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On the Correlation of Configurational Changes in the Inner Mitochondrial Membrane with Energization

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Abstract

Two reports in the literature that mitochondria isolated in special media or from a particular source do not undergo configurational changes under energizing conditions have been analyzed in detail. It could be shown that the failure to demonstrate configurational changes was a consequence of a procedure which allowed anaerobiosis to set in before the mitochondria were fixed. When fixation was achieved while the mitochondria were still under energizing conditions, the correlation between configurational change and change in the energy state could be confirmed.

In the present communication we shall be concerned with the factors which underlie the inability of Sordahl *et al.*[1] and Kuner and Beyer[2] to observe configurational changes during the energy cycle of heart and skeletal muscle mitochondria. Sordahl *et al.*[1] examined heart mitochondria from four species isolated by a variety of techniques. They found that when the mitochondria were isolated by a procedure dependent upon mechanical disruption of the heart tissue, the mitochondria failed to show configurational changes under energizing conditions, and this negative result was obtained regardless of the media in which the mitochondria were suspended. However, mitochondria prepared by digestion of heart muscle tissue with a proteolytic enzyme, consistently showed configurational changes. Sordahl *et al.* concluded that "ultrastructural studies of heart mitochondria revealed a resistance to conformational alterations, except when the mitochondria were isolated under certain conditions." It should be pointed out that these authors use the term conformation to describe the configuration of the inner membrane.

We shall be presenting evidence that the method used by Sordahl *et al.* to "freeze" their system in the configuration obtaining during energization was inadequate for this purpose. Their mitochondria became deenergized as a result of anaerobiosis before the fixative was introduced. The problem as we see it is not to explain their negative results which are predictable from the particular methods used, but to explain how they managed to get positive results at all in some of their experiments with the same methods. We have prepared heart mitochondria by exactly the same procedures and suspended

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them in exactly the same media which Sordahl *et al.* have used. These mitochondrial preparations consistently showed the normal configurational changes when exposed to energizing conditions. Our standard method of fixation was used to freeze instantly the mitochondria in the configurational state obtaining at the time of addition of the fixing reagents. In a previous communication the critical conditions that had to be met to insure preservation of the membrane configuration were described in full.[3]

Kuner and Beyer have reported that mitochondria from rat skeletal muscle do not show "the strict correlation between metabolic states and ultrastructural state in isolated rat skeletal muscle mitochondria as has been observed in isolated mitochondria from several other tissue types." They used the same centrifugal method as did Sordahl *et al.* for separating the mitochondria which had been exposed to energizing conditions—a method which is unsuited for "freezing" the configurational state before anaerobiosis can set in. We have prepared mitochondria from rat skeletal muscle and have found unambiguous evidence of configurational change in all our experiments under energizing conditions when the proper precautions were taken to fix the mitochondria while still energized. Moreover, we have fully duplicated the negative findings of Kuner and Beyer and of Sordahl *et al.* whenever we have substituted their procedure for the one we have recommended.

Experimental Methods

Preparation of mitochondria. Heavy beef heart mitochondria were prepared by the method of Crane *et al.*[4] as described by Hatefi and Lester.[5] This standard procedure which involves mechanical homogenization of the coarsely minced heart muscle was kept constant in the series of studies to be presented. The variable was the medium in which the mitochondria prepared by the above procedure were washed, suspended and tested. Three media have been examined *in extenso*—the "KEA," "CEA" and "STE" media as defined by Sordahl *et al.*[1] The composition of these respective media is as follows: KEA (0·18 M in KCl, 10 mM in EDTA and 0·5% in BSA of pH 7·2); CEA (0·18 M in choline chloride, 20 mM in Tris-Cl, 10 mM in EDTA and 1% in BSA of pH 7·4); STE (0·25 M in sucrose, 1 mM in Tris-Cl and 10 mM in EDTA, pH 7·4). The STE medium is in fact the standard medium used in our laboratory.

Rat skeletal muscle mitochondria were prepared by the method of Makinen and Lee.[6] The final suspension medium was 0·25 M in sucrose and 10 mM in Tris-Cl of pH 7·4.

Assay of oxidative phosphorylation. This assay was carried out at 30° according to the method and under the conditions described in full in a previous communication from this laboratory.[7] The method depends upon the conversion of inorganic phosphate to esterified phosphate (glucose-6-phosphate).

The ADP-induced increase in State III respiration was assayed under conditions identical for the assay of oxidative phosphorylation except for the omission of hexokinase and glucose, and for a lower concentration of ADP (0·2–0·4 mM).

Assay of the energized accumulation of inorganic phosphate. The assay system as well as the assay procedure were fully described in a previous communication by Wakabayashi *et al.*[3] After incubation of mitochondria for 60 sec in a medium containing succinate and labelled inorganic phosphate, the mixture was centrifuged for 30 sec in a Misco high speed centrifuge and the pellet was rinsed three times with a chilled sucrose-Tris-Cl

medium. The concentration of inorganic phosphate in the pellet was then determined by dissolving the pellet in 2% sodium dodecyl sulfate and measuring the radioactivity of the solution.

