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Studies on the Transition of the Cristal Membrane from the Orthodox to the Aggregated Configuration. III. Loss of Coupling Ability of Adrenal Cortex Mitochondria in the Orthodox Configuration

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Abstract

When the cristae of adrenal cortex mitochondria are stabilized in the orthodox configuration by the binding of 20–25 mμmoles/mg protein of either Ca^{2+} or free fatty acids (oleic acid), both the capacity for carrying out coupled reactions and the capacity for undergoing energized configurational transitions are lost. The coupled reactions studied include ATP synthesis, divalent cation translocation, monovalent cation translocation, and reversed electron transfer. The coupled processes and energized configurational changes are fully operative when the cristae of adrenal cortex mitochondria are in the aggregated configuration. However, two processes that have been shown to depend on conformational changes (the anaerobic–aerobic proton ejection and energized accumulation of inorganic phosphate) still proceed when mitochondria are in the orthodox configuration. When the mitochondria are initially in the orthodox configuration, addition of divalent cations (Mg^{2+} or Mn^{2+}) or albumin induces a transition of the cristae to the aggregated configuration and leads to restoration of all the coupled processes. The orthodox to aggregated transition is reversible and the modulation of this reversibility appears to be one of the key points of control in the mitochondrion and possibly of cellular functions.

Introduction

In the second communication of this series[1] evidence was presented that mitochondria of the adrenal cortex‖ could be isolated with cristae either entirely in the orthodox configuration or entirely in the aggregated configuration. The concentration of Ca^{2+} in the isolation medium was one of the determinants of the configuration of the cristae. Depending on the level of free Ca^{2+} in the albumin-free suspending medium, the isolated mitochondria were found to contain amounts of bound Ca^{2+} ranging from about 1 mμmole to about 25 mμmoles/mg protein. Approximately 95% of the mitochondria containing about 25 mμmoles of Ca^{2+}/mg protein were in the orthodox configuration, whereas

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‖ The designation "adrenal cortex mitochondria" always refers to mitochondria of the *zona fasciculata*.

about 95% of the mitochondria were in the aggregated configuration when the Ca^{2+} content was less than 2 mμmoles/mg protein. Ca^{2+} was not the only determinant of the aggregated–orthodox transition. Harding *et al.*[2] and Satre *et al.*[3] have shown that adrenal mitochondria isolated in a medium containing sucrose (0·25–0·27 M), TrisCl, and bovine serum albumin were in the aggregated configuration. When the isolation is carried out in an identical medium except for the presence of albumin, the mitochondria were found to be in the orthodox configuration.[1] We have found at least two determinants of the aggregated–orthodox transition: (a) the level of bound Ca^{2+}, and (b) the level of bound nonesterified fatty acid.

The mitochondria from the adrenal cortex are unusual in two respects. First, these mitochondria can easily be isolated in the orthodox configuration in a medium which is known to compel the aggregated configuration in mitochondrial suspensions of heart, liver, kidney, etc.[4–8] Second, adrenal cortex mitochondria initially in the orthodox configuration can readily be stabilized in that configuration even when electron transfer is proceeding. Although mitochondria from heart, liver, kidney, and other sources can also be stabilized *in vitro* in the orthodox configuration,[5–8] adrenal cortex mitochondria lend themselves more readily to this stabilization. By virtue of this ease of stabilization, we have been able to determine the differences in coupling capability between mitochondria with cristae initially in the orthodox configuration and mitochondria with cristae initially in the aggregated configuration. The present communication is addressed to this comparison and provides evidence that mitochondria with cristae stabilized in the orthodox configuration are completely uncoupled by all criteria and that coupling can be restored by imposing conditions (i.e., addition of Mg^{2+} or removal of the nonesterified fatty acids) which induce the aggregated configuration of the cristae. This totally unexpected property of the orthodox configuration may well be one of the keys to the control of cellular function.

Methods

Isolation of Adrenal Cortex Mitochondria

Two media were used for isolating adrenal cortex mitochondria in the orthodox and aggregated configurations respectively—the ST medium (0·25 M in sucrose and 10 mM in TrisCl, pH 7·8), and the STE medium (0·25 M in sucrose, 10 mM in TrisCl, pH 7·8, and 0·1 mM in Na–K–EDTA). When albumin was present in the former medium, its concentration was 0·25%. The details of the isolation procedure were fully described in a previous communication.[9] Unless otherwise stated the sucrose used in these studies contained 20 mμmoles of Ca^{2+}/250 μmoles as a contaminant.[9] When it was desirable to use Ca^{2+}-free sucrose, the sucrose solutions were treated with Amberlite MB-3 as described earlier.[9]

Preparation of Submitochondrial Particles

Mitochondria were suspended in the appropriate medium at 10 mg/ml and sonicated for 1 min in a Branson Probe Sonifier at maximal intensity. The suspension was then centrifuged for 15 min at 20,000 × *g* in a Spinco No. 40 rotor. The supernatant fluid was centrifuged for 25 min at 50,000 × *g* in a Spinco No. 50 rotor. The pellet was resuspended

in a minimal volume of ST (Ca^{2+}-free) and the Mg^{2+} content determined as described previously.[1]

Enzymic Assays

Oxidative phosphorylation was measured by the conversion of inorganic phosphate to esterified phosphate concomitant with the oxidation of succinate at 25°. The assay system (5 ml) contained 1250 μmoles of Ca^{2+}-free sucrose, 50 μmoles of TrisCl, pH 7·5, 50 μmoles K_2HPO_4 (adjusted to pH 7·4 and labelled with $^{32}P_i$), 5 μmoles of ADP, 20 μg of rotenone, 12·5 μmoles of succinate, and 20–30 mg of mitochondrial protein. Other additions or modifications are listed in the legends of the appropriate figures. The control was incubated without added succinate. When the oxygen in the closed cell was fully consumed (measured by a Clark oxygen electrode), an aliquot was removed from both the control and experimental reaction mixtures and analyzed for esterified phosphate by the method of Lindberg and Ernster.[10] The aqueous phase containing the esterified phosphate was washed twice with butanol saturated with water. Aliquots of the phase containing the esterified phosphate were added to 15 ml of Bray's scintillation fluid containing Cab-O-Sil and the radioactivity was determined in a Packard Tri-Carb Scintillation spectrometer. The esterification of inorganic phosphate was not found to be enhanced by the addition to the medium of yeast hexokinase and glucose.

The valinomycin-dependent translocation of potassium was assayed as described by Blondin *et al.*[11]

The energy-dependent reduction of pyridine nucleotides was assayed as described by Purvis *et al.*[12] in an Aminco Chance dual wavelength spectrophotometer. The assay system was identical with that described by Purvis *et al.*[12], except that $MgCl_2$ was omitted.

The anaerobic–aerobic proton jump was assayed in a medium containing 1250 mμmoles of sucrose (Ca^{2+}-free), 50 μg of rotenone, 25 mg of mitochondrial protein, 50 μg of catalase, and 12 μmoles of K-succinate in 5 ml. When the closed system was anaerobic, oxygen was introduced by the addition of 0·02 ml of H_2O_2 (0·3%). The concentration of m-ClCCP was 10^{-6} M.

The energized binding of inorganic phosphate was determined by the method of Colowick and Womack[13] as modified by Lee *et al.*[14]

The translocation of Ca^{2+}, Mn^{2+}, or Sr^{2+} was assayed essentially as described earlier.[15] In all cases Mg^{2+} was omitted from the incubation system unless otherwise stated. In the assay of Mn^{2+} and Sr^{2+} translocation, ATP and rutamycin were omitted. The incubation time for the translocation of Mn^{2+} and Sr^{2+} was set at 5 min after a 3-min preincubation. In the case of Mn^{2+} translocation the mitochondrial pellet obtained after centrifugation through sucrose was rinsed with fresh sucrose and the tube cut with a razor blade to permit placing the pellet in a plastic tube for counting in a Packard-Auto Gamma Counter.

Determination of Nonesterified Fatty Acids in the Mitochondrion

The method of Novak[16] was used to estimate the nonesterified fatty acid content.

Electron Microscopy

The fixation and treatment of the specimens were carried out exactly as described previously.[9]

Source of Chemicals

The sources of various chemicals used in the present investigation are specified in the parentheses which follow each of the listed chemicals: $^{45}CaCl_2$ (General Electric Irradiation Product Operation, P.O. Box 846, Pleasanton, Calif.); sucrose and Amberlite MB-3 resin (Mallinckrodt Chemical Works, St. Louis, Mo.); $^{54}MnCl_2$ and $^{85}SrCl_2$ (New England Nuclear Corp., Boston, Mass.); redistilled glutaraldehyde (Poly Sciences, Rydol, Pa.); rutamycin (Eli Lilly Pharmaceutical Co., Indianapolis, Ind.); rotenone (Aldrich Chemical Co., Milwaukee, Wis.); antimycin (Wisconsin Alumni Research Foundation, Madison, Wis.), valinomycin (California Corporation for Biochemical Research, Los Angeles, Calif.); and Bovine Serum Albumin low in fatty acid (Mann Research Lab, New York, N.Y.).

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

Results

Coupling Capability of Mitochondria with Cristae in the Aggregated or Orthodox Configuration

Oxidative phosphorylation. Adrenal cortex mitochondria isolated in the STE medium, and preponderantly in the aggregated configuration, couple the oxidation of succinate to the esterification of inorganic phosphate with P/O ratios in the range of 1·2–1·6 (see Table I). By contrast, mitochondria isolated from the same source in the ST medium

TABLE I. The relation between configurational state and the P/O ratio

Additions*	Configuration		P/O†
	Initial	Final	
None	Aggregated	Aggregated	1·30
$MgCl_2$ (2 mM)	Aggregated	Aggregated	1·35
None	Orthodox	Orthodox	0·08
$MgCl_2$ (2 mM)	Orthodox	Aggregated	1·11

* The addition of $MgCl_2$ was made about 1 min prior to the addition of succinate.

