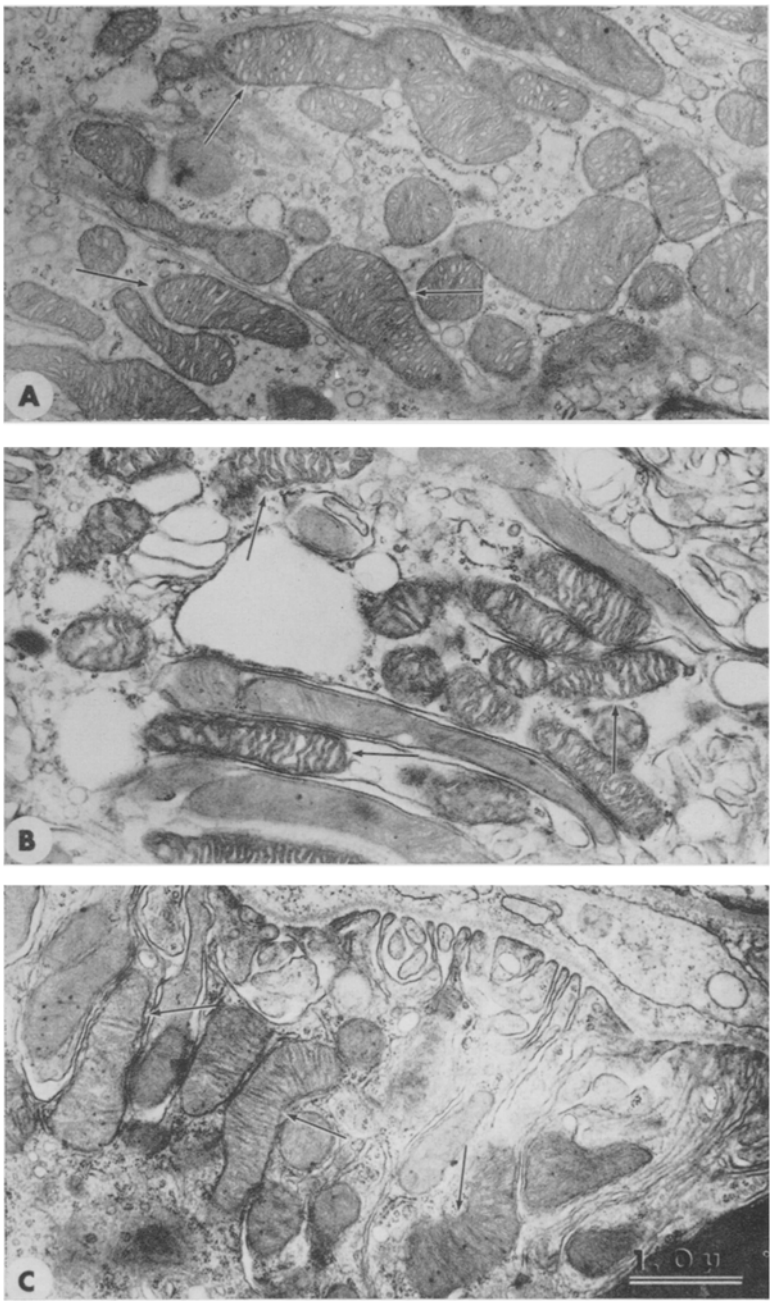


Figure 6. Configurational states of rat kidney convoluted tubule mitochondria *in situ*. The mitochondria in electron micrograph A were fixed without incubation. The arrows point to mitochondria in a non-orthodox configuration. The mitochondria in electron micrograph B were incubated in KRP with substrate plus oxygen. The arrows illustrate mitochondria that are in the energized-twisted configuration with very condensed, zigzag matrix spaces. The mitochondria in electron micrograph B were incubated in KRP with substrate, oxygen, and the uncoupler, DNP. The arrows point out the typically orthodox cristae observed in uncoupled mitochondria.

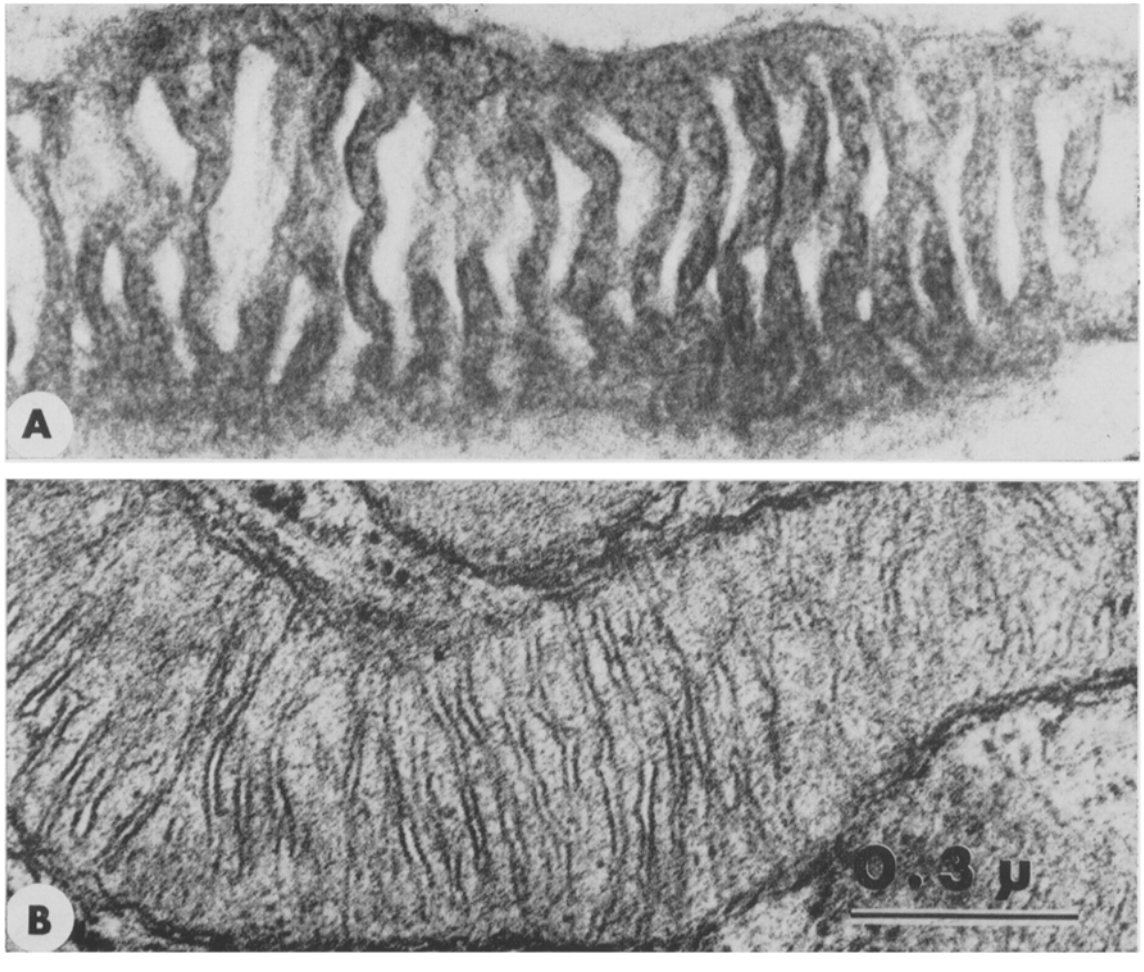
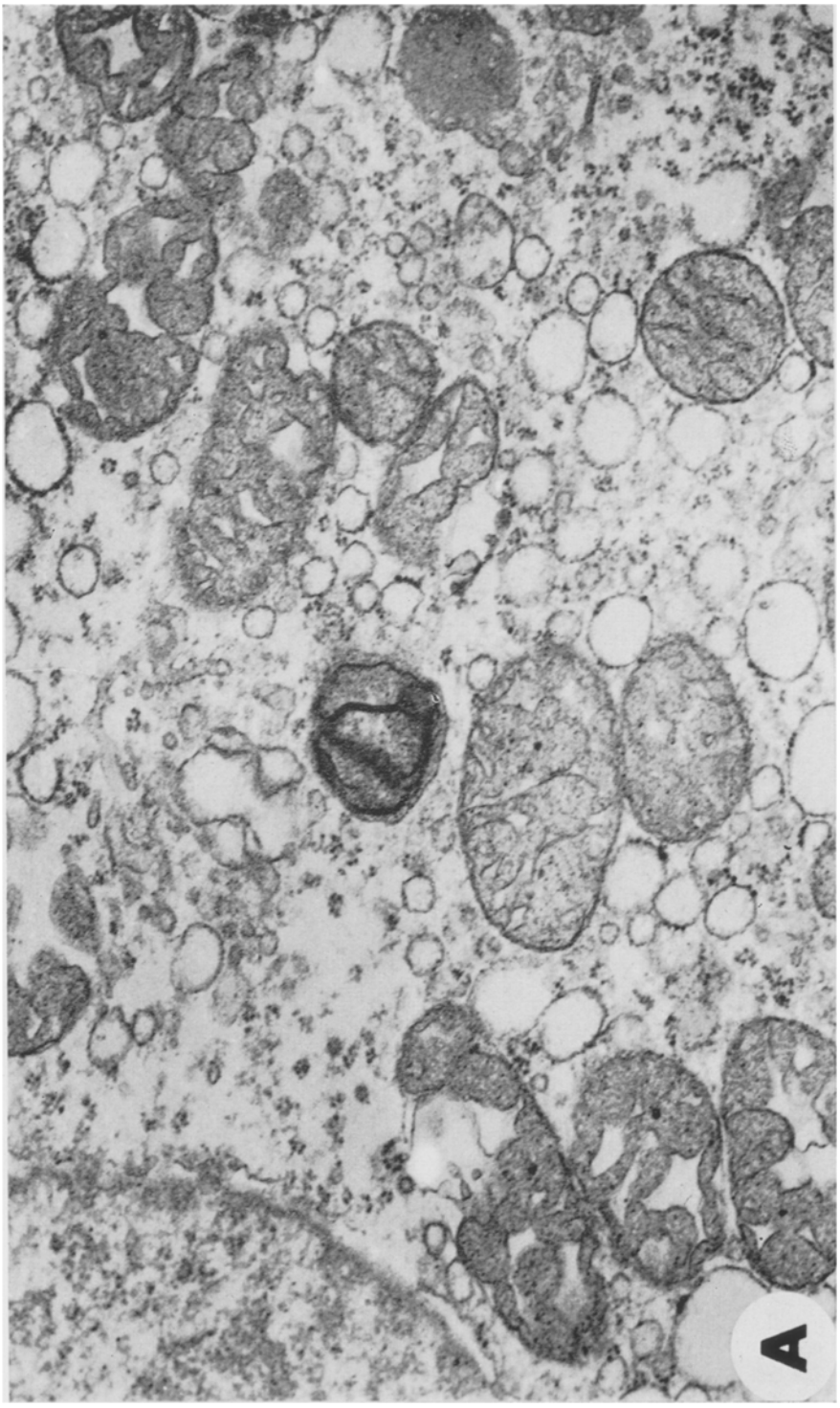