Light scattering changes. The measurement of light scattering changes in the Brice-Phoenix photometer has been described in full by Wakabayashi *et al.*[3]

Electron microscopy. Mitochondrial suspensions were fixed by mixing with an equal volume of a solution which was 4% in glutaraldehyde, 0·05 M in K cacodylate of pH 7·4 and 0·25 M in sucrose. The samples were further treated with osmium tetroxide and uranyl acetate, dehydrated and embedded as described previously.[3] Fixation of the mitochondrial configuration by direct addition of glutaraldehyde to the incubation mixture is the procedure which we routinely use and recommend. The fixation procedure used by Sordahl *et al.*[1] as well as by Kuner and Beyer[2] depends upon the centrifugal separation of mitochondria from the incubation mixture *prior* to the addition of osmium tetroxide. Moreover, glutaraldehyde was not used at all in their procedure. We have carried out the centrifugal procedure as follows. The reaction mixture was incubated at 1° for 5 min and then hydrogen peroxide was added in presence of catalase before sedimentation of the mixture in the Misco centrifuge for 20 sec at maximum speed. As soon as the rotor came to a full stop, the supernatant fluid was decanted and replaced by a solution which was 2% in glutaraldehyde or OsO_4. The mitochondrial pellet was mechanically dispersed in the fixing solution. The entire procedure carried out at 1° was completed within 60 seconds. The same fixation procedure was applied at 30° for mitochondria which were sedimented prior to fixation.

Source of chemicals. In the previous communication of Wakabayashi *et al.*[3] the sources of the chemicals used in the present investigation were specified.

Results

Coupled state of mitochondria. Both beef heart mitochondria and skeletal muscle mitochondria as prepared by the methods described in the section on Experimental Methods were well coupled as determined by three criteria—the P/O ratio, the ADP/O ratio and the respiratory control index (see Table I). Beef heart mitochondria appeared equally well coupled whether prepared in KEA, CEA or STE medium. The same high coupling efficiency was observed in skeletal muscle mitochondria whether prepared in

TABLE I. Coupling properties of beef heart and skeletal muscle mitochondria in media used for demonstrating configurational changes

Source of Mitochondria	Suspending Medium	Method of Preparation	P/O	ADP/O	Respiratory Control Index
Beef heart	STE	Hatefi & Lester[5]	1·6	1·7	3·8
Beef heart	KEA	Sordahl *et al.*[1]	1·8	1·6	3·1
Beef heart	CEA	Sordahl *et al.*[1]	1·8	1·7	3·6
Skeletal muscle	STE	Kuner & Beyer[2]	1·8	1·8	4·3

The substrate for electron transfer in all these experiments was succinate.

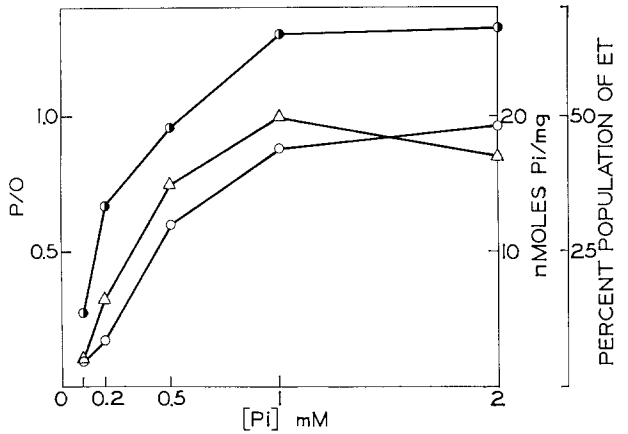


Figure 1. The three variables, P/O (◑——◑), per cent of the population in the energized twisted configuration (△——△), and the amount of Pi taken up under energizing conditions (o——o), are plotted as a function of the concentration of inorganic phosphate in the medium.

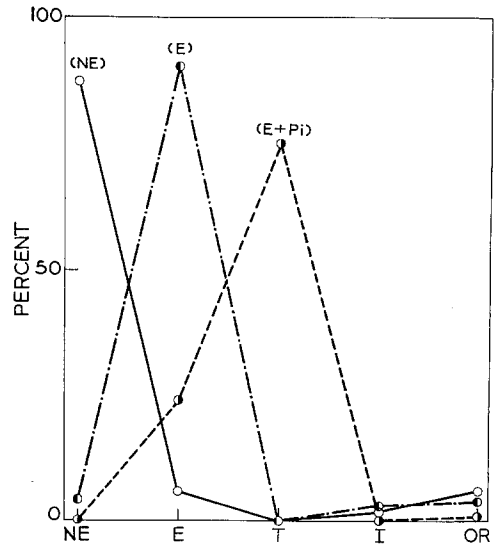
Figure 2. Histograms of the three configurational states of mitochondria under various experimental conditions.

Nonenergized conditions (NE) (o——o). Mitochondria were incubated in a medium (STMR) which was 0·25 M in sucrose, 10 mM in Tris-Cl, pH 7·4 and 3 mM in $MgCl_2$, and contained 2 μg of rotenone per ml of medium. The reaction was carried out for 5 min at 30° before fixative was added.