† The substrate used was succinate.

and preponderantly in the orthodox configuration show negligible capacity for oxidative phosphorylation. The P/O ratios for such mitochondria ranged from 0·01 to 0·18 (see Table I). In both cases Mg^{2+} was omitted from the incubation systems. When Mg^{2+} (2 mM) was added to the assay medium, the P/O ratios for mitochondria initially in the aggregated configuration remained unchanged or were slightly increased. However, after addition of Mg^{2+} (2 mM) to mitochondria initially in the orthodox configuration, the P/O ratios then observed approached the ratios for mitochondria in the aggregated configuration. The requirement for exogenous Mg^{2+} in mitochondria in the orthodox configuration for oxidative phosphorylation is not due to a deficiency of endogenous Mg^{2+}, since mitochondria isolated in either configuration contained essentially the same

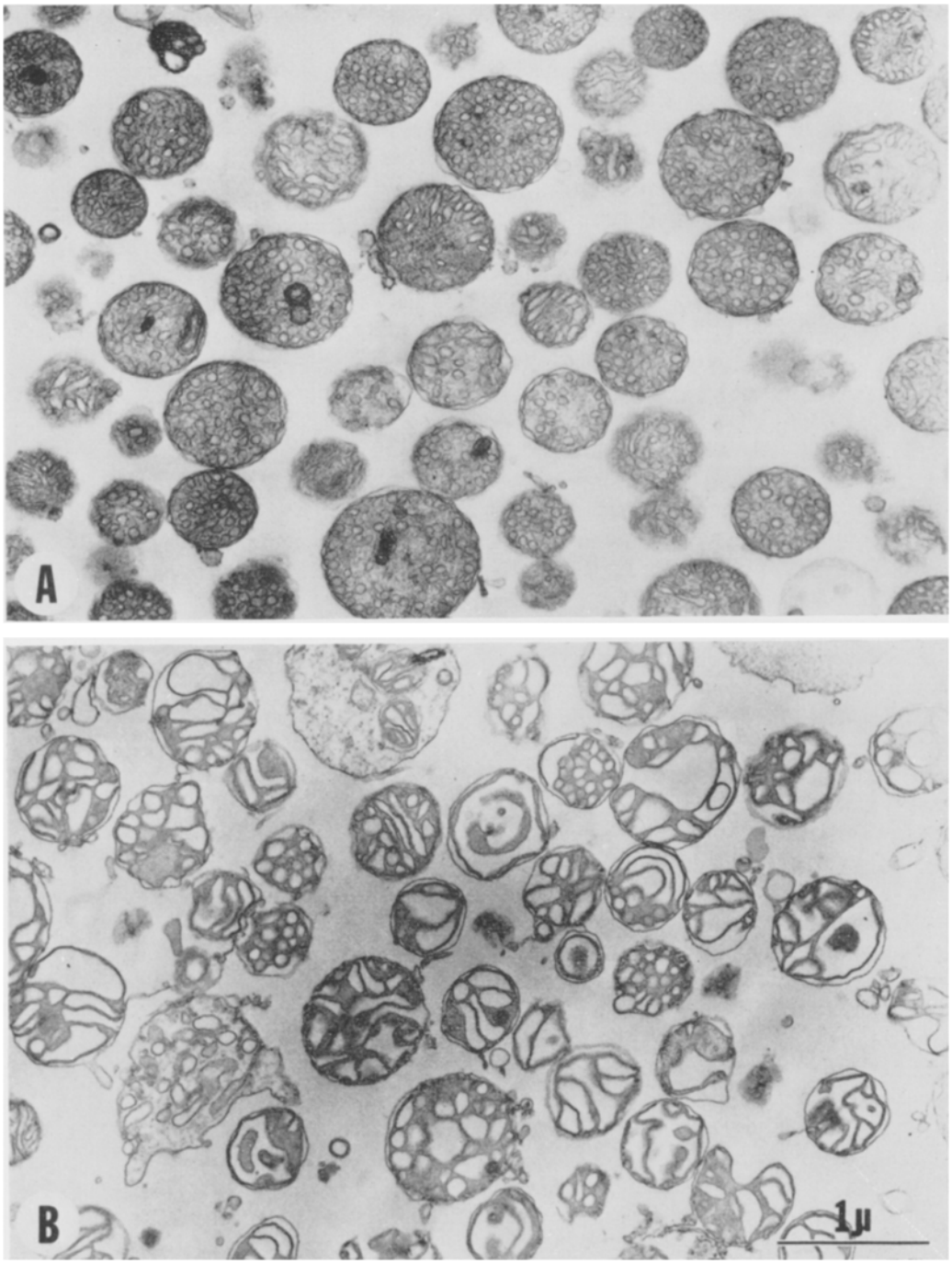

Figure 1. Electron micrographs of adrenal cortex mitochondria isolated in the STE medium. (A) Mitochondria (4 mg/ml) were incubated at 25° in 5 ml of ST–Ca^{2+}-free medium containing 10 mM phosphate, pH 7·5, 50 μg of rotenone and 2·5 mM succinate for 1 min prior to fixation with glutaraldehyde (38546). (B) Mitochondria incubated under the above conditions, except that $MgCl_2$ (2 mM) was added to the incubation medium (38958).

amount of bound Mg^{2+}.[1] Therefore, the requirement of oxidative phosphorylation for exogenous Mg^{2+} most probably has to be explained in terms other than variation in the level of endogenous Mg^{2+}. As shown in a previous communication,[1] the addition of Mg^{2+} or Mn^{2+} to mitochondria with cristae in the orthodox configuration transformed these cristae to the aggregated configuration, but these ions had no effect on the configuration of mitochondria in the aggregated configuration. We then examined the ultrastructure of orthodox mitochondria before and after addition of Mg^{2+}. The effect of exogenous Mg^{2+} on the configurational transition is illustrated in Fig. 1. Mitochondria initially in the orthodox configuration [Fig. 1(A)] had a P/O ratio of 0·08, whereas addition of exogenous Mg^{2+} (2 mM) induced the formation of the aggregated configuration [Fig. 1(B)] and restored the P/O ratio to the normal level (in this experiment the P/O ratio was 1·2). It should be noted that the oxidation rates (with succinate) were slightly increased in the orthodox mitochondria compared to those of the mitochondria in the aggregated configuration. The decline in the rate of phosphorylation parallels the decline in the P/O ratio.

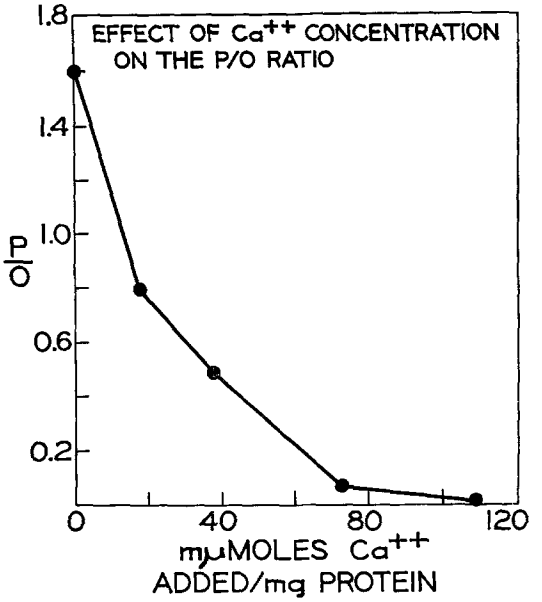

Figure 2. The effect of addition of $CaCl_2$ on the P/O ratio. Mitochondria initially in the aggregated configuration were assayed for oxidative phosphorylation as described in the methods section, except that graded amounts of $CaCl_2$ were added prior to the addition of succinate.

There is an alternative method of demonstrating the uncoupled state of orthodox mitochondria. We have previously shown that the addition of graded amounts of $CaCl_2$ to mitochondria initially in the aggregated configuration (even in the presence of rotenone, antimycin and rutamycin) will compel an increasing proportion of the mitochondria to be in the orthodox configuration.[1] When mitochondria in the aggregated configuration were titrated with $CaCl_2$, a progressive reduction in the P/O ratio (Fig. 2), and the rate of phosphorylation was observed. When the level of bound Ca^{2+} was 20–25 mμmoles/mg protein (this level was achieved by the addition of 75 mμmoles of $CaCl_2$ to the medium for each milligramme of mitochondrial protein), the P/O ratio declined to about one-twentieth of its original value, i.e., to 0·08 as compared to 1·5. It is to be noted that prior to the addition of Ca^{2+}, the mitochondria showed no requirement for added Mg^{2+} in the assay of oxidative phosphorylation. Although not shown in Table I, the addition of Mg^{2+} to mitochondria in the orthodox configuration by virtue of the prior addition of Ca^{2+}, restored the capability for coupled phosphorylation (P/O ratios between 1·0 and 1·4). The concentration of added Ca^{2+} required for uncoupling of ATP synthesis is also modulated by the sucrose concentration. For example, when mitochondria were suspended in 0·25 M sucrose the addition of 75 mμmoles of Ca^{2+}/mg protein resulted in 95% inhibition of ATP synthesis, whereas only 35 mμmoles of

Ca^{2+}/mg protein were required for 95% inhibition when the sucrose concentration of the suspending medium was 0·15 M.