Figure 7. Configurational states of rat kidney convoluted tubule mitochondria at high magnification. The mitochondrion in electron micrograph A was incubated in KRP with addition of substrate plus oxygen. Note the condensed zigzag appearance of the cristae. The mitochondrion in electron micrograph B was incubated in KRP with substrate, oxygen, and the uncoupler, DNP. Note the orthodox configuration of this mitochondrion.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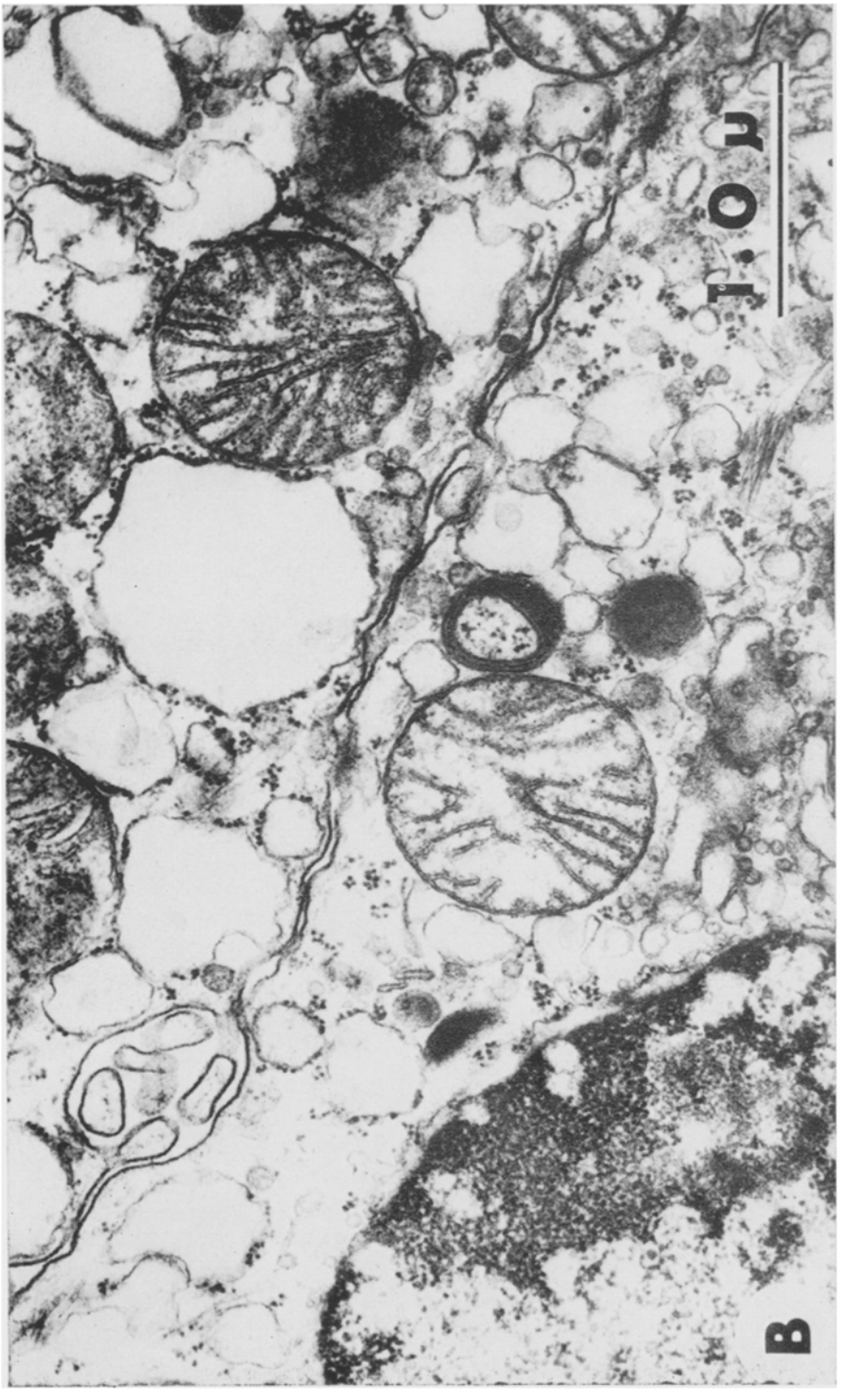


Figure 8. Configurational states of perinuclear rat kidney mitochondria *in situ*. The mitochondria in electron micrograph A were incubated in KRP with substrate plus oxygen. The arrows illustrate the condensed appearance of the cristae of the mitochondria. The mitochondria in electron micrograph B were incubated in KRP with substrate, oxygen, and the uncoupler, DNP. Note the orthodox configuration of *all* the mitochondria in the micrograph.