Energized conditions (E) (◐—·—◐). The conditions were the same as those for (NE), except for the presence of K-succinate (5 mM) and for a time of incubation of 2 min rather than 5 min.

Energized conditions in presence of Pi(E + Pi) (◑-----◑). The conditions were the same as those for (E), except for the presence of K-phosphate (10 mM).

NE = aggregated configuration in the nonenergized form
E = aggregated configuration in the energized form
T = twisted configuration in either nonenergized or energized form
OR = orthodox configuration in either nonenergized or energized form
I = aggregated configuration but indeterminate with respect to energy form



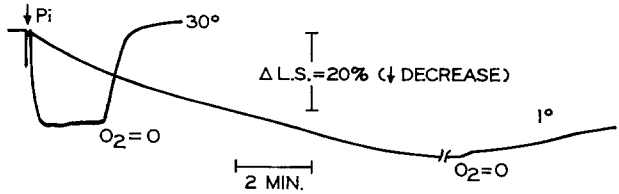
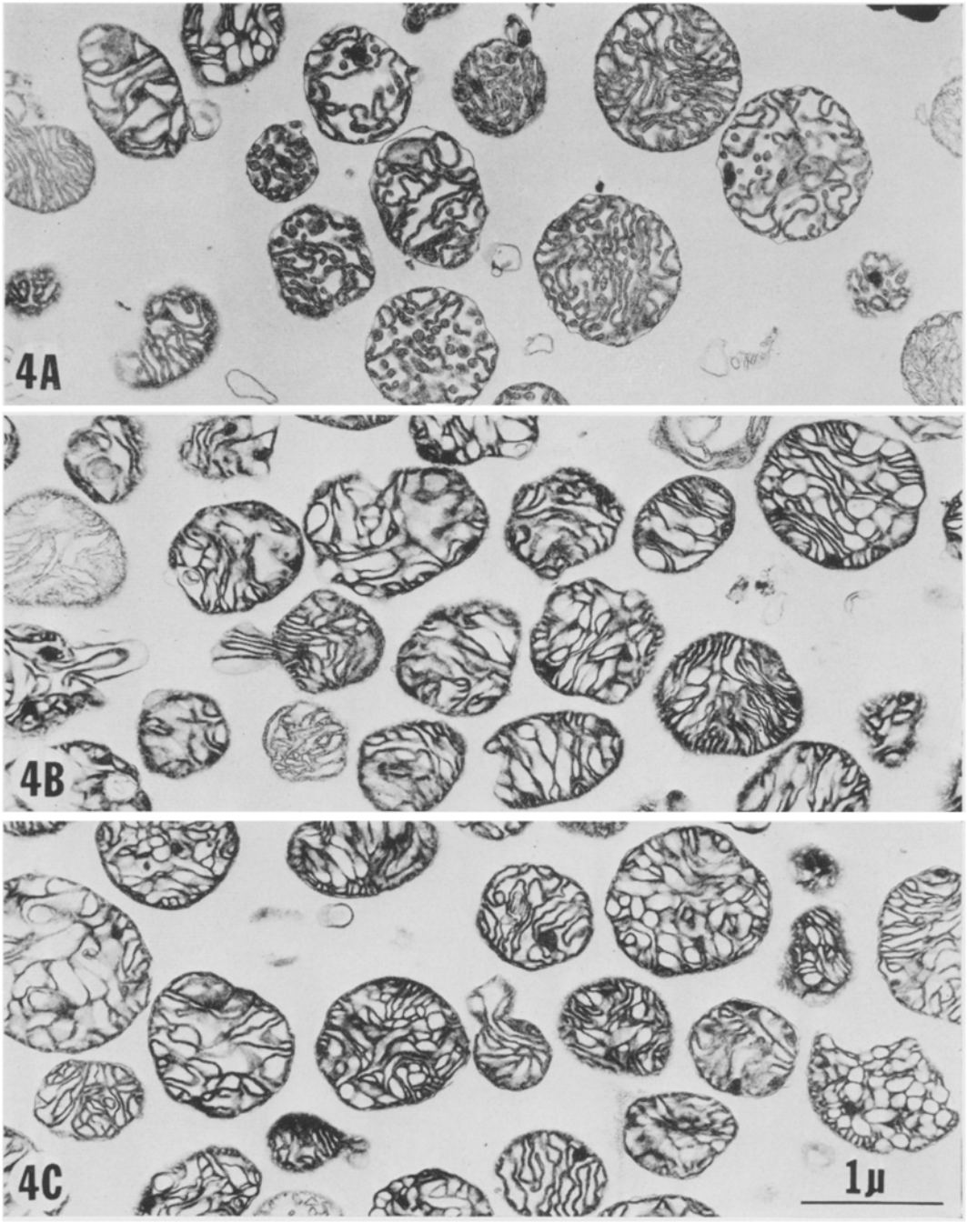
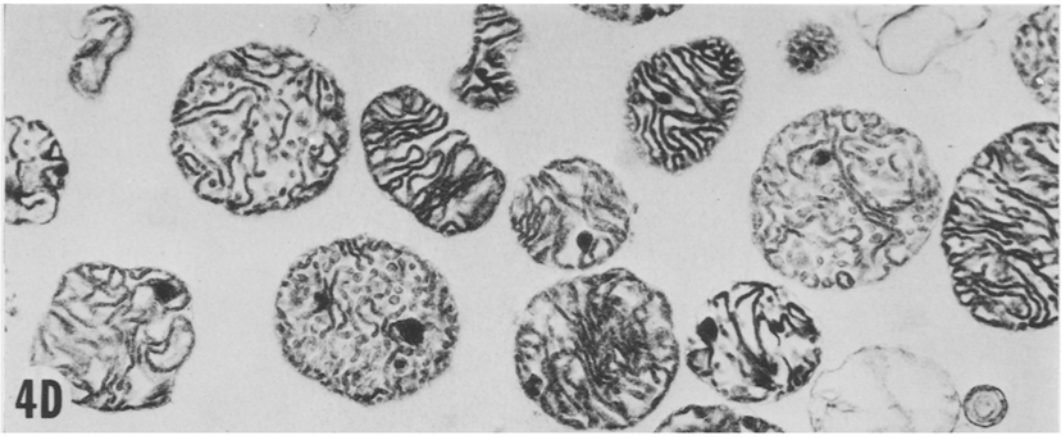


