Bioenergetics (1970) **1**, 87–107.

Studies on the Transition of the Cristal Membrane from the Orthodox to the Aggregated Configuration. II: Determinants of the Orthodox-Aggregated Transition in Adrenal Cortex Mitochondria

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Abstract

Bovine adrenal cortex mitochondria when isolated in a medium 0·25 M in sucrose contaminated (with calcium) have tubular cristae which are periodically expanded to spherical vesicles and contracted to flattened connecting sections. This scalloped tubular form of the cristae corresponds to the nonenergized orthodox configuration—a configuration in which the matrix space is maximally expanded. When adrenal cortex mitochondria are isolated in media in which the free calcium content is relatively low, e.g., a medium 0·25 M in sucrose and 0·1 mM in EDTA, the cristae assume the aggregated configuration—a nonenergized configuration in which the matrix space is maximally contracted. The composition of the isolation medium determined the configuration. Procedures have been described for isolating bovine adrenal cortex mitochondria (a) predominantly in the orthodox configuration, (b) predominantly in the aggregated configuration, or (c) in a mixed (1 : 1) population of configurations. The concentration of Ca^{2+} bound to the mitochondrion was found to be a determinant of the nonenergized configuration. When the level of bound Ca^{++} was 20–25 μmoles/mg protein, the cristae of the mitochondria were entirely in the orthodox configuration. Addition of Ca^{2+} could induce the transition of cristae from the aggregated to the orthodox configuration whereas addition of Mg^{2+} could induce the transition of cristae from the orthodox to the aggregated configuration. The configurational transition could be followed by any of several methods—a change in 90° light scattering, a change in $O.D._{520\,m\mu}$, a change in pH, or examination by electron microscopy. The orthodox to aggregated transition is energy-independent since it proceeds even in presence of inhibitors both of electron transfer and of ATP hydrolysis. The binding of Ca^{2+} is independent of the binding of Mg^{2+}; this independent binding is consistent with the opposite effects induced by Ca^{2+} and Mg^{2+}, respectively. Whereas Ca^{2+} induces a proton release, a decrease in 90° light scattering and a decrease in $O.D._{520\,m\mu}$ (when the cristae are initially in the aggregated configuration), Mg^{2+} induces equal and opposite changes (when the cristae are initially in the orthodox configuration).

Introduction

An extensive literature has grown up on the atypical topology of the cristal membrane of adrenal cortex mitochondria‖ (see ref. 1 for a review) and on the dramatic changes in the

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‖ In this communication the description "adrenal cortex mitochondria" always refers to mitochondria of the *zona fasciculata*.

topology under various physiological conditions.[2-7] The electron microscopic evidence of unusual transitions in the ultrastructure of the cristal membranes of adrenal cortex mitochondria was the starting point for the present investigation. It soon became apparent to us from the studies of Williams *et al.*[8] in our laboratory that the ultrastructural transitions that had been observed in adrenal cortex mitochondria[2-7] were probably variations of a theme common to the energy cycle of the mitochondria generally and that we were not dealing with a problem peculiar to adrenal cortex mitochondria. Williams *et al.*[8] showed that as a general rule the cristae of mitochondria *in situ* are in the orthodox configuration, in contrast to the cristae of isolated mitochondria which are generally in the aggregated configuration.[8-12] By definition the matrix space is maximally expanded in the orthodox configuration and maximally contracted in the aggregated configuration. As the intracristal space expands, the apposition of one crista with its neighbors (above, below, and to the side) is inevitable; hence, the term aggregation. When mitochondria *in situ* (under nonenergizing conditions) are examined by standard electron microscopic methods, the cristae almost always are in the orthodox configuration. This uniformity of appearance of mitochondria *in situ* examined by electron microscopy is merely a token of the fact that the anaerobiosis which inevitably sets in after removal of the tissue from the animal induces the orthodox configuration of the cristae.[8]

The present communication will be dealing with some of the factors that determine the transition of these mitochondria from one nonenergized configuration to the other. The first communication of this series dealt with the topology of orthodox adrenal cortex mitochondria.[13] In the third communication of this series,[14] the coupling properties of mitochondria in each of these two configurations of the cristae will be documented. While the transition from the orthodox to the aggregated configuration is, indeed, intrinsic to the energy cycle, nonetheless, conditions can be found in which the adrenal cortex mitochondrion can be "frozen" in the orthodox configuration. In this way it has become possible for the first time to recognize profound differences in coupling capability between mitochondria in the orthodox and aggregated configurations.

The conventional medium for suspension of isolated mitochondria is 0·25 M in sucrose or in some other nonpermeable solute. This high concentration of solute that is impermeable to the matrix space of the suspending medium compels the transition from the orthodox to the aggregated configuration.[15] Adrenal cortex mitochondria are atypical in two respects; first, these mitochondria remain in the orthodox configuration during isolation in a medium 0·25 M in sucrose; second, they can be maintained in this configuration even under energizing conditions, providing the level of Ca^{2+} is properly regulated.[14]

The present communication is the second of a series addressed to the reversible transition of various types of mitochondria from the orthodox to the aggregated configuration. While the transition is relevant to the energy cycle of all mitochondria, the ease with which it can be controlled physiologically is variable. The transition is a key control point in many cells. A considerable number of mitochondrial phenomena are intimately tied up with this ultrastructural transition-phenomena, such as the loss and recovery of respiratory control, the antagonism between Ca^{2+} and Mg^{2+}, and the mode of action of some drugs and toxins.