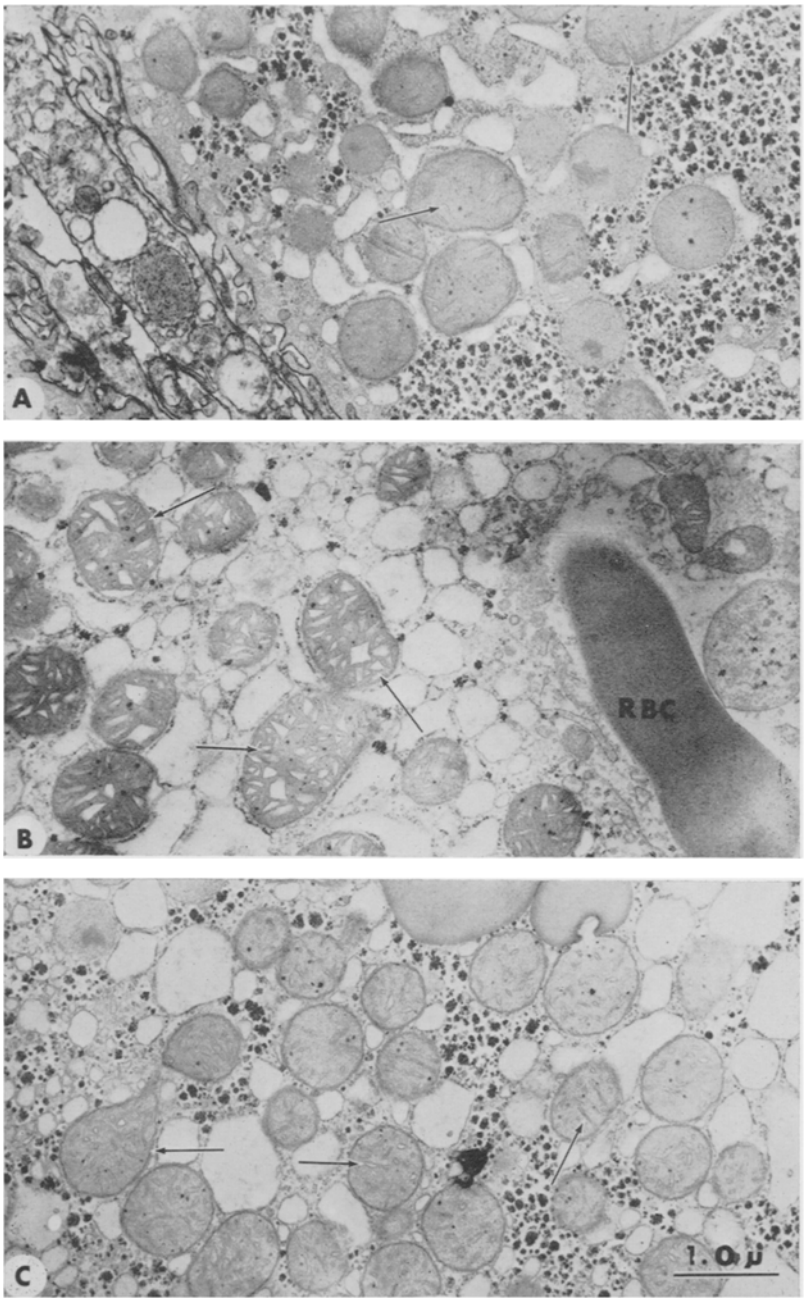


Figure 9. Configurational states of rat liver mitochondria *in situ*. The mitochondria in electron micrograph A, were fixed immediately without incubation. The arrows illustrate the typically orthodox configuration of the cristal membrane. The mitochondria in electron micrograph B were incubated in KRP with substrate plus oxygen. The arrows point to the condensed angular cristae typical of energized liver mitochondria *in situ*. The mitochondria in electron micrograph C were incubated in KRP with substrate, oxygen, and the uncoupler, DNP. Note the uniformity of the orthodox configuration in these uncoupled mitochondria.

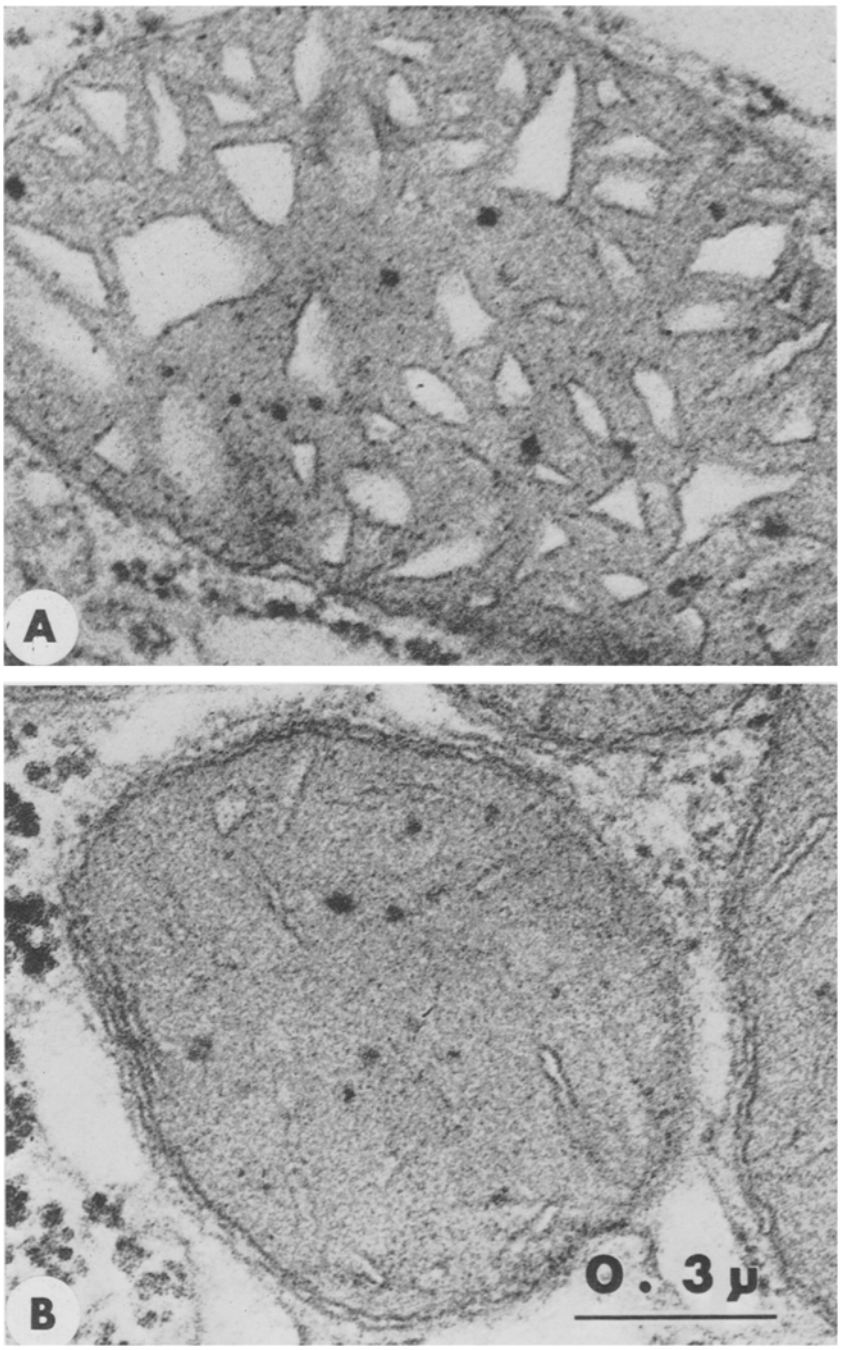
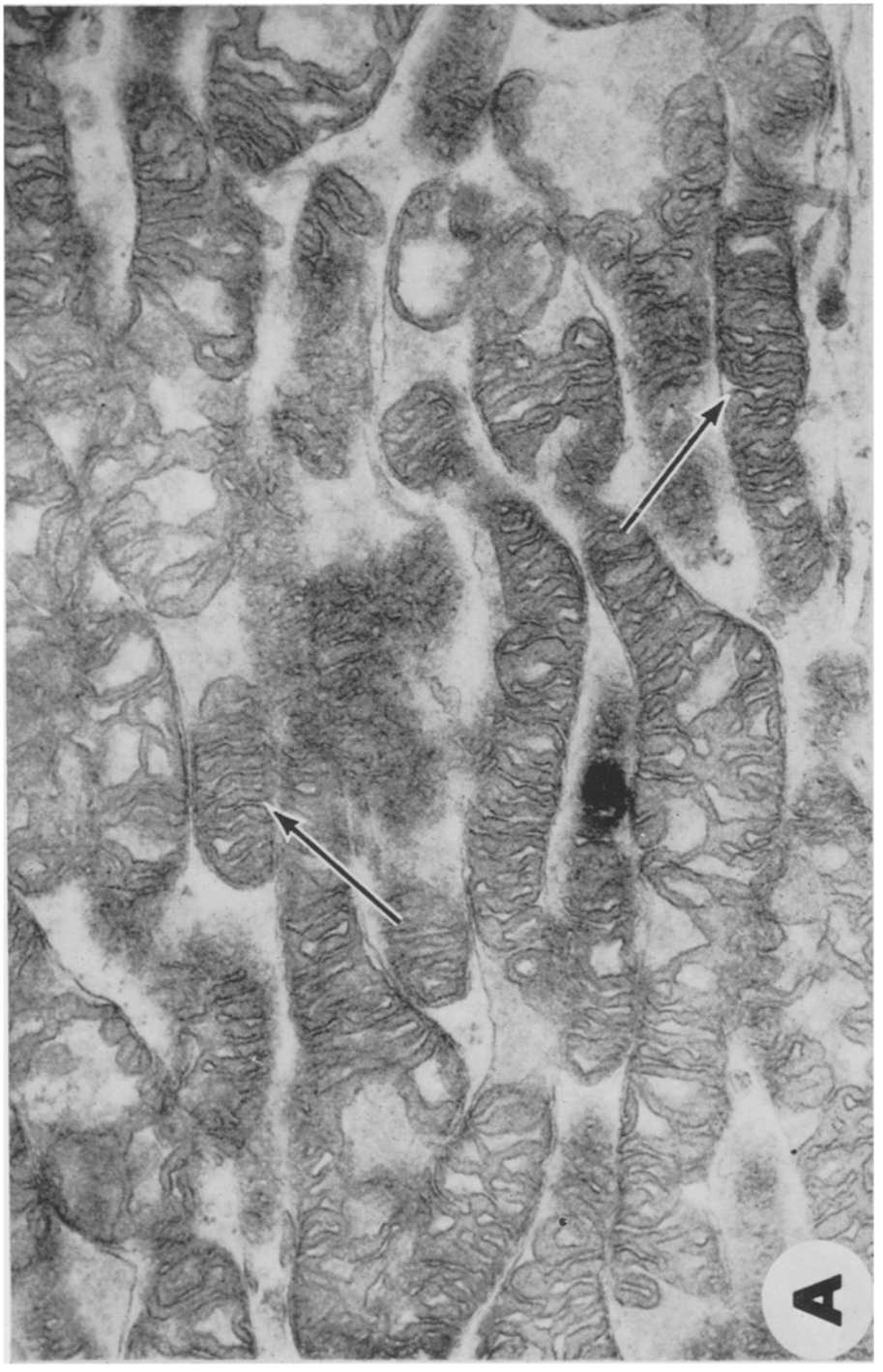