Figure 3. Light scattering (L.S.) changes induced in beef heart mitochondria under energizing conditions by addition of inorganic phosphate.

The composition of the suspending medium was that of STMR. The substrate for electron transfer was succinate. The medium became anaerobic at the point indicated by the notation "O_2 = O".


4A
4B
4C
1μ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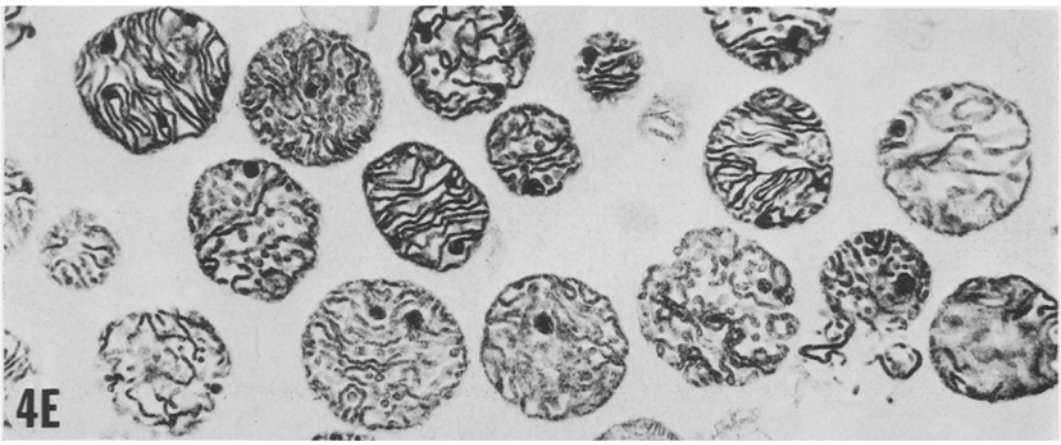
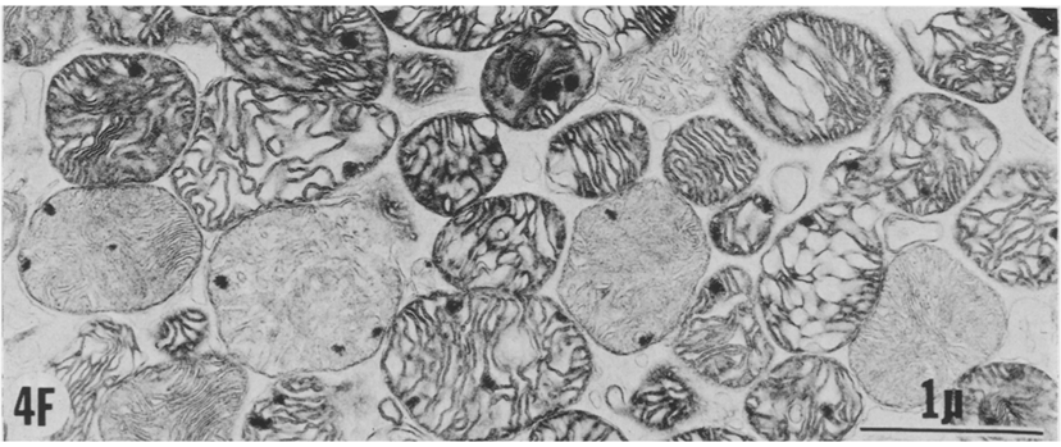

4E


4F
1μ

0·25 M sucrose or in 0·15 M KCl. The point at issue is that the mitochondria which we have used for the configurational studies to be described were uniformly well coupled. Moreover, the procedures which were used by Sordahl *et al.*[1] for preparation of the mitochondria were as effective as our standard procedure in maintaining coupling capacity.