A similar correlation can be established between the uncoupling of ATP synthesis by the addition of Zn^{2+} to mitochondria initially in the aggregated configuration and the transformation of the cristae to the orthodox configuration. For example, addition of $ZnCl_2$ (200 mμmoles/mg protein) reduced the P/O ratio from 1·2 to 0·24. The addition of Zn^{2+} at this level to mitochondria in the aggregated configuration was shown by us earlier to result in the formation of the orthodox configuration.[1]

The high coupling efficiency seen after the addition of Mg^{2+} or Mn^{2+} to mitochondria in the orthodox configuration was also observable after the addition of serum albumin which was low in fatty acid (see Fig. 3). The addition of albumin had only a small stimulatory effect on the P/O ratio of mitochondria in the aggregated configuration. This was similar to the small stimulatory effect of Mg^{2+} on the P/O ratio in mitochondria in the aggregated configuration. The ability of albumin to restore normal P/O ratios in the mitochondria initially in the orthodox configuration suggested that the concentration of nonesterified fatty acids might be different in the two types of mitochondria. The nonesterified fatty acid content was determined in mitochondria isolated in either the ST or STE media: the nonesterified fatty acid concentration was found to be 16·0 mμmoles/mg protein in mitochondria prepared in STE and 25·5 mμmoles/mg protein in mitochondria prepared in ST. These values were the average of three determinations. When mitochondria initially in the aggregated configuration were exposed to oleic acid at a concentration of 10–15 mμmoles/mg protein, the P/O ratio was reduced to 0·05 times the original values, i.e., to about 0·05. The uncoupling by exogenous fatty acids was not limited to unsaturated fatty acids, since addition of palmitic acid gave similar results. The addition of $MgCl_2$ to mitochondria exposed to oleate completely reversed the uncoupling presumably by forming an insoluble salt of the oleate (see Fig. 4). Harding *et al.*[2] and Satre *et al.*[3] have observed that adrenal cortex mitochondria isolated

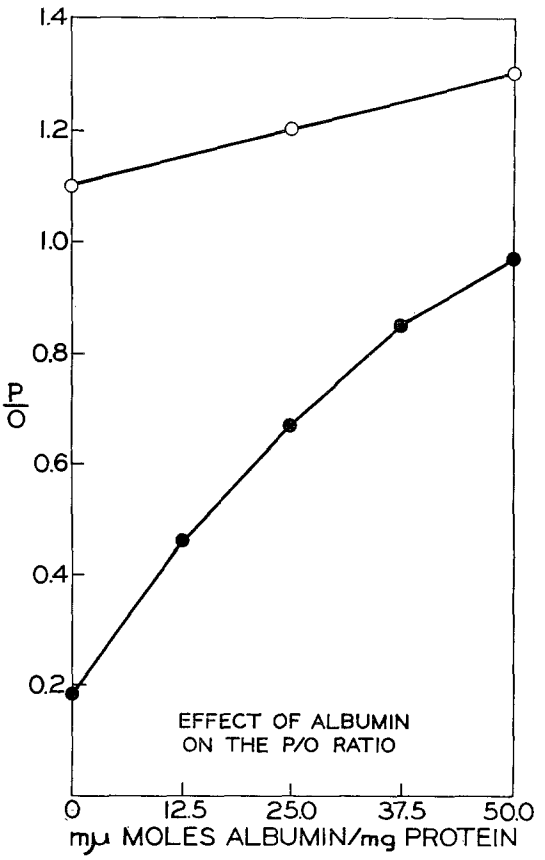

Figure 3. The effect of bovine serum albumin on the P/O ratio. The conditions were the same as indicated in the legend of Fig. 2, except that graded amounts of albumin was added prior to succinate. The open circles represent the P/O ratio obtained when the mitochondria tested were initially in the aggregated configuration. The solid circles correspond to the P/O ratios for mitochondria which were initially in the orthodox configuration. Note that the sample of albumin used in these experiments was low in fatty acid content.

in the presence of albumin (and in absence of EDTA) were in the aggregated configuration. We also have observed that replacement of EDTA by albumin in the isolation medium lowers the concentration of nonesterified fatty acids from 25 mμmoles/mg protein to 10 mμmoles/mg protein, but has no effect on the Ca^{2+} level; the mitochondria thus isolated were in the aggregated configuration. The fact that the configuration of the mitochondria was affected by the level of bound nonesterified fatty acids was the stimulus for determining whether the addition of Ca^{2+} to mitochondria, low in both Ca^{2+} and nonesterified fatty acids, would increase the level of nonesterified fatty acids. When Ca^{2+} (100 mμmoles/mg protein) was added, in the presence of rotenone, antimycin, and rutamycin, to mitochondria in the aggregated configuration, there was an immediate (within 10 sec) increase in the nonesterified fatty acid content from 10 mμmoles to 30 mμmoles/mg protein. Thus, the induction of the orthodox configuration by Ca^{2+} may in fact be due to the generation of nonesterified fatty acids followed by the formation of the orthodox configuration. The nonenergized proton ejection previously observed following the addition of Ca^{2+} to the mitochondria in the aggregated configuration[1] was also observable following the addition of oleate. The data in Table II summarize the levels of Mg^{2+}, Ca^{2+}, and nonesterified fatty acids observed in mitochondria in either configuration.

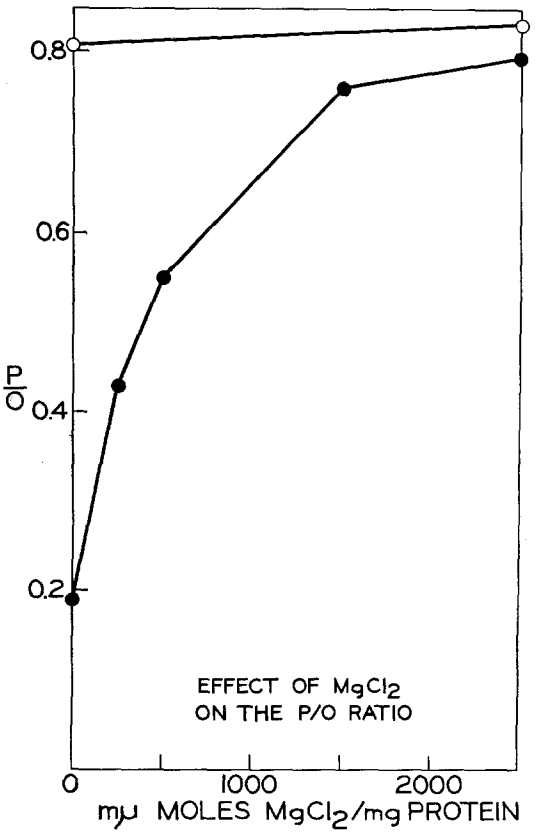

Figure 4. Effect of $MgCl_2$ on the P/O ratio of mitochondria previously exposed to oleate. The conditions were the same as those indicated in the legend of Fig. 2, except that 15 mμmoles of oleate per milligramme protein were first added to mitochondria in the aggregated configuration and then later graded amounts of $MgCl_2$ were added prior to initiation of the assay by addition of succinate. The open circles represent the P/O ratios of the mitochondria which were not pretreated with oleate. The solid circles represent the P/O ratio for mitochondria previously exposed to oleate.

Although we have not observed a significant change in the level of Mg^{2+} in the two configurations, there was a significant change in the Mg^{2+} content of submitochondrial particles (ETP) prepared from mitochondria in each of the two configurations. When the mitochondria were in the orthodox configuration (either by isolation in the ST medium or by addition of Ca^{2+} to mitochondria in the aggregated configuration), the Mg^{2+} content of the ETP particles derived therefrom was between 25–30 mμmoles/mg protein. However, when the ETP particles were prepared from mitochondria in the aggregated configuration (prepared in either STE or ST + BSA), the Mg^{2+} content of ETP was about 65–70 mμmoles/mg protein. Thus, some Mg^{2+} in mitochondria with cristae in the orthodox configuration might have been dislodged by the high fatty acids.

TABLE II. The content of Mg^{2+}, Ca^{2+}, and nonesterified fatty acids (mμmoles/mg protein) in mitochondria in either the aggregated or orthodox configurations

Configuration of the mitochondrion	Mg^{2+}	Ca^{2+}	Nonesterified fatty acids
Orthodox (ST)*	21·8	22·5	25·5
Orthodox (STE + Ca)	22·0	25·0	30·0
Aggregated (STE)	18·0	2·4	15·0
Aggregated (ST + BSA)	20·8	24·3	10·5

* The abbreviations in parentheses describe the composition of the isolation medium.

Energized configurational changes in adrenal cortex mitochondria. It has been well documented that isolated mitochondria undergo configurational changes concomitant with changes in the energy state of the mitochondria.[5–8, 17–19] In all these studies of configurational changes, the mitochondria, whether of heart muscle or liver, were invariably in the aggregated configuration.[5] The possibility of studying configurational changes in isolated mitochondria initially in the orthodox or aggregated configuration was first presented by adrenal cortex mitochondria. It was demonstrated that adrenal cortex mitochondria initially in the orthodox configuration did not undergo configurational change when energized by succinate in presence of inorganic phosphate and then later exposed to the action of uncoupler (see Fig. 5). The orthodox configurational pattern was maintained whether substrate plus phosphate were present or not, or whether uncoupler was present or not. If there were any changes in configuration induced by energizing conditions, they would have to be of a subtle nature. However, mitochondria

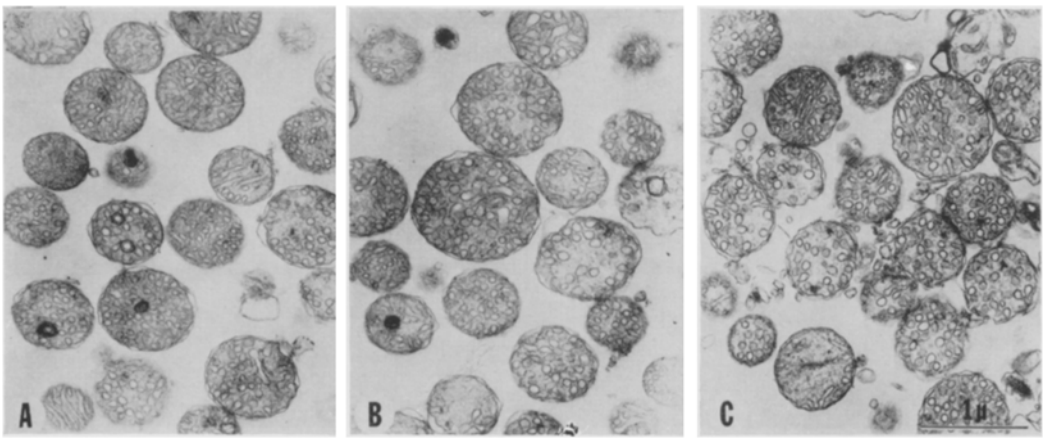

Figure 5. Configurational changes in mitochondria initially in the orthodox configuration. (A) Mitochondria (4 mg/ml) were incubated at 25° for 2 min in the presence of 0·25 M sucrose (Ca^{2+}-free), 10 mM TrisCl, pH 7·5, 50 μg of rotenone, 50 μg of antimycin, and 50μg of rutamycin (35063). (B) Mitochondria (4 mg/ml) were incubated at 25° for 2 min in the presence of 0·25 M sucrose (Ca^{2+}-free), 10 mM TrisCl, pH 7·5, 10 mM KPO_4, pH 7·5, 2·5 mM succinate and 50 μg of rotenone (35071). (C) The same as (B), except that m-CLCCP (10^{-6} M) was added at the end of the 2-min incubation and the incubation continued for 1 min longer (35070).