Figure 10. Configurational state of rat liver mitochondria *in situ* at high magnification. The mitochondrion in electron micrograph A was incubated in KRP with substrate plus oxygen. Note the condensed, angular cristal membranes which are representative of liver mitochondria in the energized state. The mitochondrion in electron micrograph B was incubated in KRP with substrate, oxygen, and the uncoupler, DNP.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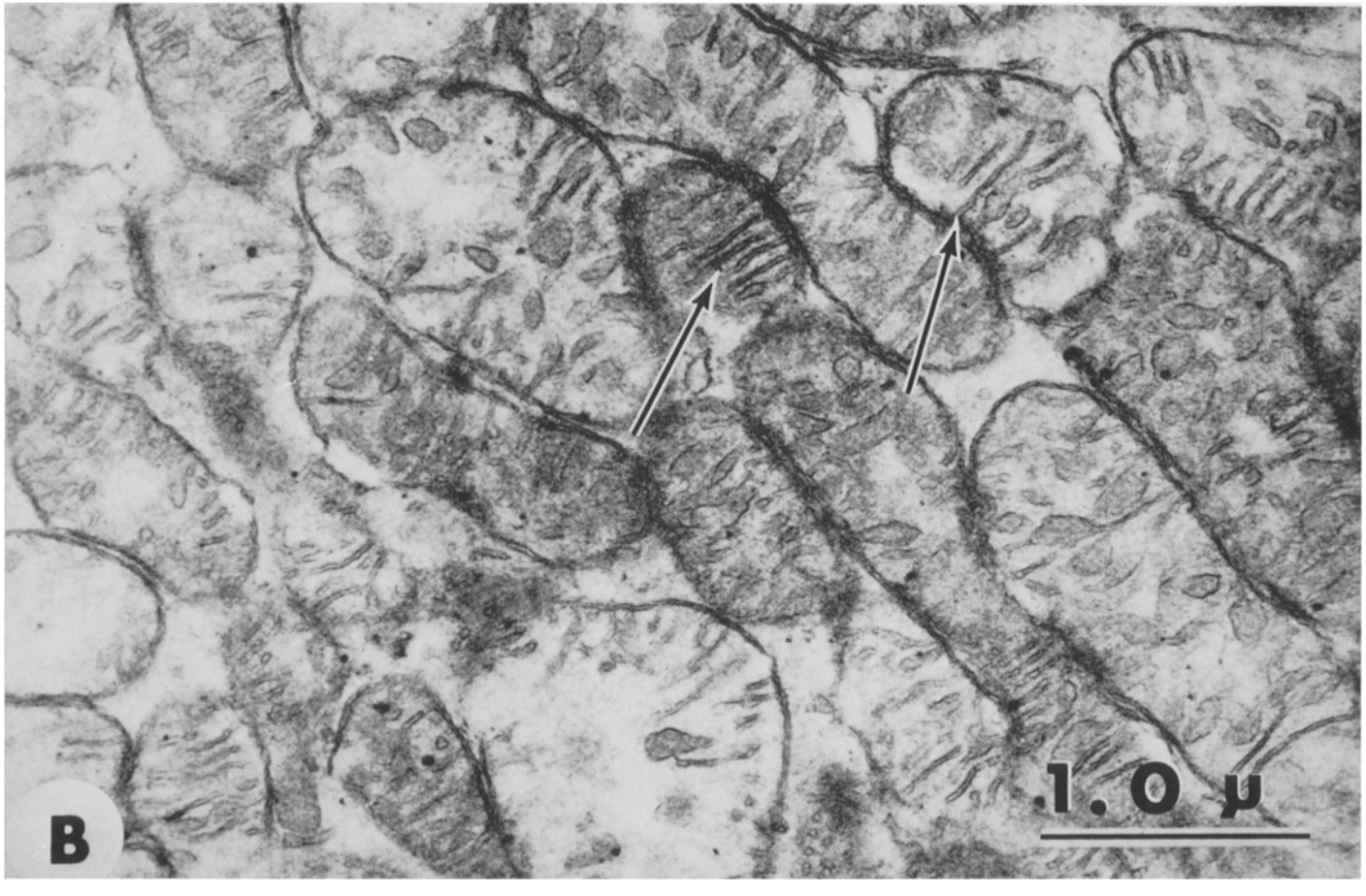


Figure 11. Configurational states of bovine retinal cone mitochondria *in situ*. The mitochondria in electron micrograph A were incubated in modified KRP with substrate plus oxygen. The arrows illustrate the typically energized-twisted configuration of the condensed cristal membranes. The mitochondria in electron micrograph B were incubated in modified KRP with substrate, oxygen, and the uncoupler, DNP. The arrows illustrate typically orthodox cristae observed in these mitochondria.

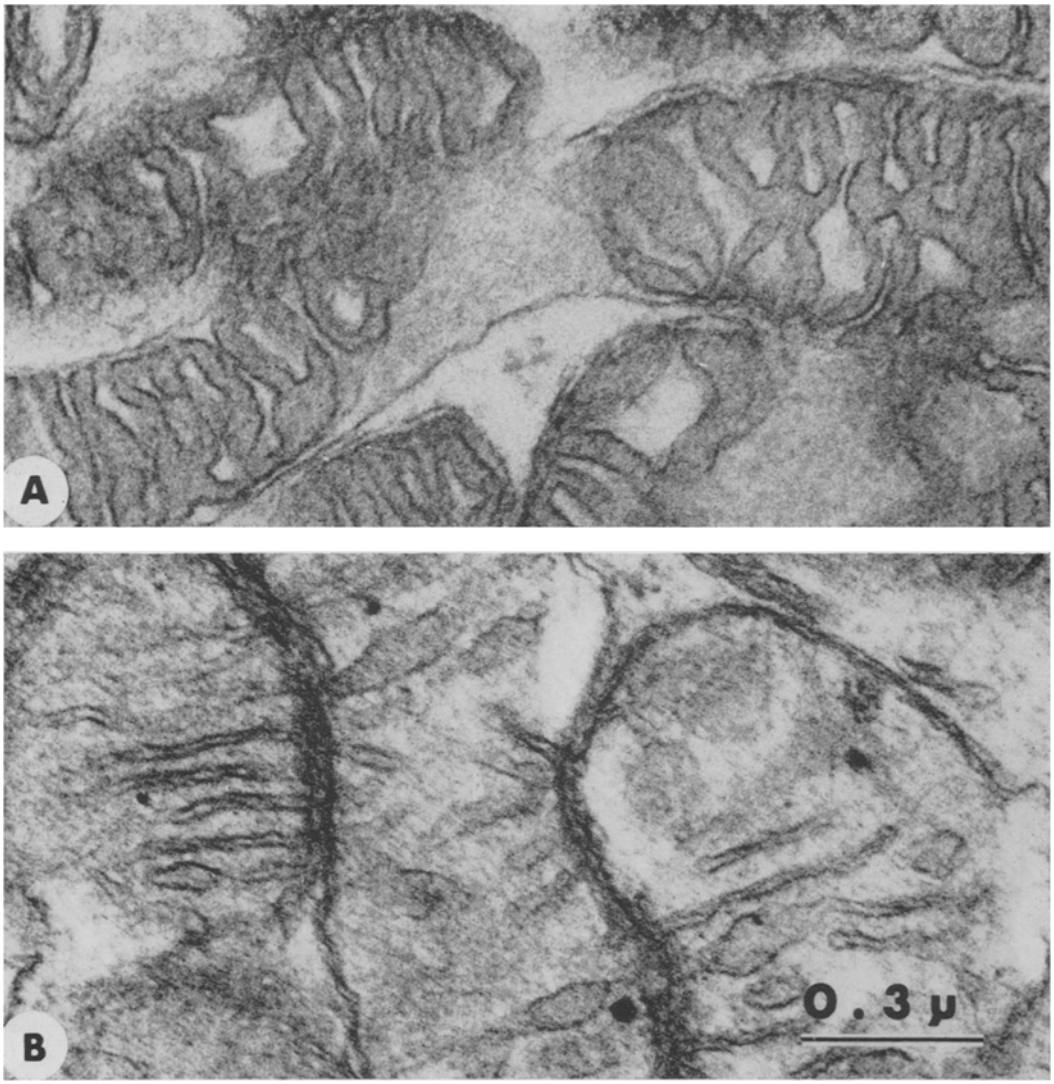


Figure 12. Configurational states of bovine retinal cone mitochondria at high magnification *in situ*. The mitochondria in electron micrograph A were incubated in modified KRP with substrate plus oxygen. Note the energized-twisted configuration of the cristal membrane. The mitochondria in electron micrograph B were incubated in modified KRP with substrate, oxygen, and the uncoupler, DNP. Note the orthodox appearance of the cristal membrane.