Correlation between P/O, configurational change and energized uptake of phosphate. The correlative study described in this section points up a new facet of the relation between configurational change and energized processes in mitochondria. In Fig. 1, three quantities are plotted as functions of the concentration of inorganic phosphate in the medium—the P/O ratio, the amount of phosphate taken up during energization, and the per cent of mitochondria in the twisted configuration during energization. The three curves are very similar. This would suggest that the level of inorganic phosphate in the medium affects an event which is intrinsic to all three energized processes.

Figure 2 is a histogram of the different configurational states of the mitochondria when energized in presence and absence of inorganic phosphate. There are only three configurational states that need be considered—the aggregated configuration in both the nonenergized and energized forms and the twisted configuration (no distinction has been made between the two forms of the twisted configurational state because these are indistinguishable electron microscopically). At the level of inorganic phosphate which is optimal for energized configurational change, the mitochondrial population is predominantly in the twisted configuration, whereas prior to energization the predominant configurational state is aggregated.

Demonstration of the aggregated to twisted configurational transition. The light scattering studies shown in Fig. 3 help to define how the aggregated to twisted configurational transition can be followed. When an aliquot is removed from the mixture at the time the light scattering change is maximal, and mixed immediately with the fixing solution containing glutaraldehyde, electron micrographs of such a sample will show that the mitochondrial population is entirely in the twisted configuration (see Fig. 4, A). However, if the aliquot is removed after anaerobiosis has set in, and after the light

Figure 4. Electron micrographs of beef heart mitochondrial suspensions fixed in different ways and at different temperatures.

All samples were energized with succinate in presence of inorganic phosphate (10 mM). The mitochondria were suspended in a medium 0·25 M in sucrose, 10 mM in Tris-Cl of pH 7·4, and 3 mM in $MgCl_2$, and contained 2 μg of Rotenone per mg of protein.

Plate	Temperature	Fixation Procedure	Comment
A	30°	Direct addition of glutaraldehyde to the incubation medium (1 min after addition of Pi)	Aerobic at the time of fixation
B	30°	Direct addition of glutaraldehyde to the incubation medium	Anaerobic at the time of fixation (1 min after the system became anaerobic)
C	30°	Addition of glutaraldehyde after centrifugation of mitochondria	Anaerobic at the time of fixation
D	1°	Direct addition of glutaraldehyde to the incubation medium (5 min after addition of Pi)	Aerobic at the time of fixation
E	1°	Addition of glutaraldehyde after centrifugation of mitochondria. (Just before centrifugation, H_2O_2 was added in presence of catalase)	Aerobic at the time of fixation
F	1°	Addition of OsO_4 after centrifugation of mitochondria. (Just before centrifugation, H_2O_2 was added in presence of catalase)	Aerobic at the time of fixation

scattering change has been reversed, the electron micrographs will show only the aggregated configuration (see Fig. 4, B). The rate of electron transfer of the mitochondria at 30° is so rapid that when the centrifugal method of Sordahl *et al.* is used—a method which depends upon the separation of the mitochondria from the medium by centrifugation before mixing with the fixative solution—the sedimented pellet is already anaerobic by the time the centrifugation is completed, i.e., *before* the fixative is added. The centrifugal method requires at least 30 seconds before fixative can be added to the pellet, and during this time the mitochondrial pellet becomes anaerobic particularly at 25°. At this temperature 1 mg of mitochondrial protein consumes oxygen at a rate of at least 0·25 mμ atoms oxygen per second. Since the pellet of mitochondria contains at most 3 μl of water per mg protein in isotonic media,[8] the amount of oxygen dissolved in the pellet cannot maintain aerobic conditions for longer than 12 seconds. Figure 4, C establishes that an aliquot of the same incubation mixture which clearly showed preponderantly the twisted configuration when the sample was immediately fixed with the glutaraldehyde-containing medium failed to show this configuration when the mitochondria were centrifuged before fixation. Our thesis is that the sedimentation procedure is perfectly valid provided that the system does not become anaerobic before the fixative is added. At 30° the prevention of anaerobiosis during sedimentation is not easy to achieve because of the rapid rate of oxygen consumption.* But at 1°, the rate of oxygen uptake is reduced to 1–2% of the rate at 30°. All changes are slower but at least onset of anaerobiosis during centrifugation is avoided (see Fig. 4, D obtained by standard fixation). The rate of oxygen uptake at 1° is slow enough that the pellet collected after 20 seconds of centrifugation in the Misco centrifuge is still aerobic. Thus, when the procedure of Sordahl *et al.* is applied at 1° and the additional precaution is taken of adding H_2O_2 plus catalase to ensure aerobiosis during the centrifugation, and when the pellet thus obtained by centrifugation is resuspended in the glutaraldehyde-containing fixative solution, then the electron micrographs clearly show that the configuration is entirely twisted as would be predicted (see Fig. 4, E). But note that we have used glutaraldehyde to fix the mitochondria and not osmium tetroxide as was used by Sordahl *et al.* When we used osmium at the concentration which they employed, then we found a negligible proportion of the mitochondrial population in the twisted configuration (see Fig. 4, F). We have already presented a body of evidence that osmium above a critical concentration itself induces a transition from the twisted to the aggregated configuration.[3] Thus there were two major limitations in the procedure of Sordahl *et al.*—that of anaerobiosis setting in before fixation could freeze the configurational state, and that of osmium at a concentration of 2% inducing spontaneously a configurational transition leading to the disappearance of the twisted configuration. As we stated in the introduction, the negative findings are entirely predictable from the limitations of the fixation procedure used by Sordahl *et al.*; the surprising thing and something that still needs explaining is how they succeeded in observing configurational changes with mitochondria isolated by the Nagarse method using the same procedure.