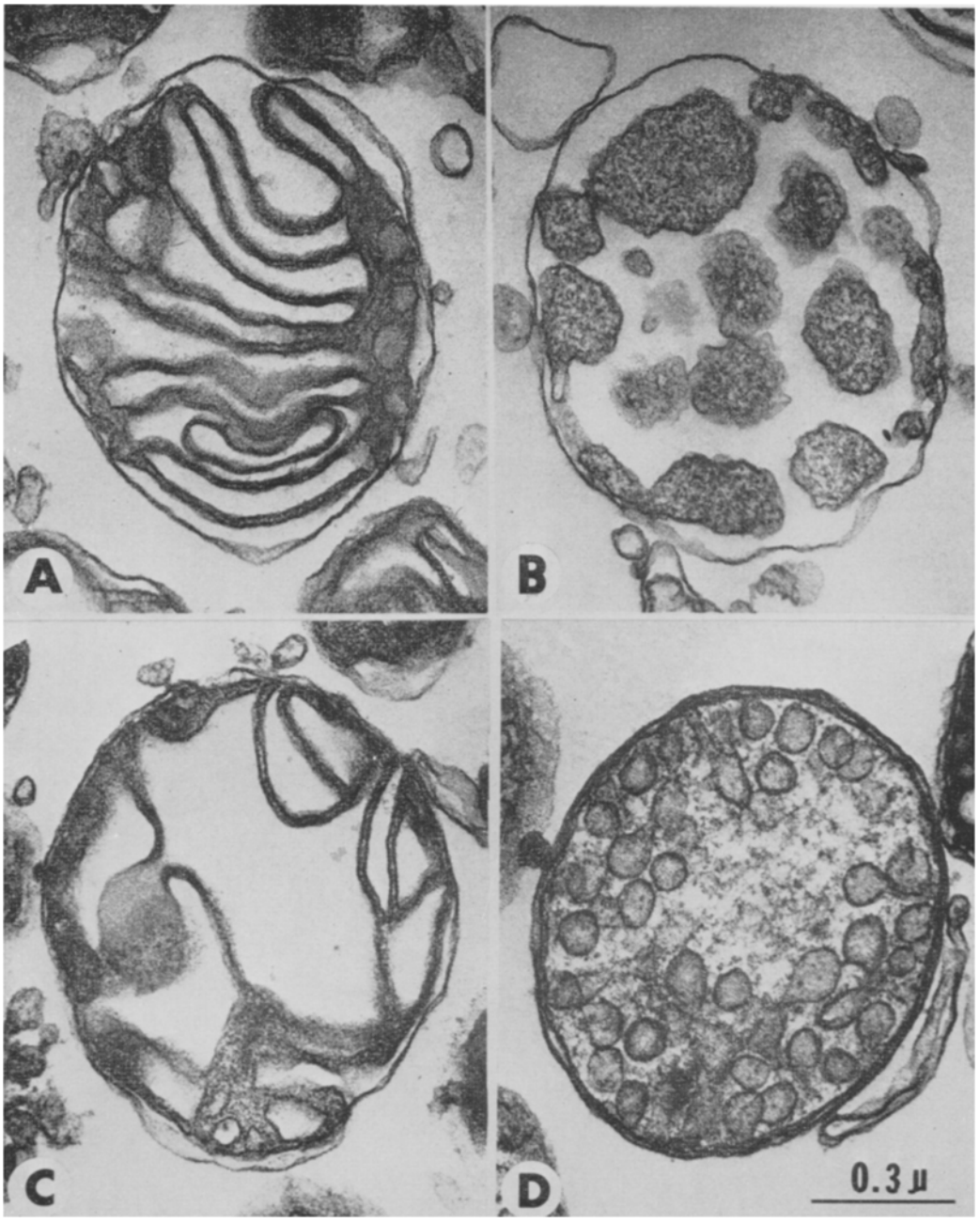

Figure 6. Configurations of mitochondria initially in the aggregated configuration. (A) A single mitochondrion in the nonenergized configuration (38958). (B) A single mitochondrion in the energized–twisted configuration (38801). (C) A single mitochondrion in the nonenergized configuration (38787). (D) A single mitochondrion in the orthodox configuration (38759).

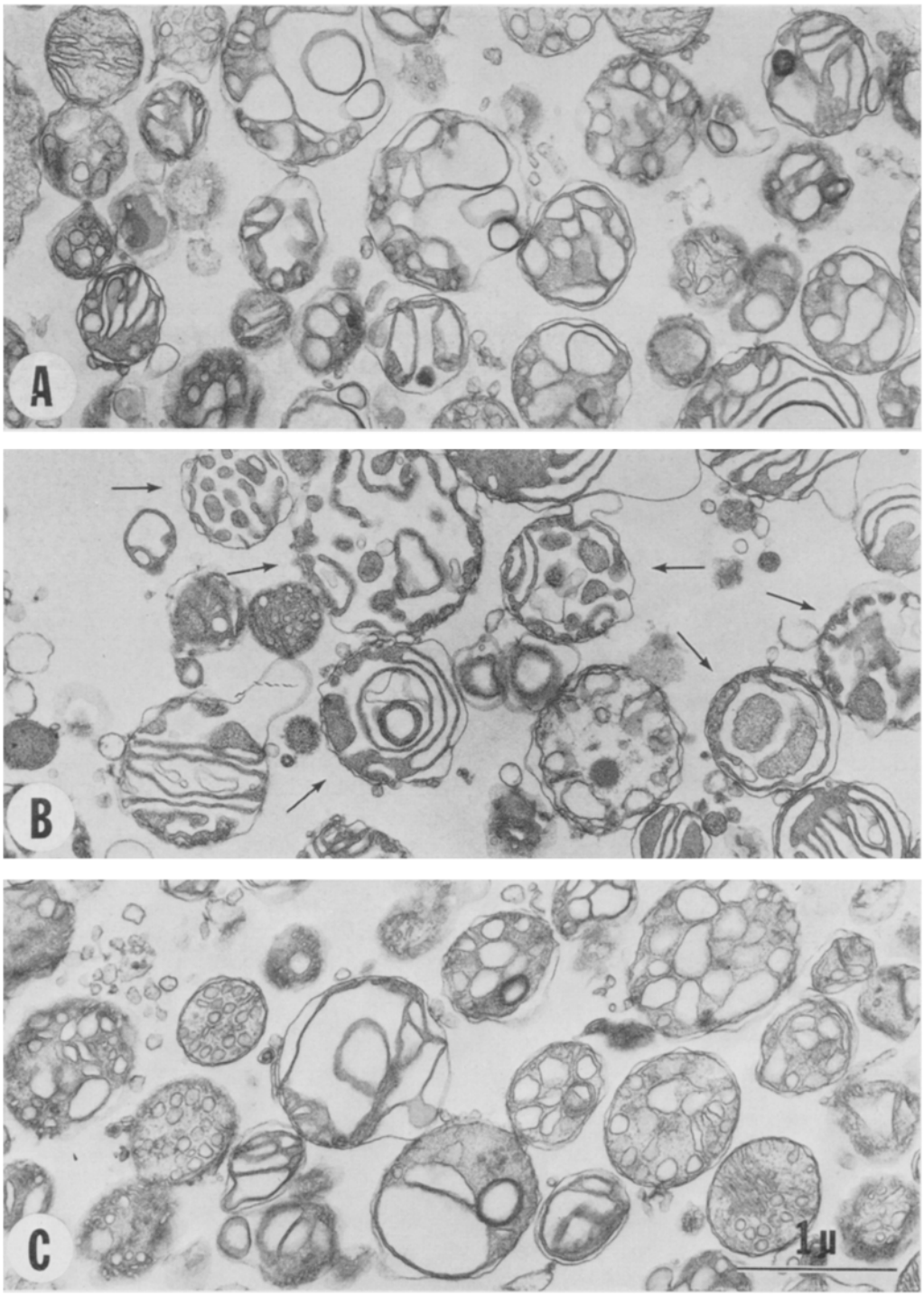

Figure 7. Configurational changes in mitochondria initially in the aggregated configuration. The conditions for incubation of the three samples were the same as those described in the legend of Fig. 5. (A) Incubated in the presence of rotenone, antimycin, and rutamycin (31180). (B) Incubated in the presence of rotenone, succinate, and phosphate (38801). (C) Incubated in the presence of rotenone, succinate, and phosphate and then exposed to m-ClCCP (38763).

initially in the nonenergized aggregated configuration [Figs. 6(A) and 7(B)] undergo a transition to the energized-twisted configuration [Figs. 6(A) and 7(B)] when exposed to substrate in presence of inorganic phosphate. When adrenal cortex mitochondria in the energized–twisted configuration are exposed to the action of uncoupler, about half the mitochondrial population is discharged to the nonenergized, aggregated configuration [Figs. 6(C) and 7(C)] and the rest to the nonenergized, orthodox configuration [Figs. 6(D) and 7(C)]. The large fields of mitochondria are shown in Fig. 7. The individual mitochondria shown in Fig. 6 are representative of the large field. Whatever the technical difficulties in demonstrating the configurational cycle in the aggregated configuration, the important point is that no such cycle can be observed when mitochondria are initially in the orthodox configuration. Since these mitochondria are uncoupled, it was not surprising that they did not show any configurational changes. The orthodox mitochondria will undergo energized configurational changes only after transformation to the aggregated configuration by the addition of reagents such as Mg^{2+}, Mn^{2+} or albumin. These experiments illustrate that the cycle of configurational change from nonenergized to energized–twisted and then back again to the nonenergized configuration is observable only in mitochondria that are capable of coupled oxidative phosphorylation and is not observable in mitochondria that are uncoupled.