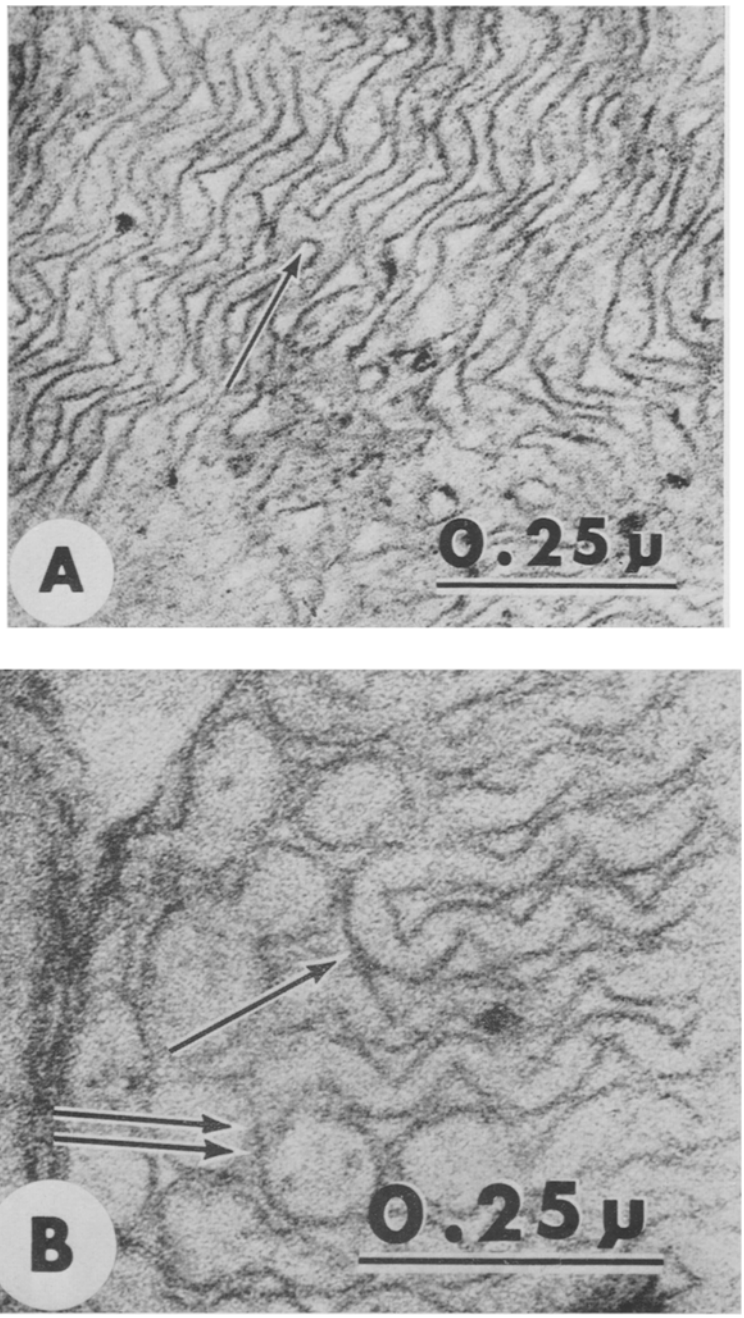
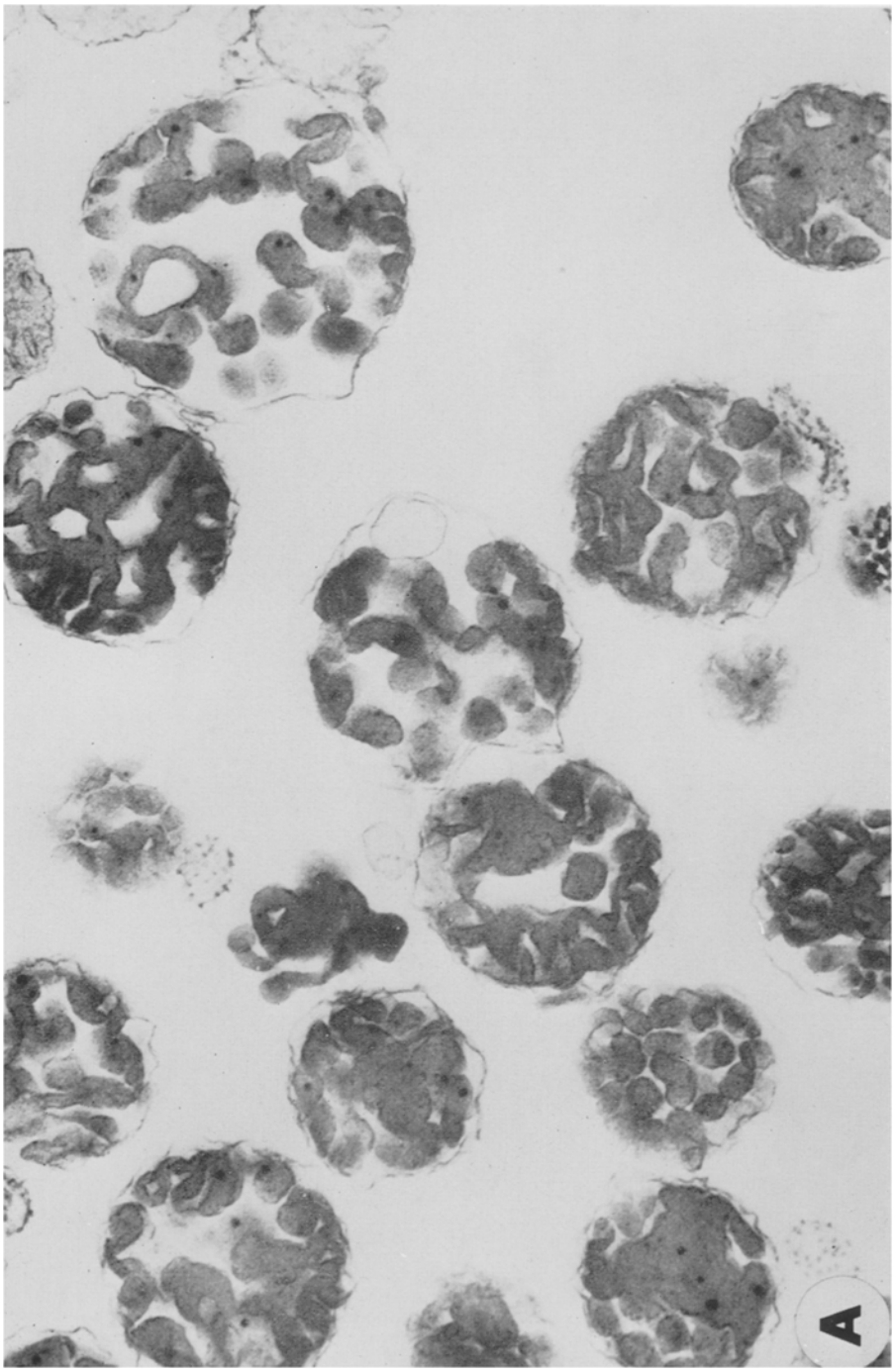


Figure 13. Paracrystalline arrays in heart mitochondria.

(A) Canary heart. The mitochondrion in electron micrograph A is from canary heart muscle incubated in KRP with substrate plus oxygen. Note the zigzag arrangement of the cristal membranes which accounts for the paracrystalline appearance. The arrow illustrates an apparent anastomoses between matrix spaces which is regularly found in canary heart mitochondria.