The nature of the isolation medium whether CEA or KEA or STE has no influence on the demonstration of configurational change. The same results were obtained in any of these media (Fig. 5). In other words, the problem is entirely one of fixing the

* It should be pointed out that Hackenbrock originally adapted the centrifugal procedure for the fixation of rat liver mitochondria,[9] but these mitochondria respire relatively slowly.

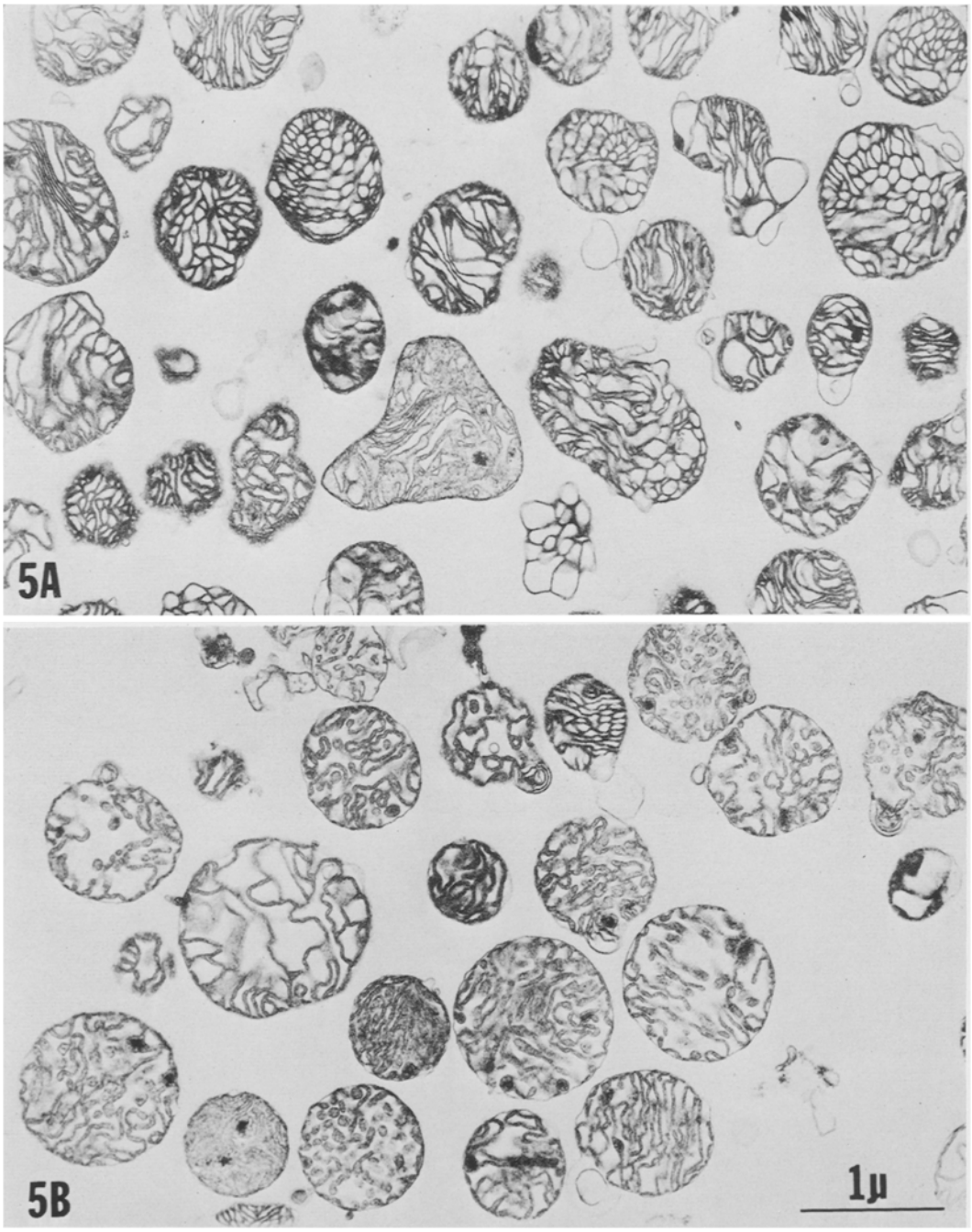


Figure 5. Configurational changes in beef heart mitochondria prepared by mechanical disruption of heart tissue and exposed to energizing conditions in presence of inorganic phosphate.