Assay of Other Coupled Processes

Translocation of divalent cations. The translocation of divalent cations such as Mg^{2+}, Ca^{2+}, Mn^{2+}, and Sr^{2+} in heart and liver mitochondria is energized either by electron transfer or ATP hydrolysis.[20–23] When attempts were made to demonstrate active translocation of Ca^{2+} (either substrate or ATP driven) in adrenal cortex mitochondria, it was found that only small amounts of Ca^{2+} were translocated unless $MgCl_2$ (3–10 mM) was added to the medium (see Table III). The addition of Mg^{2+} resulted in a ten- to fifteen-fold increase in Ca^{2+} translocation regardless of the initial configuration of the mitochondria. In a previous section we have presented evidence that mitochondria in the aggregated configuration exhibit no requirement of exogenous Mg^{2+} for oxidative phosphorylation, whereas mitochondria in the orthodox configuration do. However, exogenous Mg^{2+} was required for Ca^{2+} translocation whether the mitochondria were initially in the aggregated or orthodox configuration. What would account for this difference in the Mg^{2+} requirement for these two coupled processes? The answer emerged from the examination of the ultrastructure of the mitochondria before and after initiation of Ca^{2+} translocation.

TABLE III. Ca^{2+} translocation as a function of the configurational state

Additions	Configuration of the mitochondrion		Ca^{2+} translocation (mμmoles/2 min/mg protein)
	Before Ca^{2+} addition	After Ca^{2+} addition	
None	Aggregated	Orthodox	22
$MgCl_2$ (10 mM)	Aggregated	Aggregated	352
None	Orthodox	Orthodox	18
$MgCl_2$ (10 mM)	Aggregated*	Aggregated	342

* These mitochondria were initially in the orthodox configuration prior to addition of $MgCl_2$.

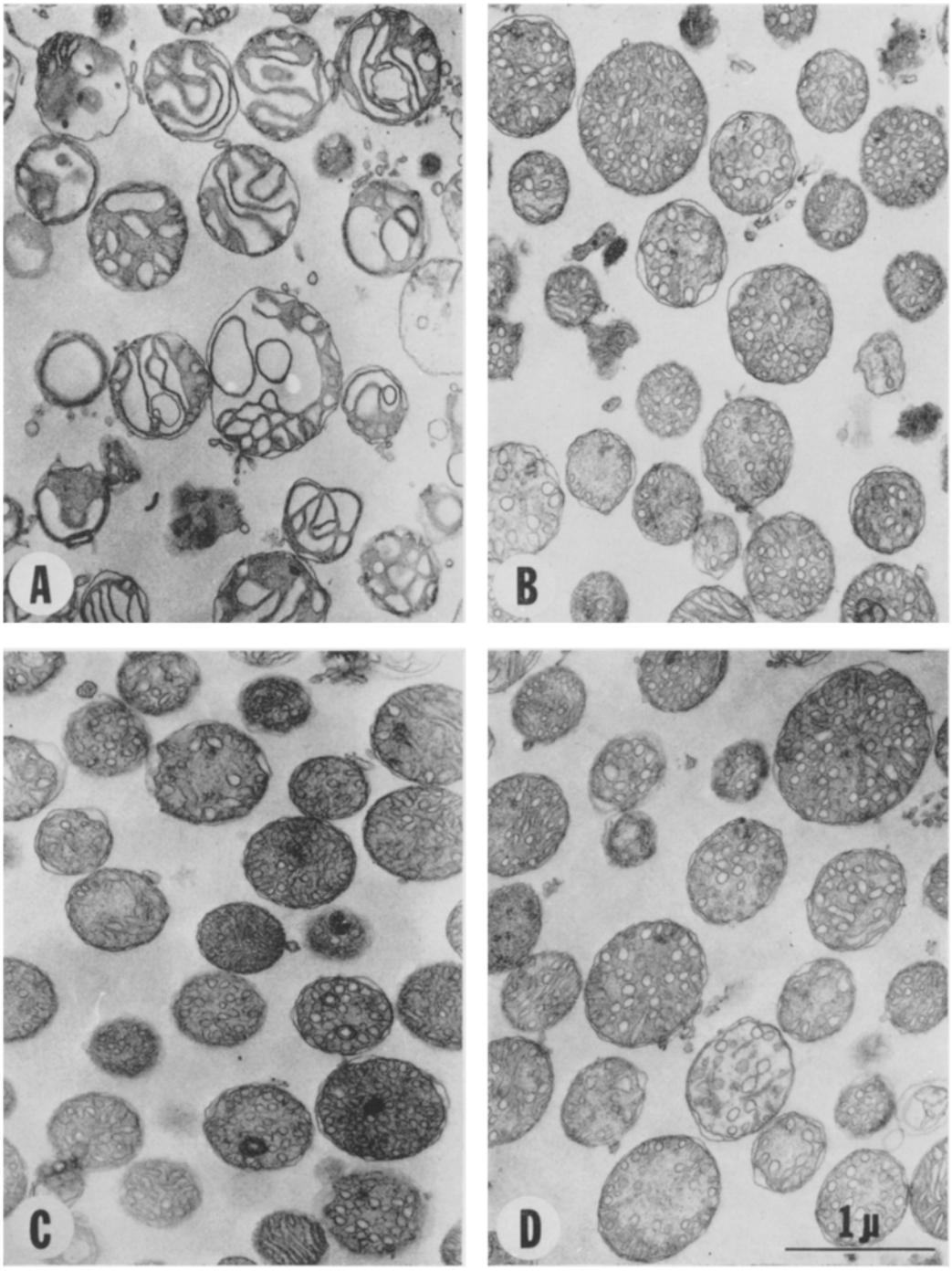

Figure 8. Electron micrographs of mitochondria incubated under the same conditions as were used for testing Ca^{2+} translocation. (A) Mitochondria initially in the aggregated configuration after incubation for 3 min at 30° in the absence of Mg^{2+} and Ca^{2+} (38544). (B) Mitochondria as in (A), 2 min after addition of $CaCl_2$ (1 mM) (38957). (C) Mitochondria initially in the orthodox configuration after incubation for 3 min at 38° in the absence of Mg^{2+} and Ca^{2+} (38950). (D) *Mitochondria as in* (C), 2 min after addition of $CaCl_2$ (1 mM) (38963). See the method section for assay conditions.