(B) Rat heart. The mitochondrion in electron micrograph B is from a specimen of rat liver tissue incubated in KRP with substrate plus oxygen. Note the twisted tubular structure of the cristal membranes in longitudinal and cross section. The zigzag arrangement of the cristal membrane with the cross section of a tube (double arrow) forms a structure similar to the Star of David.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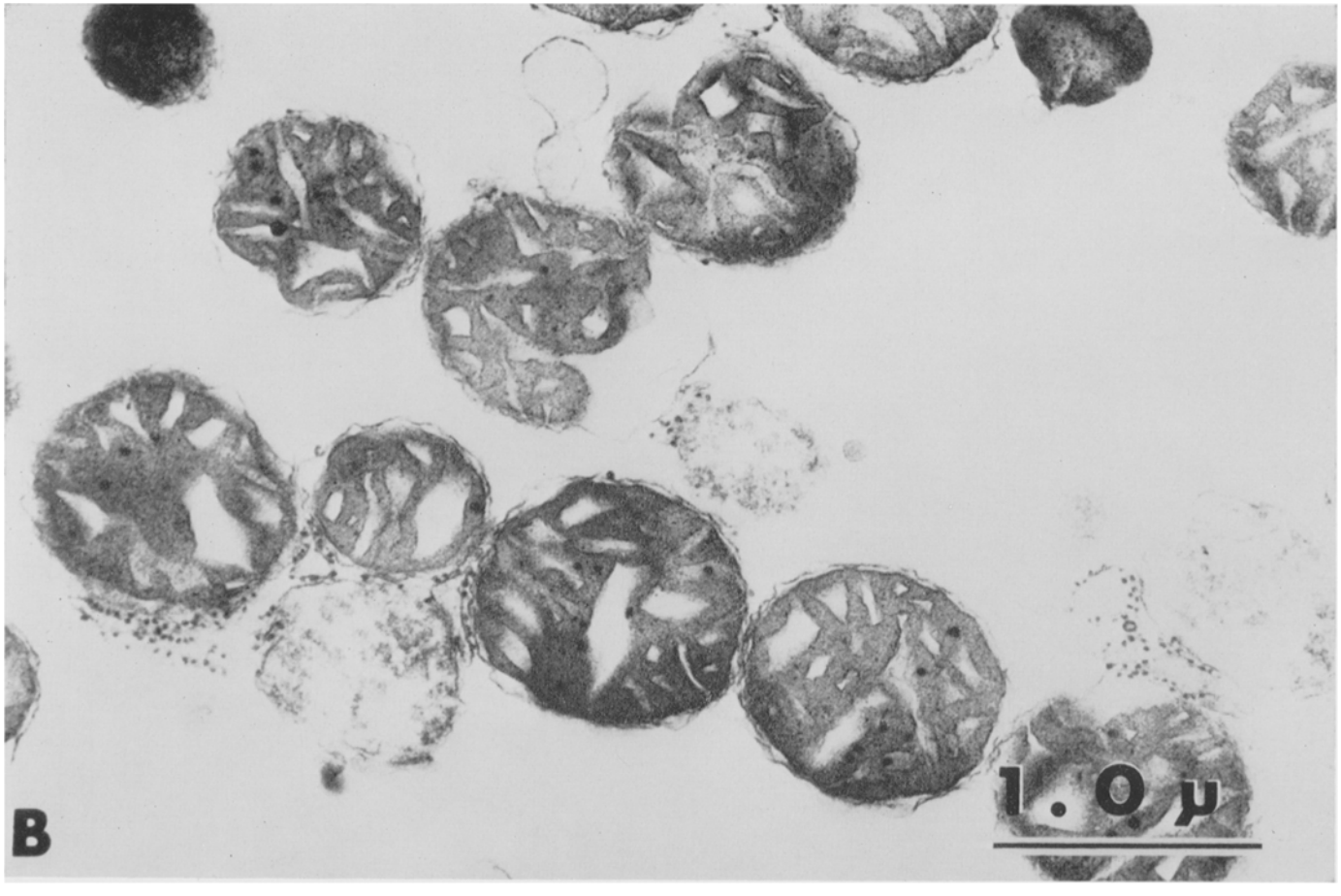

Figure 14. Configurational states of isolated rat liver mitochondria. Electron micrograph A is of rat liver mitochondria incubated in 0·25 M sucrose supplemented with pyruvate plus malate as substrate and with inorganic phosphate and oxygen. Note the condensed, twisted-tubular configuration of the cristal membrane.

Electron micrograph B is of rat liver mitochondria incubated in 0·25 M sucrose supplemented with 5 mM pyruvate plus malate as substrate, 10 mM inorganic phosphate, oxygen, and the respiratory inhibitor (1 μg/mg protein) antimycin. Note the nonenergized, aggregated (condensed) configuration of the cristal membrane.

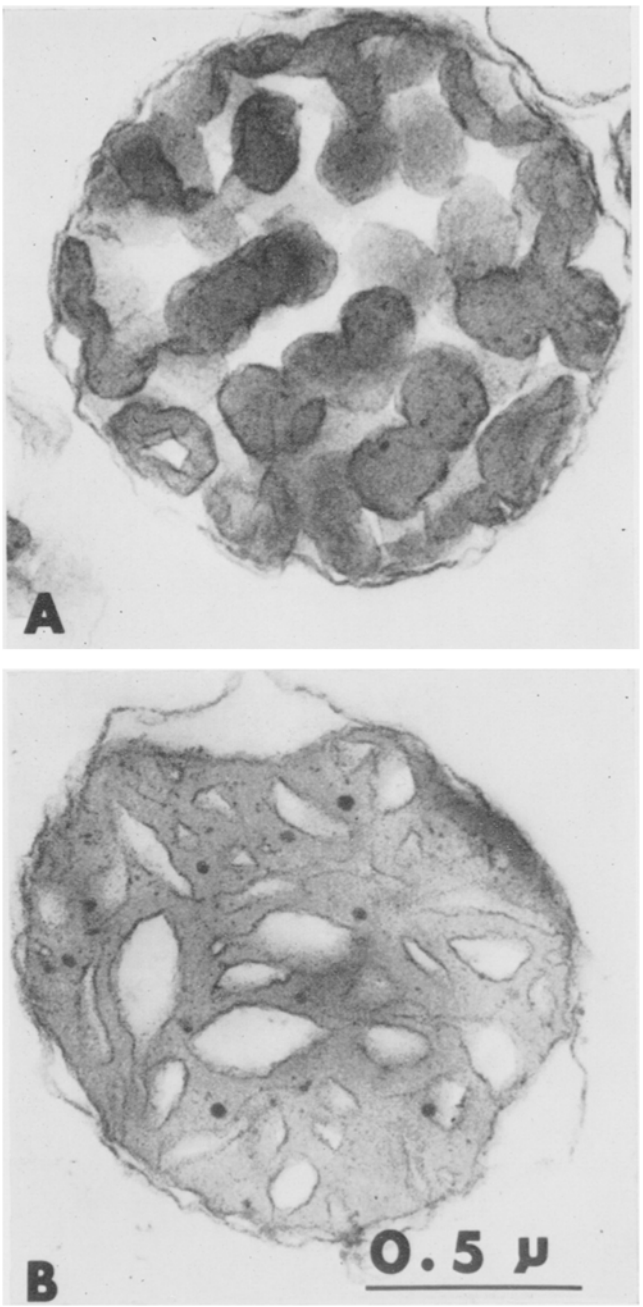

Figure 15. Configurational states of isolated rat liver mitochondria at high magnification. Electron micrograph A is of a rat liver mitochondrion incubated in 0·25 M sucrose supplemented with 5 mM pyruvate plus malate as substrate, 10 mM inorganic phosphate and oxygen. Note the energized-twisted configuration of the cristal membranes. Electron micrograph B is of a rat liver mitochondrion incubated in 0·25 M sucrose supplemented with 5 mM pyruvate plus malate as substrate, 10 mM inorganic phosphate, oxygen, and (1 μg/mg) antimycin. Note the nonenergized (aggregated) configuration of the cristal membranes.

mitochondria in which the zigzag character of the energized-twisted configuration is readily recognizable, and that of liver mitochondria in which the energized-twisted configuration is very difficult to visualize *in situ*. Canary flight muscle mitochondria [Fig. 3] conforms more closely to the model of liver mitochondria. In these "in between" mitochondria the cristae have a more condensed appearance than the cristae of heart mitochondria, but the zigzag character of the cristae shows through more clearly than it does in the cristae of liver mitochondria.

As a baseline or starting point for these studies, each tissue studied was fixed immediately after being excised and finely divided but before being incubated in the aerobic media. If the processing of the tissue was very rapid, i.e., rapidly fixed with the ice-cold

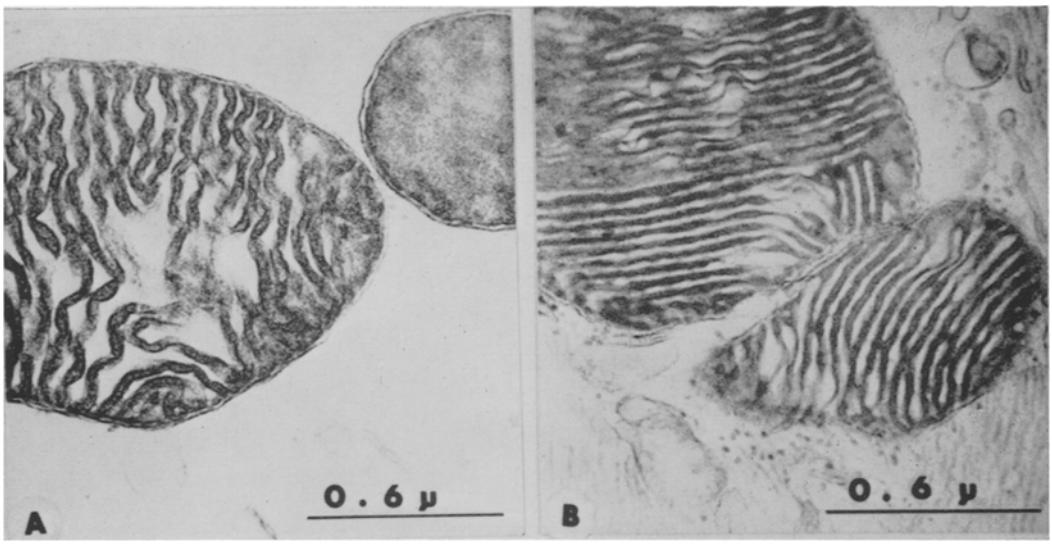

Figure 16. Configurational states of isolated canary heart mitochondria. Electron micrograph A shows the energized-twisted configuration of the cristae of mitochondria incubated in 0·25 M sucrose supplemented with 10 mM inorganic phosphate, oxygen, and 5 mM pyruvate plus malate. Electron micrograph B shows the nonenergized, aggregated configuration of mitochondria which had been incubated in 0·25 M sucrose supplemented with 10 mM inorganic phosphate, oxygen, 5 mM pyruvate plus malate, and (1 μg/mg) antimycin.

fixative solution, the mitochondria were frequently "trapped" in the energized or energized-twisted configuration [see Fig. 6(A)]. Usually the mitochondria were observed to be in the nonenergized, orthodox configuration because of the inevitable anaerobiosis which occurred [see Fig. 4(A)]. This observation probably accounts for the fact that the orthodox configuration is the one seen by the great majority of electron microscopists using standard procedures with no control over the state of aerobiosis of the tissue.