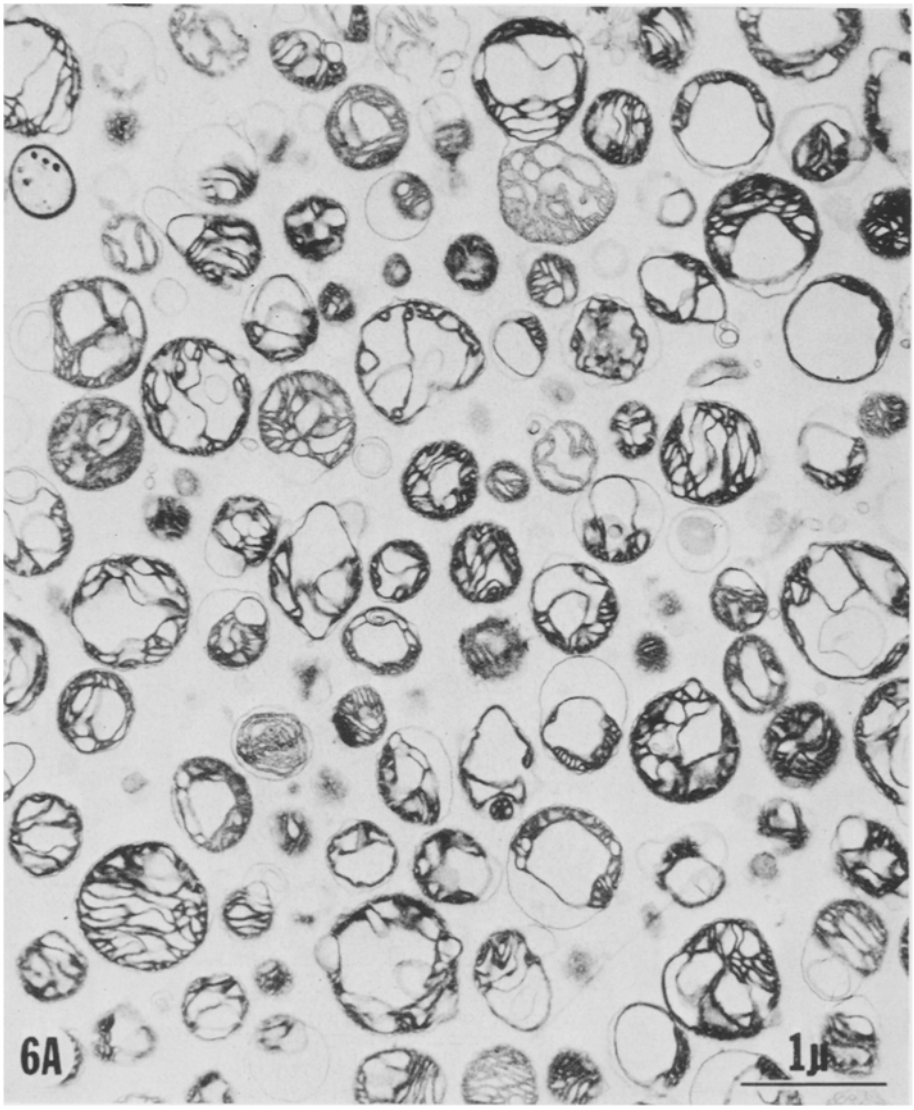
The mitochondria were energized by succinate in presence of inorganic phosphate (10 mM). The suspension medium was KEA.

A: Before energization with succinate.

B: After energization with succinate.

configuration while energizing conditions still obtain. When the fixation procedure is adequate, configurational change is demonstrable regardless of the nature of the suspending medium and procedure used in the isolation.

Configurational changes in rat skeletal muscle mitochondria. The method which Kuner and Beyer[2] used to demonstrate configurational changes in mitochondria of rat skeletal muscle mitochondria is essentially indistinguishable from that used by Sordahl *et al.* and suffers from the same two disabilities pointed out above. We have isolated rat skeletal muscle mitochondria by the method of Makinen and Lee[6] and have been able to show consistently the aggregated to twisted configurational change under energizing conditions (see Fig. 6). Fixation was achieved by direct addition of glutaraldehyde to



the mitochondrial suspension previously exposed to energizing conditions in presence of inorganic phosphate. The use of 2% osmium tetroxide for purposes of fixation was avoided. The configurational changes observed with rat skeletal muscle mitochondria were obtained in both sucrose- and salt-containing media.