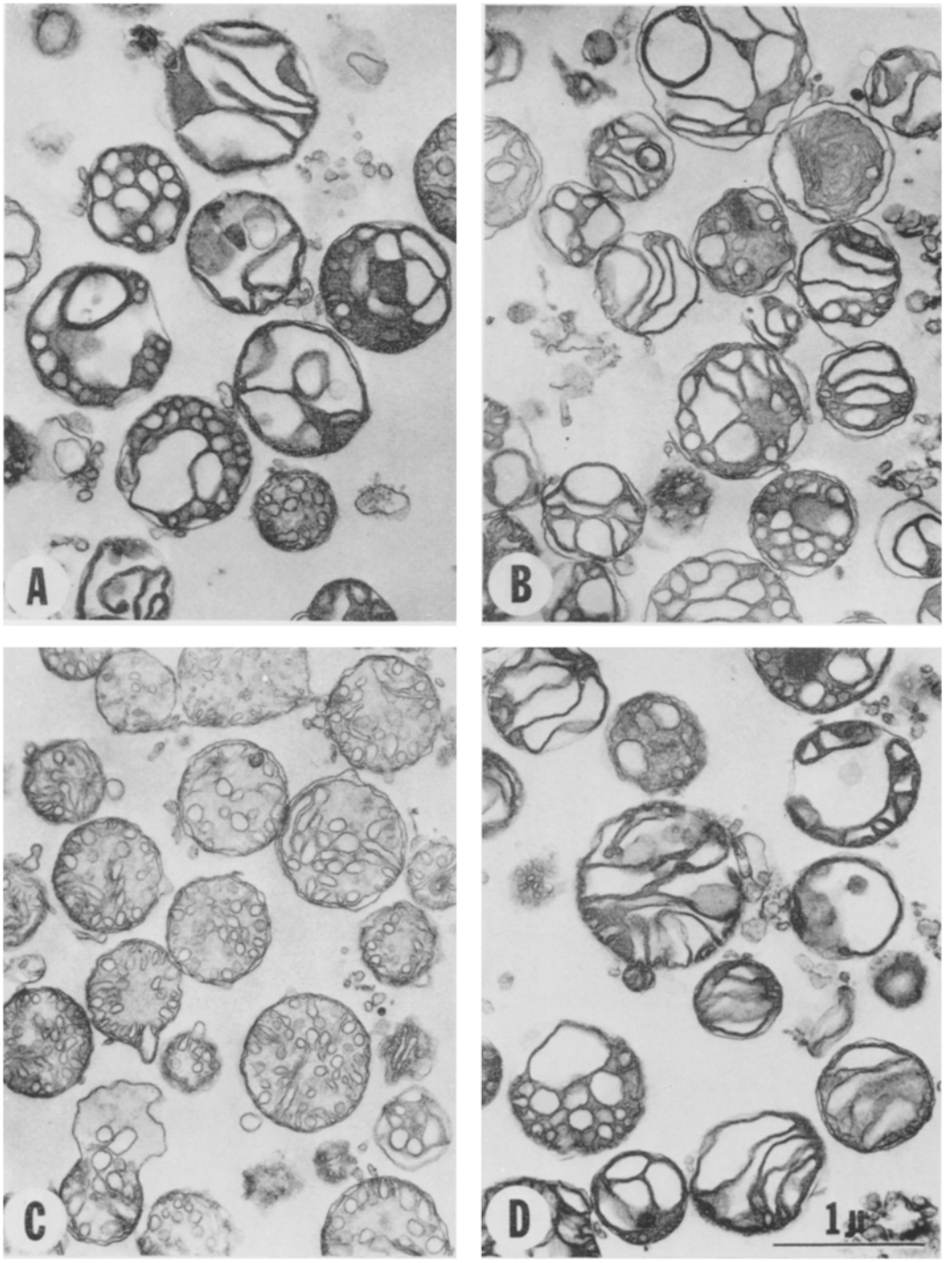

Figure 9. Electron micrographs of mitochondria incubated under the same conditions as were used for assay of Ca^{2+} translocation with and without supplementation by $MgCl_2$. (A) Mitochondria initially in the aggregated configuration after incubation for 3 min at 38° in the absence of $MgCl_2$ (10 mM) (38908). (B) Mitochondria as in (A), 2 min after addition of $MgCl_2$ (10 mM) and $CaCl_2$ (1 mM) (38906). (C) Mitochondria initially in the orthodox configuration incubated for 3 min in the absence of $MgCl_2$ (10mM) (38960). (D) Mitochondria as in (C), 2 min after addition of $MgCl_2$ and $CaCl_2$ (38905).

When mitochondria initially in the aggregated configuration were incubated in the absence of Mg^{2+} and Ca^{2+}, the mitochondria remained in the aggregated configuration [Fig. 8(A)]. After addition of the $CaCl_2$ to initiate Ca^{2+} translocation, the mitochondria underwent transformation to the orthodox configuration [see Fig. 8(B)]. When mitochondria initially in the orthodox configuration were incubated in the absence of Ca^{2+} and Mg^{2+}, the mitochondria remained orthodox [see Fig. 8(C)]. Moreover, the addition of Ca^{2+} to these mitochondria did not lead to a change in the configuration [Fig. 8(D)]. When Ca^{2+} translocation was assayed under identical conditions (i.e., in absence of added Mg^{2+}), only about 10–20 mμmoles of Ca^{2+} was translocated per milligramme protein whether the mitochondria were initially in the orthodox or aggregated configuration. Thus, the inability of adrenal cortex mitochondria to translocate Ca^{2+} could be correlated with the orthodox configuration. Addition of $MgCl_2$ (10 mM) to the assay system resulted in a ten- to fifteen-fold increase in Ca^{2+} translocation and the formation of the aggregated configuration. The micrographs of Fig. 9 illustrate that the addition of $MgCl_2$ maintains the mitochondria in the aggregated configuration even after the addition of $CaCl_2$ (1 mM).

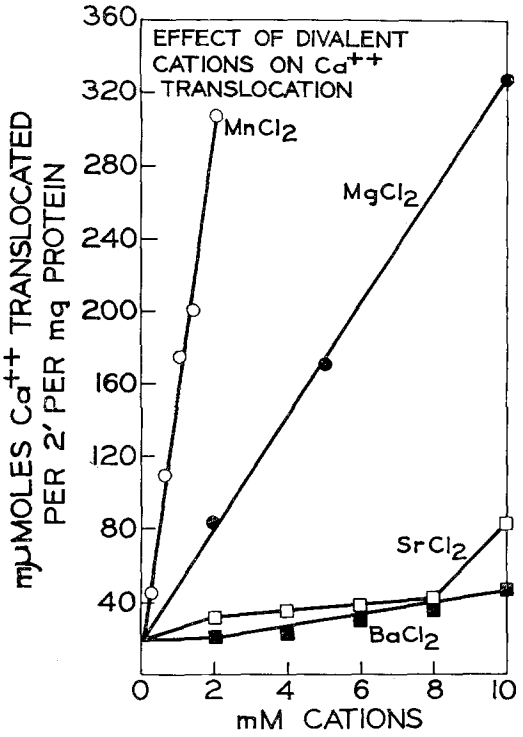

Figure 10. Effect of Mg^{2+}, Mn^{2+}, Sr^{2+}, and Ba^{2+} on Ca^{2+} translocation. Mitochondria in the orthodox configuration were assayed for Ca^{2+} translocation. The addition of Mg^{2+}, Mn^{2+}, Ba^{2+}, or Sr^{2+} were made at the start of the preincubation (i.e., 3 min prior to the addition of $CaCl_2$).

TABLE IV. $MnCl_2$ translocation in adrenal cortex mitochondria

Initial configuration of the mitochondrion	Additions	Mn^{2+} Translocated (mμmoles/min/mg protein)
Orthodox	Succinate	79·0
Orthodox	Succinate, $MgCl_2$ (10 mM)	79·0
Orthodox	Succinate, $CaCl_2$ (3 mM)	20·0
Orthodox	Succinate, $CaCl_2$ (3 mM), $MgCl_2$ (5 mM)	45·0
Aggregated	Succinate	120
Aggregated	Succinate, $MgCl_2$ (10 mM)	79·0
Aggregated	Succinate, $CaCl_2$ (3 mM)	21·0
Aggregated	Succinate, $CaCl_2$ (3 mM), $MgCl_2$ (5 mM)	65·0

The concentration of Mn^{2+} in all experiments was 1mM.

Other divalent cations can replace Mg^{2+} in inducing the aggregated configuration[1] and supporting Ca^{2+} translocation. Figure 10 illustrates the effectiveness of Mn^{2+}, Ba^{2+}, and Sr^{2+} in supporting Ca^{2+} translocation. It can be seen from this data that Mn^{2+} is much more effective than Mg^{2+} in supporting Ca^{2+} translocation, whereas Sr^{2+} and Ba^{2+} are much less effective. We would infer from these observations that Sr^{2+} and Ba^{2+} are not as effective as Mn^{2+} or Mg^{2+} in inducing the aggregated configuration.

In a previous communication[1] evidence was presented that Mn^{2+} could induce the transition of cristae from the orthodox to aggregated configuration. The data in Table IV establish that Mn^{2+} can be translocated regardless of the initial configurational state of the mitochondria and that exogenous Mg^{2+} is not required for this translocation. Addition of $CaCl_2$ [at a molar ratio (Ca^{2+}:Mn^{2+}) of 3:1] resulted in a 70–80% decrease in the extent of Mn^{2+} translocation. Under these conditions the addition of Mg^{2+} was required to achieve maximal translocation of Mn^{2+}.

TABLE V. $SrCl_2$ translocation in adrenal cortex mitochondria

Initial configuration	Additions	Sr^{2+} Translocated (mμmoles/min/mg protein)
Orthodox	Succinate	1·5
Orthodox	Succinate, $MgCl_2$ (1·66 mM)	56·8
Orthodox	Succinate, $CaCl_2$ (0·3 mM)	0·5
Aggregated	Succinate	49·5
Aggregated	Succinate, $MgCl_2$ (1·66 mM)	49·5
Aggregated	Succinate, $CaCl_2$ (0·3 mM)	0·5

The concentration of Sr^{2+} used in all these experiments was 1 mM.

The translocation of Sr^{2+} was unaffected by the addition of Mg^{2+} when mitochondria were initially in the aggregated configuration, whereas Mg^{2+} was required when the mitochondria were initially in the orthodox configuration (see Table V). As previously documented, Sr^{2+} is not as effective as Mg^{2+} or Mn^{2+} in inducing the aggregated configuration. Therefore, it was not unexpected that the translocation of Sr^{2+} (1 mM final concentration) required the presence of Mg^{2+} in the medium. It should be noted that the Mg^{2+} concentration required for maximal Sr^{2+} translocation was several times lower than that required for Ca^{2+} translocation (see Fig. 10).

Translocation of K^+. As with other mitochondria (e.g., heart and liver), adrenal cortex mitochondria are capable of translocating K^+ under energizing conditions in the presence of valinomycin and a permeant anion (e.g., acetate). Just as for the other coupled processes (divalent cation translocation and ATP synthesis) the capacity for translocation of K^+ was dependent on the initial configuration of the cristae. When the mitochondria were initially in the aggregated configuration about 1100 mμmoles of K^+ were translocated per milligramme protein. The extent of translocation of K^+ was reduced to about 60 mμmoles/mg protein when the mitochondria were initially in the orthodox configuration. However, the addition of $MgCl_2$ (2 mM) to mitochondria in the orthodox configuration increased the extent of K^+ translocation to about 600 mμmoles/mg protein.

The inhibition of K^+ translocation by the addition of Ca^{2+} to mitochondria initially in the aggregated configuration is illustrated in Fig. 11.

Reversed electron transfer. Several investigators[2,12] have observed that adrenal cortex mitochondria are capable of carrying out an energy-dependent reduction of pyridine nucleotides by succinate. In order to demonstrate this process consistently it was necessary sometimes to include bovine serum albumin in the assay medium.[12] As shown above, this is the very condition that maintains mitochondria in the aggregated configuration. It was also noted by Harding *et al.*[2] that mitochondria aged at room temperature lose the capacity for reversed electron transfer. Aging has been shown to lead to the formation of the orthodox configuration.[3] In view of the sensitivity of reversed electron transfer to the aging of mitochondria, we assayed this coupled process in mitochondria in each of the two configurational states. The data in Fig. 12 illustrates that adrenal cortex mitochondria in the aggregated configuration catalyze an extensive reduction of pyridine nucleotides by succinate in the presence of ATP. However, mitochondria in the orthodox configuration lack this catalytic capability when assayed under the same conditions. Thus, reversed transfer is another coupled process that does not proceed when mitochondria are in the orthodox configuration.