We have found, in general, that the entire mitochondrial population has a uniform configurational appearance when in a given energy state (see Figs. 4, 6, 9 and 11). When we have used for illustrative purposes one particular electron micrograph, it is not that the electron micrograph is atypical but rather that it shows certain ultrastructural features more elegantly than the large number of other electron micrographs

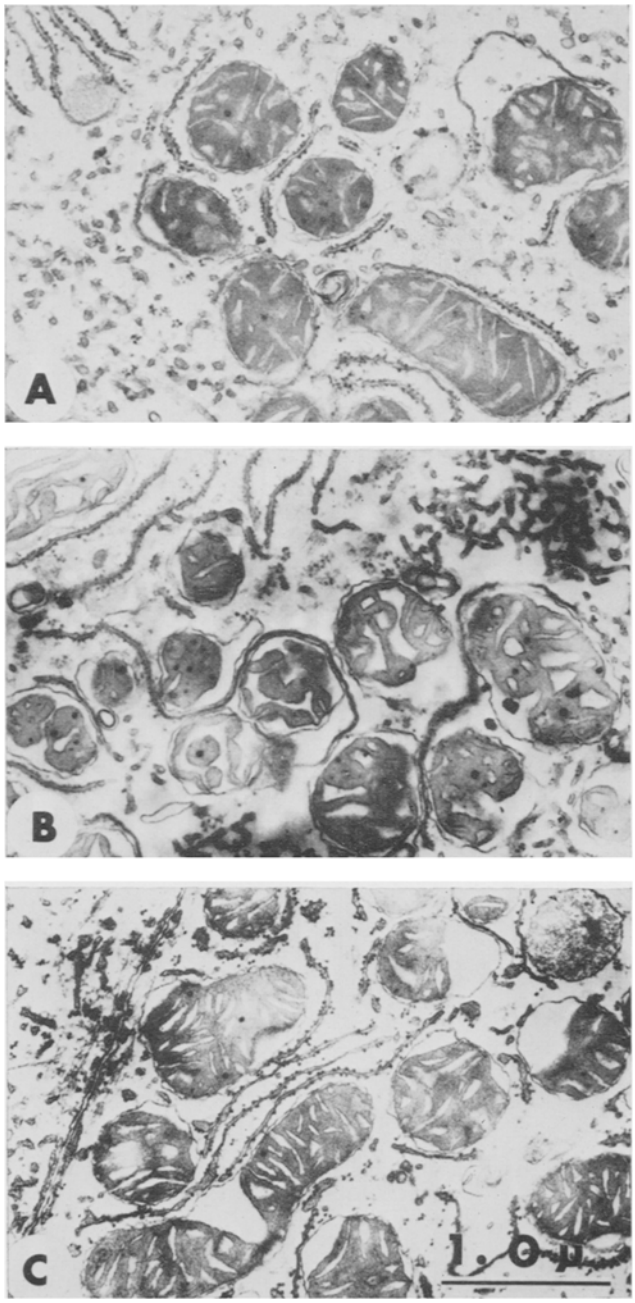

Figure 17. Configurational states of *in situ* rat liver mitochondria incubated in sucrose. Electron micrograph A is of mitochondria in rat liver tissue incubated in a KRP medium 0·25 M in sucrose under anaerobic conditions (nitrogen atmosphere). Note the condensed appearance of the cristae. Electron micrograph B shows the energized-twisted configuration of rat liver tissue mitochondria incubated in a KRP medium 0·25 M in sucrose, under energizing conditions. Note the twisted-tubular structure of the cristae. Electron micrograph C is of rat liver tissue mitochondria incubated in a KRP medium, 0·25 M in sucrose and supplemented with the uncoupler, DNP. Note the nonenergized aggregated appearance of the cristal membranes.

accumulated from forty *in situ* experiments. We have been able to repeatedly achieve the same result when the experiments were repeated with the same tissues from different animals.

II. *The Determinants of the Configurational Form of the Cristae of Rat Liver Mitochondria In Situ*

It has been pointed out in the previous sections that the cristae of nonenergized mitochondria *in situ* (suspended in KRP medium) were invariably found to be in the orthodox configurational mode. They were always in the orthodox mode when the tissue was exposed to aerobic conditions in the presence of dinitrophenol. Is this a property of mitochondria *in situ* or of the osmotic pressure of the medium in which the mitochondria are suspended?

When the suspending KRP medium is supplemented with sucrose (0·1 or 0·25 M final concentration), *then under nonenergizing conditions* (anaerobiosis), the cristae are found invariably in the aggregated or condensed configuration [see Fig. 17(A)]. Clearly the osmotic pressure of the medium is the critical determinant of the mode of the nonenergized configuration.

The effect of sucrose in modulating the configurational form of the cristae has been documented by Crane[19] for beef heart mitochondria *in situ* and by Allmann *et al.*[20] for adrenal cortex mitochondria *in situ*. In both cases sucrose induced the aggregated configurational mode of the cristae under nonenergizing conditions. The modulation of configurational change by changing the osmotic pressure of the suspending medium has been systematically explored in our laboratory.[6, 20]

III. *Paracrystalline Arrays in Energized Mitochondria*

An extensive literature has developed which deals with paracrystalline arrays in mitochondria.[21, 22] The nature of these striking structures has been rather baffling. One particular paracrystalline pattern is that of the "Star of David" which has been observed in the mitochondria of the jumping muscle of the locust.[23] This particular pattern is found in rat heart and canary heart mitochondria *in situ* when they are energized [see Figs. 13(A) and 13(B)]. These patterns have been analyzed three-dimensionally by Korman *et al.*[18] and have been shown to be the consequences of the way in which the apposed energized cristal membranes stack, fold, and coalesce.

IV. *The Configurational Cycle in Isolated Mitochondria*

The observations reported in Section III laid the groundwork for rationalizing the configurational changes of isolated liver mitochondria—changes which hitherto have been difficult to fit into the frame of the clear picture obtained for isolated beef heart mitochondria. The method of Schneider[17] was followed for the preparation of rat liver mitochondria. The suspending medium was 0·25 M sucrose. Figure 14 shows the configurational pattern of the mitochondria under nonenergizing conditions (see 14B) and under energizing conditions (see 14A). In Figure 14B the cristae are in the nonenergized, aggregated configuration; and in Figure 14A, the cristae are in the energized-twisted configuration. The time of fixation is a crucial determinant of the configurational mode. When exposure to energizing conditions is relatively short ($<1{\cdot}5$ min), the energized-twisted configuration is clearly demonstrable. When the exposure to energizing conditions is prolonged, the cristae no longer show the energized-twisted configuration but

rather the non-energized configuration. We have electron microscopic results from a series of experiments with *intact* beating rat heart cells grown in tissue culture (experiments were performed in Dr. Harary's laboratory*) which clearly show the energized-twisted configuration of the cristal membranes of the mitochondria. The beating rat heart cells in tissue culture (approximately one cell layer thick) were fixed by pouring ice cold fixative solution into the culture medium. These results provide further support for our thesis that the configurational cycle is operative at the cellular level and is not an artefact of preparation or fixative.

Although the energized-twisted tubules formed by the cristae of rat liver mitochondria under energizing conditions are unmistakable, nonetheless these tubules are significantly different from the energized-twisted tubules of beef heart mitochondria. This difference appears to be referable to the high concentration of matrix protein in liver mitochondria and the relatively low concentration of matrix protein in heart mitochondria. In general, there is a correlation between the bulbar (overstuffed) appearance of energized-twisted tubules in mitochondria and the high concentration of matrix protein.

V. *Critical Factors in the Demonstration of Energized Configuration in Mitochondria In Situ*

We have conducted a series of experiments to determine which factors are indispensable for the visualization of the energized configurations in mitochondria *in situ*. Two requirements must be met: (a) oxygen to insure an aerobic environment, and (b) rapid fixation. All other variables in our incubation mixture are less critical. The inhibitors we routinely use (rotenone, rutamycin, and iodoacetate) are not always required for the demonstration of the energized configuration, but there are instances in which their presence produced sharper results. The possibility of side reactions which could cause the dissipation of the energized state or endogenous substrate serving as the energy source is reduced by the use of these inhibitors. In many experiments we have found that endogenous substrate is present at a concentration sufficient to energize the mitochondria.