The transition from aggregated to twisted configuration in rat skeletal muscle mitochondria is easily recognizable electron microscopically. But the transition from the

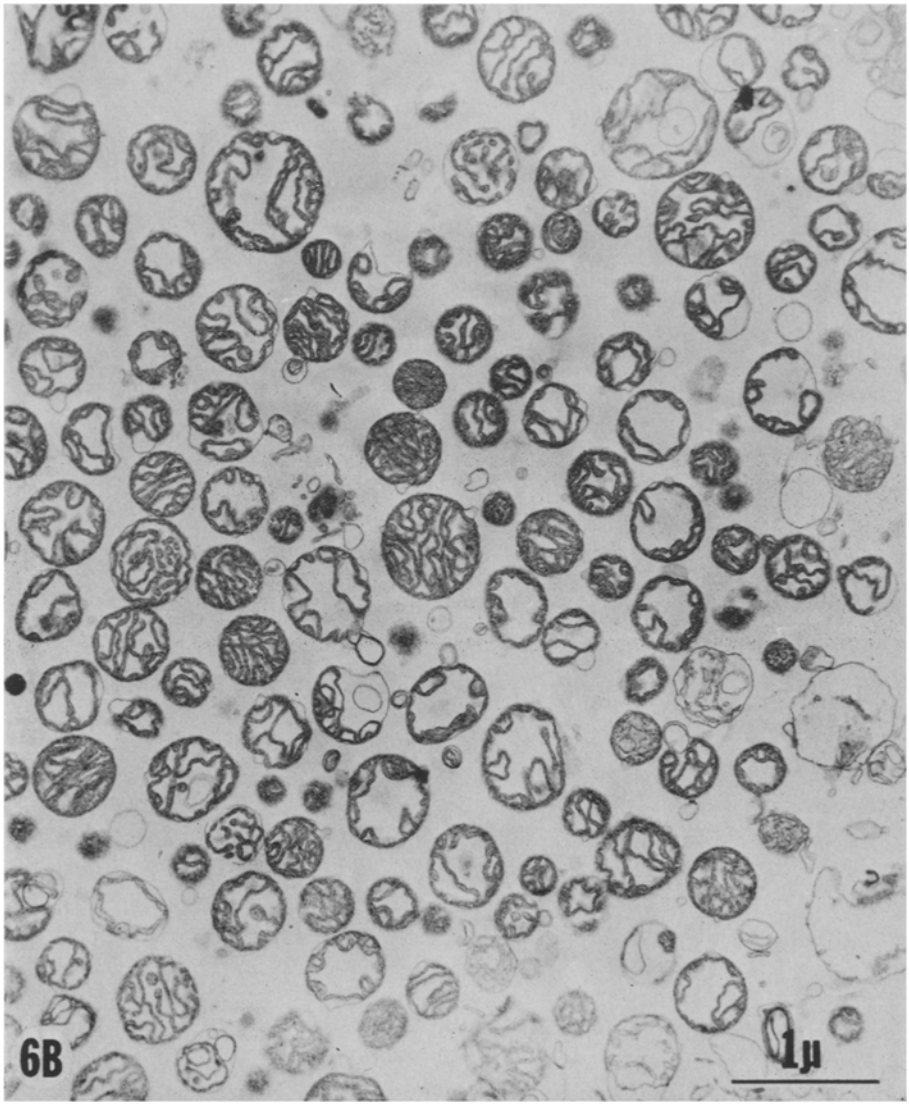


Figure 6. Configurational changes induced in rat skeletal muscle mitochondria under energizing conditions in presence of inorganic phosphate.
The mitochondria were energized by succinate in presence of inorganic phosphate (10 mM).
A: Before energization with succinate.
B: After energization with succinate.

nonenergized to the energized form of the aggregated configuration is more subtle and less obvious by casual inspection of a large field. For this reason we have not presented evidence on this other transition although we have ample evidence that this transition always takes place under energizing conditions.

Discussion

It has been the postulate of our laboratory that when mitochondria are energized under well defined experimental conditions, configurational changes are always observed electron microscopically and these changes reflect events which are intrinsic to the coupling mechanism. In the present communication we have gone to great pains to show that the two reports in the literature, of exceptions to this postulate of the invariance of configurational change during energization of mitochondria under specified conditions, were based on methodology unsuited for the purpose intended.

Our views about the meaning and interpretation of configurational change during energization have undergone some revision since our first formulation of the conformational model. We now consider configurational change as primarily an expression of conformational change in the matrix system. While indeed electron transfer induces conformational changes in the repeating units of the inner membrane, these changes are low amplitude in nature and are not directly observable electron microscopically. However, energization of the inner membrane induces large amplitude conformational changes in the proteins of the matrix network system. It is these extensive conformational changes in the matrix proteins which underlie configurational changes in mitochondria under energizing conditions. The invariance of the correlation between configurational change and energization now has to be interpreted in terms of the matrix proteins behaving as conformational probes and registering the energized state of the inner membrane repeating units by undergoing conformational transitions. These views will be developed elsewhere *in extenso* by Green *et al.*[10]

Elsewhere R. Capaldi and J. Smoly in our laboratory will be reporting on a mechanism for control of the geometric configurational mode of the inner membrane. One of the important components of the control mechanism is a nonmembranous structured system in the matrix space which can undergo changes in state under energizing conditions by interacting with ions such as Pi, Ca^{++}, Mg^{++}, etc. In Fig. 1, we have presented evidence that the Pi-requiring aggregated to twisted configurational transition shows the same dependence on the concentration of Pi as does the P/O ratio for oxidative phosphorylation. We interpret this identity of the dependency curve as an indication of the intimate relation between the configuration of the inner membrane and the ion-dependent state of the nonmembranous structured system.

Acknowledgments

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