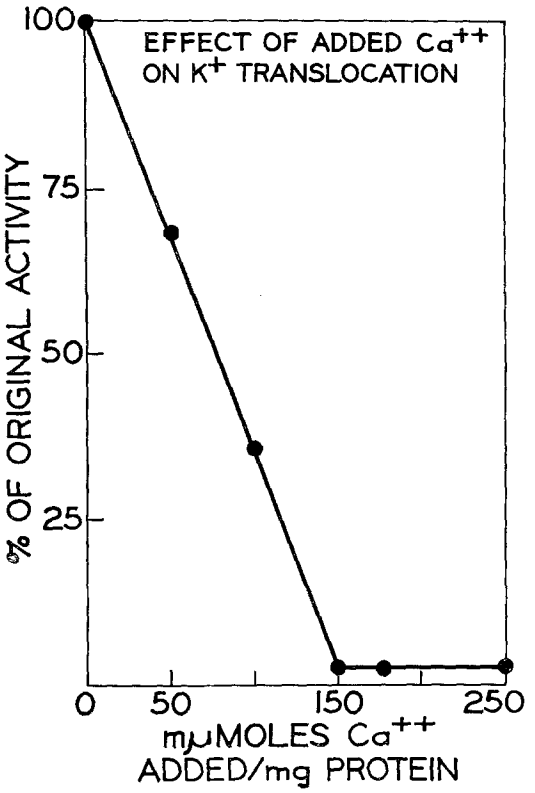

Figure 11. Effect of addition of Ca^{2+} on K^+ translocation. Mitochondria initially in the aggregated configuration were assayed for K^+ translocation in the presence of KOAc, valinomycin, malate, and glutamate as described by Blondin *et al.*[11] The addition of $CaCl_2$ was made 1 min prior to the addition of valinomycin.

Energized Conformational Changes

Anaerobic to aerobic proton jump. When oxygen is introduced into anaerobic suspension of beef heart mitochondria containing substrate, protons are ejected. This substrate-dependent ejection of protons is accounted for in terms of a conformational change undergone by the tripartite repeating unit during the nonenergized to energized transition.[24] In view of the inability of adrenal cortex mitochondria in the orthodox configuration to undergo configurational change or to carry out coupled reactions when exposed to substrate, it was necessary to ascertain whether the proton jump was affected by the orthodox to aggregated transition. The data illustrated in Fig. 13 show that when the mitochondria are initially in the orthodox configuration, the proton jump is actually greater than the proton jump when the mitochondria are initially in the aggregated configuration. A similar result was found to obtain for liver mitochondria in the orthodox configuration.[25]

Energized phosphate binding. Heart mitochondria have been shown to accumulate

inorganic phosphate during the energized to energized–twisted configurational change.[25] The energized accumulation of phosphate has been explained in terms of the exposure of binding sites accompanying the substrate-dependent conformational change. As was the case with the proton jump, mitochondria with cristae in the orthodox configuration bound more phosphate under energizing conditions than did mitochondria in the aggregated configuration. The increment in the concentration of inorganic phosphate during energizing was 30 mμmoles/mg protein for orthodox mitochondria and 15 mμmoles/mg protein for aggregated mitochondria. Thus, two events indicative of energized conformational change—the ejection of protons and the binding of inorgainc phosphate—are actually enhanced when the cristae are in the orthodox configuration.

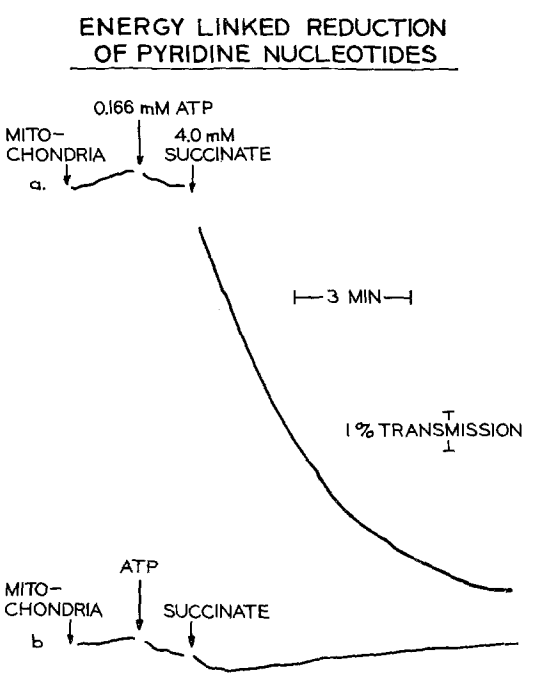

Figure 12. Effect of the mitochondrial configuration on the extent of reversed electron transfer. (A) The succinate-dependent reduction of pyridine nucleotides in mitochondria isolated in the STE medium. (B) the succinate-dependent reduction of pyridine nucleotides in mitochondria isolated in the ST medium. A downward deflection indicates reduction.

Discussion

Regulation of the Aggregated–Orthodox Transition

The studies described in this and previous communications[1,9] show that Ca^{2+}, Mg^{2+}, and nonesterified fatty acids can influence the coupling capability by regulating the configurational state. The addition of Ca^{2+} to adrenal cortex mitochondria with cristae in the aggregated configuration induces the following events: (a) an increase in the level of tightly bound Ca^{2+}, (b) a nonenergized ejection of protons, (c) an increase in the level of bound nonesterified fatty acids, and (d) the movement of water from the intracristal space into the matrix space with the formation of the orthodox configuration. The addition of oleate to adrenal cortex mitochondria in the aggregated configuration leads to events similar to those produced by the addition of Ca^{2+}, except that there is no increase in the level of bound Ca^{2+}. Moreover, the addition of Mg^{2+} to mitochondria with cristae in the orthodox configuration leads to the following events: (a) a nonenergized proton uptake, (b) an increase in the level of bound Mg^{2+}, and (c) movement of water from the matrix space into the intracristal space with the formation of the aggregated configuration. The addition of albumin accomplishes the same end results as does addition of Mg^{2+}, but the albumin effect is referable to the removal of nonesterified fatty acids. From these observations what can we conclude about the primary determinant of the orthodox to aggregated configurational change? We postulate that the determinant of the configurational change is the level of bound Mg^{2+}. We have not been able as yet to determine which

ligands Mg^{2+} is bound to or where Mg^{2+} is bound in intact mitochondria. Some light on this problem has been shed by the Mg^{2+} concentration of submitochondrial particles. When we prepared ETP particles, we found a difference in the level of bound Mg^{2+}, depending whether the mitochondria were initially orthodox or aggregated. In ETP particles prepared from mitochondria in the aggregated configuration, bound Mg^{2+} was between 65–70 mμmoles of Mg^{2+}/mg protein. When the mitochondria were in the orthodox configuration, the Mg^{2+} of the particles derived therefrom was found to be between 25–30 mμmoles/mg protein. Although the level of Mg^{2+} in intact mitochondria remains relatively constant, regardless of configuration, there must have been a labilizing of some of the Mg by Ca^{2+} or nonesterified fatty acids in order to account for the above results.

It has been assumed for a number of years that free fatty acids were uncouplers *per se*.[27–30] On the basis of the studies reported in this communication, we propose that free fatty acids achieve uncoupling by modulation of the configuration of the mitochondria. Depending on the level of bound Mg^{2+} and nonesterified fatty acids, the mitochondria will be either in the orthodox (uncoupled) configuration or the aggregated (coupled) configuration. In this modulation there is an interplay of free fatty acid and Mg^{2+}. The latter in sufficient amount can overcome the "uncoupling" action of the former.

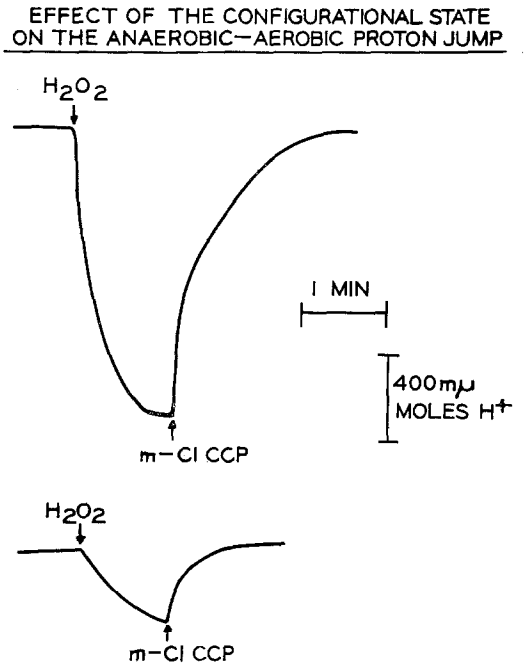

Figure 13. The anaerobic to aerobic proton jump in adrenal cortex mitochondria. The upper trace represents the proton ejection in mitochondria isolated in the ST medium and the lower trace represents the proton ejection isolated in the STE medium. A downward deflection represents proton ejection.

The Role of Exogenous Mg^{2+} in Coupling

The studies described in this communication compel a reassessment of the meaning of a requirement for exogenous Mg^{2+} in coupled mitochondrial processes. In all cases examined in this study the requirement for exogenous Mg^{2+} is relevant to the establishment of the aggregated configurational state of the crista, and not referable to the participation of Mg^{2+} in any enzymic reaction. There is only a requirement for exogenous Mg^{2+} when the mitochondria are in the orthodox configuration. For example, there ia a requirement of Mg^{2+} (or Mn^{2+}) for Ca^{2+} translocation, since Ca^{2+} induces the orthodox configuration. However, the translocation of Mn^{2+} or Sr^{2+} by mitochondria already in the aggregated configuration does not require the addition of Mg^{2+}, since Mn^{2+} and Sr^{2+}[1] do not induce the orthodox configuration.