The temperature of the mitochondrial suspension at the time of fixation is critical. We have had the best results when the fixative was ice cold when poured into the incubation flasks. The osmolarity of the fixative solution does not appear to be a critical factor in the demonstration of energized configurations. We have reduced the osmolarity of the fixative solution to about one-third of the recommended osmolarity (200 instead of 1000 milliosmoles) and found no significant difference in the results. These results are in good agreement with the observations of Sjostrand.[24] Stoner and Sirak[25] are of the opinion that the ultrastructural changes we have observed in mitochondria during the energy cycle are referable to the osmotic effect of the fixative. In all our *in situ* experiments the mitochondria were fixed with exactly the same fixative solution. Since the conditions of fixation were constant whether the mitochondria were nonenergized or energized, there is no basis in fact for relating the configurational changes to the fixative concentration.

Discussion

The essence of the conformational hypothesis is that the free energy of electron transfer is transduced into conformational energy and that in turn conformational energy is transduced into the bond energy of ATP. This hypothesis requires that each repeating unit

*M. W. Seraydarian, I. Harary, E. Sato, *Biochim. Biophys. Acta*, **162** (1968), 414-423.

should exist in two conformational states—nonenergized and energized. When the repeating units are largely or entirely in the same conformation then the configuration of the membrane provides a measure of the conformation of the repeating units. If we assume this correspondence of configuration and conformation under conditions which insure synchrony of the repeating units, then the studies presented above establish that there is an exact parallelism between the conformation of the repeating units and the energy state in mitochondria of five different cell types and of three different species.

The mode of the nonenergized configuration of mitochondria *in situ* under anaerobic conditions is invariably the orthodox mode simply because the osmotic pressure of the cell sap which bathes the mitochondrion is insufficient to compel the expansion of the cristal membranes, i.e., of the intracristal space. When sucrose is added to the suspension medium, the osmotic gradient between the intracristal and matrix spaces can be raised to the point at which this expansion is effectuated. In such a medium, the mode of the nonenergized configuration then becomes the aggregated mode.

There are in fact two energized configurations—the energized and energized-twisted. Inorganic phosphate induces the transition of cristae in the energized to the energized-twisted configuration.[6] We have made no effort experimentally to select conditions which lead exclusively to one or the other energized configuration. That is to say, the particular energizing conditions which we have used (endogenous plus added substrate and inorganic phosphate, in presence of oxygen) will lead to a mixture of energized and energized-twisted configurations. This duality of energized configurational states was not only expected but observed. However, a yet unsuspected option was discovered with respect to the modes of the energized-twisted configuration. This could be tubular as in isolated mitochondria or zigzag as in mitochondria *in situ*. When the osmotic pressure of the suspending medium is increased by addition of sucrose, the mode of the energized-twisted configuration of the cristae of mitochondria *in situ* becomes tubular rather than zigzag. Even within the tubular mode of the energized-twisted configuration, there is yet another option. In mitochondria with little matrix protein, e.g., heart mitochondria, the tubules are highly regular and not much wider than orthodox cristae; whereas in mitochondria which have a high concentration of matrix protein, e.g., liver mitochondria, the tubules are irregular and many times wider than orthodox cristae.

Matrix protein modulates not only the form of the energized-twisted tubules but also the form of the aggregated and energized configurations. When the cristae of mitochondria with high matrix protein (liver) are in the nonenergized aggregated or energized aggregated configurations, there is no simple way to distinguish between these two configurations. In both cases the mitochondria show highly condensed cristae. However, it is possible to distinguish easily the condensed from either the orthodox or the energized-twisted configuration of the cristae in liver mitochondria.

Studies with liver mitochondria *in situ* have brought to light the rapid decay of the energized-twisted configuration as a function of time. The imposition of this state is rapidly accompanied by a set of changes (low amplitude swelling or active transport) which eventually lead to the discharge of the energized-twisted configuration. This critical time period was not appreciated by several workers who have attempted to correlate configurational change and energy states in mitochondria.

Hackenbrock[26–28] has carried out an extensive series of experiments on configurational

changes in liver mitochondria under various experimental conditions. While in general he accepts and endorses the thesis of the conformational basis of energy transduction, he has found it difficult to interpret his results in terms of the nonenergized to energized configurational transition so clearly seen in heart mitochondria.[1-3] The various factors which account for Hackenbrock's observations have been considered in detail in the experimental section. These include: (a) the orthodox to aggregated transition; (b) the rapid decay of the energized-twisted configuration to the aggregated; (c) the role of sucrose in modulating the mode of each configurational state; and (d) the ambiguity of the condensed state of the crista with respect to the configurational mode (nonenergized aggregated and energized aggregated are indistinguishable). When these factors are properly taken into account, we find correspondence between our results and those of Hackenbrock.

Goyer and Krall[29] have observed in isolated rat kidney mitochondria that the cristae were in the tubular configuration when fixed at 1 min and in the orthodox configuration when fixed after 5 min incubation. The time factor for the decay of the energized-twisted configuration may be related to the rate of release of free fatty acids during aerobic incubation of mitochondria. Allmann (unpublished studies) has shown that free fatty acids can catalyze the transition from aggregated to orthodox cristae. Thus, release of free fatty acid would automatically lead to the discharge of the energized-twisted configuration and the formation of the nonenergized orthodox configuration.

Undoubtedly, the orthodox to aggregated configurational transition has contributed most heavily to the confusion attending configurational change in liver mitochondria and to the difficulty in specifying which configuration is energized or nonenergized. For example, when liver mitochondria *in situ* suspended in KRP solution are energized, the cristae undergo a transition from the orthodox to the energized aggregated configuration (condensed). When sucrose is added to another aliquot of the liver tissue mitochondria under nonenergizing conditions, the cristae undergo a transition from the orthodox to the aggregated configuration (condensed). Thus, addition of sucrose to the medium achieves a result comparable to that achieved by imposing energizing conditions, namely, expansion of the intracristal space and contraction of the matrix space. There are thus two ways of expanding the intracristal space—by addition of sucrose under nonenergizing conditions or by imposing energizing conditions. Thus, while the transition from the orthodox to condensed state is part of the configurational cycle under energizing conditions, it can nonetheless proceed even in the absence of energizing conditions.

Isolated beef heart mitochondria in 0·25 M sucrose are invariably in the aggregated mode of the cristae. However, addition of uncoupler in relatively high concentrations, or of Ca^{2+}, or of the endotoxin of *Bordetella bronchiseptica*[30] can induce the transition of the cristae of these mitochondria in 0·25 M sucrose from the aggregated to the orthodox configuration.

We have presented a large amount of evidence that the condensed or aggregated state is the energy-conserving configuration in mitochondria in tissues, and that this state is assumed under aerobic conditions. After completing most of the experiments described in this report, we found that our results were in agreement with those reported by Jasper and Bronk[31] and Fawcett and McNutt.[16] Jasper and Bronk[31] observed the condensed energized-twisted configuration in the mitochondria of mouse intestinal

mucosal cells which were actively accumulating amino acids. When DNP was added to their tissue preparations, the mitochondria shifted to the orthodox configuration and the ability to accumulate amino acids was lost. Fawcett and McNutt[16] observed energized-twisted mitochondria in cat papillary muscle that was incubated under aerobic conditions. Both of these groups fixed the tissues under aerobic conditions and Jasper and Bronk indicated that they used a cold fixative. These are exactly the conditions we have found to be *essential* for preserving the energized state of the mitochondria. Recent experiments by Meszler and Gennaro[32] established that the cristae of the mitochondria of radiant heat receptors *Agkistrodon p. pisceverous* undergo configurational changes which parallel the sensory activity of the receptor organ. When the receptor organ is quiescent (no heat stimulus), the mitochondria are in the energized-twisted configuration whereas when the receptor organ is stimulated (heat stimulus), the mitochondria are in the discharged orthodox mode.

Our experiments indicate that the orthodox configurational state represents the nonenergized state of the cristal membrane as evidenced by the fact that this state was observed in the presence of the uncoupler DNP. We can now ask the questions: How does the orthodox configuration enhance the dissipation of energy or alternatively prevent the conservation of energy by the cristal membrane? What is the mechanism by which the aggregation of the cristal membranes provides an energy-conserving environment rather than an energy-dissipating environment? Perhaps the orthodox to aggregated transition is one of the control mechanisms by which mitochondria can generate either heat or ATP. Brown fat tissue shows a high degree of heat production under physiological conditions.[35] Mitochondria isolated from active brown fat tissue are generally uncoupled, or at best very loosely coupled.[34] If the transition to the orthodox configuration represents physiological uncoupling then future investigations should be able to correlate metabolic uncoupling with the ultrastructure of the cristal membrane of the mitochondria.

The method we have described for studying configurational changes in mitochondria *in situ* opens the door to a wide variety of physiological investigations such as the effect of drugs, anesthetics, hormones, and metabolic diseases on mitochondrial configurational changes.

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