The requirement of mitochondria for exogenous Mg^{2+} during energized Ca^{2+} translocation was also reported by Haugaard *et al.*[31] They found that Ca^{2+} was translocated by liver mitochondria when the Mg^{2+} concentration was low (less than 2 mM), but the

translocation was short-lived and the translocated Ca^{2+} was then extruded. However, the addition of higher levels of Mg^{2+} either initially or after the initial burst of Ca^{2+} translocation resulted in extensive and sustained translocation of Ca^{2+}. These results can be explained as follows: (a) initially there was Ca^{2+} binding and uptake when the mitochondria were in the aggregated configuration; (b) in consequence of the uptake of Ca^{2+} the orthodox configuration was induced; (c) the mitochondria became uncoupled and Ca^{2+} was extruded; (d) the cristae underwent a transition from the orthodox to the aggregated configuration following the addition of Mg^{2+} and coupled translocation of Ca^{2+} increased. It should be noted that in all experiments reported by Haugaard *et al.*[31], and in the present study, there is a critical molar ratio of Mg^{2+} to Ca^{2+} for translocation of Ca^{2+}. When this ratio exceeds 5 : 1, translocation is maximal, but when this ratio is decreased below 5 : 1, less Ca^{2+} is translocated. Hackenbrock and Caplan[32] recently presented evidence that when the Mg^{2+} : Ca^{2+} ratio was high, translocation of Ca^{2+} ensued, and the rat liver mitochondria remained in the aggregated configuration, whereas when the Mg^{2+} : Ca^{2+} ratio was low the mitochondria were in the orthodox configuration and lost the ability to translocate Ca^{2+}.

The Relationship of Configurational Change and Coupling

The uncoupled state of the crista in the orthodox configuration has profound significance for the theory of energized coupling. The tripartite repeating units of the crista in the orthodox configuration do not lack the capacity for electron transfer. The anaerobic–aerobic proton jump which accompanies the electron transfer process in coupled mitochondria is not lost in mitochondria with cristae in the orthodox configuration. The conformationally dependent accumulation of phosphate which is observed on the addition of inorganic phosphate to coupled mitochondria energized by substrate also is demonstrable in mitochondria with cristae in the orthodox configuration. All the requirements for coupling appear to be satisfied and yet mitochondria in the orthodox configuration are completely uncoupled. What is the nature of the lesion? The lesion of the orthodox mitochondrion may be the loss of the capacity to undergo configurational change. Any reagent or set of conditions which induces the orthodox configuration will also prevent coupling (uncoupler, endotoxin, Ca^{2+}, fatty acid, silicomolybdate). We may extend this generalization by saying that reagents or set of conditions which prevent any one of the three configurational transitions and the corresponding conformational changes will also prevent coupled reactions. For example, high levels of sucrose[26] and fluorescein mercuric acetate[33] inhibit the energized to energized–twisted configurational transition and correspondingly inhibit oxidative phosphorylation. Oligomycin prevents the energized–twisted to nonenergized configurational transition and correspondingly inhibits oxidative phosphorylation.[5] This is not to say that configurational change rather than conformational change is primary. It is our view that while conformational change is undoubtedly the primary event in the transduction of energy, the conformational cycle cannot be complete in mitochondria, unless concomitant configurational change takes place. The delivery of inorganic phosphate to the headpieces, the control of the internal pH, the movement of ions through the membrane are just a few of the many processes which can be controlled by configurational change. It is probably for these logistic reasons that the capacity for configurational change is as crucial as the capacity for conformational change. In orthodox mitochondria we have clear evidence

that the coupling process is abortive not because of the loss in the capacity for conformational change but rather because of the loss in the capacity for configurational change.

A number of investigators believe that for one reason or another the observed energized configurational changes may not bear any relationship to energy conservation.[34–37] The data presented in this communication support the thesis that the energized configurational changes which accompany coupling are indeed intrinsic to energy conservation. The energized configurational changes were observable only when mitochondria were in the aggregated configuration and therefore coupled. The inability of mitochondria in the orthodox configuration to undergo observable energized configurational change is consistent with the loss of coupling. It is possible to observe energized configurational changes with mitochondria in the orthodox configuration only after the addition of Mg^{2+}, Mn^{2+}, or albumin.

The energized configurational changes observed with adrenal cortex mitochondria *in vitro* are essentially the same as those observed *in situ* for heart, liver, and kidney mitochondria.[4] That is, the cristae of mitochondria *in situ*, in the nonenergized orthodox configuration are transformed either to the energized or to the energized–twisted configuration after the addition of oxygen, substrate, and phosphate. The same configuration transition can be induced in orthodox mitochondria *in vitro* by addition of oxygen, substrate, inorganic phosphate, and albumin or Mg^{2+}. The addition of an uncoupler discharged the energized configuration to the orthodox configuration. The transition of the orthodox (nonenergized) to the energized configuration *in situ* may involve one or more of the following events: (a) reesterification of the nonesterified fatty acids, (b) movement of Mg^{2+} or Mn^{2+} from the extramitochondrial space into the mitochondria, and (c) loss of bound mitochondrial Ca^{2+}. These events would induce the conversion of cristae from the orthodox to either the aggregated or energized–twisted configuration.

It is possible that the aggregated–orthodox transition is also applicable to submitochondrial particles. For example, in the preparation of well-coupled electron transfer particles (ETP_H) the presence of Mg^{2+}, Mn^{2+}, succinate, and ATP in the isolation medium is required.[38] If our thesis relating the configuration and coupling capacity is correct, we would predict that these supplements are required to maintain the level of bound Mg^{2+} in the submitochondrial particle. We have observed that ETP_H prepared in the presence of Mg^{2+} and Mn^{2+} has a level of bound Mg^{2+} of about 80 mμmoles/mg protein. When Mg^{2+} and Mn^{2+} are not present in the preparation, the coupling capacity of the resulting particles (ETP) is greatly reduced.[38] The level of Mg^{2+} in such particles was about 30 mμmoles/mg protein. Thus, we may equate the configuration of ETP_H with that of the aggregated configuration and the configuration of ETP with that of the orthodox configuration.

Mg^{2+} as the Ultimate Determinant of the Orthodox–Aggregated Transition

The experiments we have described in the present communication have led us to propose the following interpretation of the conformational changes implicit in the orthodox–aggregated transition. The repeating units of the cristal membrane can exist in one of two nonenergized conformations—orthodox or aggregated. The level of Mg^{2+} bound to the basepiece may be the determinant of this conformational transition. When the level of bound Mg^{2+} exceeds a critical value, the conformation of the repeating unit

would be aggregated; when the level of Mg^{2+} is below that critical value, the conformation would be orthodox. Repeating units in the orthodox conformation are uncoupled, whereas repeating units in the aggregated conformation are coupled. When all the repeating units of the cristae are in the orthodox conformation, the cristae undergo compression as water moves out into the matrix space, and the orthodox configuration of the membranes results. Conversely, when all the repeating units of the cristae are in the aggregated conformation, the cristae balloon out as water moves from the matrix space into the intracristal space, and the aggregated configuration of the membranes results. It is implicit in this formulation that the geometry of the basepiece is profoundly influenced by the change in conformation of the tripartite repeating unit in going from the nonenergized to the energized (aggregated) configuration.

The Orthodox–Aggregated Transition as a Possible Basis for Control of Mitochondrial and Cellular Function

The orthodox–aggregated transition may provide an exceedingly delicate control point not only for mitochondria but also for the cell. For example, when heat production rather than synthesis of ATP is required physiologically, as would be the case in cold-adapted animals, a variety of means could be available for accomplishing a switch (the level of Ca^{2+}:Mg^{2+} ratio, etc.). In the adrenals, the switch from steroidogenesis to ATP synthesis appears to be controlled by the orthodox–aggregated transition, since steroidogenesis proceeds much more rapidly in orthodox than in aggregated mitochondria.[39]

Harris *et al.*[40] have previously shown that the endotoxin of *Bordetella bronchiseptica* induces the orthodox configuration of heart mitochondria and simultaneously abolishes all coupled processes except one (translocation of Mg^{2+}). At the time these investigations were reported, it was not appreciated that Mg^{2+} could reverse the action of the endotoxin by inducing the aggregated configuration. Thus, the endotoxin of *Bordetella bronchiseptica* is yet another reagent for inducing the orthodox configuration in mitochondria. We have observed the same inducing effect of this endotoxin on adrenal cortex mitochondria.

The endotoxin of *Bordetella bronchiseptica* is a lipopolysaccharide which readily forms membranous vesicles.[40] Very likely it could act as a scavenger for Mg^{2+} and in this way could duplicate the action of free fatty acid in inducing the orthodox configuration. The membrane-forming capability could account for its ready penetration into the mitochondrion through the outer membrane.

Some years ago Jacobs and Sanadi[41] in our laboratory demonstrated that complex polyanions such as silicomolybdate promoted uncoupled respiration, and that this uncoupling action was reversed by Mg^{2+}, Mn^{2+}, Sr^{2+}, and Ba^{2+}, but not by Ca^{2+}. These authors appear to have inadvertently discovered the orthodox–aggregated transition and the uncoupled state of the orthodox configuration. The negatively charged polyanions such as silicomolybdate indeed can bind to the mitochondrion and indeed can induce the orthodox configuration, as we shall show in a subsequent communication. The point to be made is that a wide spectrum of reagents are now known which can control a key mitochondrial transition, and we must anticipate the extension of this list of reagents as work proceeds.

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