Methods

Isolation of Bovine Adrenal Cortex mitochondria

The isolation of the mitochondria was described in the previous communication.[13] The isolation of the mitochondria was accomplished in one of four media: (A) a solution which was 0·25 M in sucrose, 10 mM in TrisCl, pH 7·8, and 0·1 mM in NaK–EDTA (STE); (B) a solution which was 0·25 M in sucrose* and 10 mM in TrisCl, pH 7·8 (ST); (C) a solution which was 0·25 M in sucrose (Ca^{2+}-free), 10 mM in TrisCl, pH 7·8 (ST-Ca^{2+}-free); and (D) a solution which was 0·21 M in mannitol, 0·07 M in sucrose, 5 mM in TrisCl, pH 7·8, and 0·1 mM in NaK-EDTA (MSTE). The abbreviations list all the ingredients of the medium. Media A and D were equivalent in respect to maintaining mitochondria in the aggregated configuration. Medium A was generally used for this objective. Medium B was used to maintain the mitochondria in the orthodox configuration whereas medium C yielded a mitochondrial preparation which was composed of some mitochondria in the orthodox configuration and some in the aggregated configuration. Ca^{2+}-free sucrose was prepared as described previously.[13] The protein concentration was determined by the biuret method of Gornall *et al.*[16] in the presence of deoxycholate.

Measurement of 90° Light Scattering

These measurements were carried out in a Phoenix light-scattering photometer at 546 mμ. The sample (3 ml), containing 1 mg of mitochondrial protein per milliliter, was continuously stirred during the measurement at 25°. Additions were made directly into the reaction vessel (see legends for figures for further details on the concentration of reagents added to the reaction vessel during the light scattering measurements).

Measurement of Changes in Optical Density

$CaCl_2$-induced optical density changes were measured in a Beckman DU spectrophotometer at 520 mμ. The assay was carried out at 25° in a total volume of 3 ml containing 0·5 mg mitochondrial protein. The suspending medium was ST (Ca^{2+}-free).

Measurement of Proton Release and Uptake

These measurements were made with a Beckman Expando-Matic pH meter and with a micro-combination pH electrode. The assay system (4 ml) was 0·25 M in sucrose (Ca^{2+}-free) and contained 25 μg of rotenone, 25 μg of antimycin, 25 μg of rutamycin and 10–25 mg of mitochondrial protein. The proton equivalence for known excursions of the recording pen was determined by calibration of the system by additions of known amounts of a standard solution of HCl.

Measurement of Ion Binding

The binding of Ca^{2+} to adrenal cortex mitochondria (4 mg) suspended in 4 ml of ST (Ca^{2+}-free) medium was determined by measurement of the radioactivity of the mitochondrial pellet obtained by filtering the suspension through a 0·45 μ millipore filter to which $^{45}Ca^{2+}$ had been added, and washing the residue with 10 ml of the same calcium-free medium. The incubation was carried out at 25° for a specified time (see legends of the

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

appropriate tables). There was essentially no retention of radioactivity when the mitochondria-free incubation medium containing $^{45}Ca^{2+}$ was filtered. The millipore filter was found by prior test to retain 100% of the mitochondria suspension. The dried filter was dissolved in 20 ml of Bray's scintillation fluid containing Cab-O-Sil. The resulting solution was counted in a Packard Tri-Carb scintillation counter.

Binding of Mn^{2+} to the mitochondrion was determined with $^{54}Mn^{2+}$ by measuring the radioactivity either of the filtrate or of the mitochondrial pellet retained by the millipore filter. The details for these measurements were exactly the same as those for the binding of calcium, except that the washing of the filtered mitochondrial pellet was carried out with 4 ml (not 10 ml) of the suspending medium ST (Ca^{2+}-free). The samples containing $^{54}Mn^{2+}$ were counted in a Packard Gamma Counter.

Determination of Ca^{2+}, Mg^{2+}, K^+, and Na^+ by Atomic Absorption

The various cations were determined in the Perkin-Elmer atomic absorption spectrophotometer as follows. **Ca^{2+}**—The mitochondrial protein (20–50 mg) was extracted twice with a total of 4 ml of 1 M perchloric acid. One milliliter of the extract was then mixed with 3·0 ml of water, 0·5 ml of a 10% $SrCl_2$ solution and 0·5 ml of 1 M perchloric acid. **Mg^{2+}**—Extraction of the protein was carried out as above, except that an equivalent amount of water was added in place of the $SrCl_2$ solution. **K^+ or Na^+**—The mitochondrial protein (5 mg) was dissolved in a mixture 0·1 N in HCl and 0·01 M in cetyl pyridinium chloride in a total volume of 10 ml. The extract was used directly for measurement in Perkin-Elmer atomic absorption spectrophotometer.

Electron Microscopy

The fixation and treatment of the specimens were identical with that described previously.[13]

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.); $^{54}MnCl_2$ (New England Nuclear Corp., Boston, Mass.); redistilled glutaraldehyde (Poly Sciences, Rydal, Pa.); rutamycin (Eli Lilly Pharmaceutical Co., Indianapolis, Ind.); rotenone (Aldrich Chemical Co., Milwaukee, Wis.); antimycin (Wisconsin Alumni Research Foundation, Madison, Wis.).

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

Results

Isolation of Adrenal Cortex Mitochondria with Cristae in Either the Orthodox or Aggregated Configuration

When the isolation medium was 0·25 M in sucrose and 0·01 M in TrisCl, pH 7·8 (ST), at least 95% of the adrenal cortex mitochondria* isolated were in the orthodox configuration (see Fig. 1) whereas when the isolation medium was identical except for the addition

* The method of isolation from adrenal tissues which we have used routinely does not lead to a homogeneous mitochondrial preparation. The presence of membrane fragments which are non-mitochondrial is clearly recognizable in some electron micrographs of our mitochondrial preparations. In evaluating the transitions which mitochondria undergo, the reader should ignore both the membranes which are clearly non-mitochondrial and the membranes of fragmented mitochondria which are no longer ultrastructurally intact.

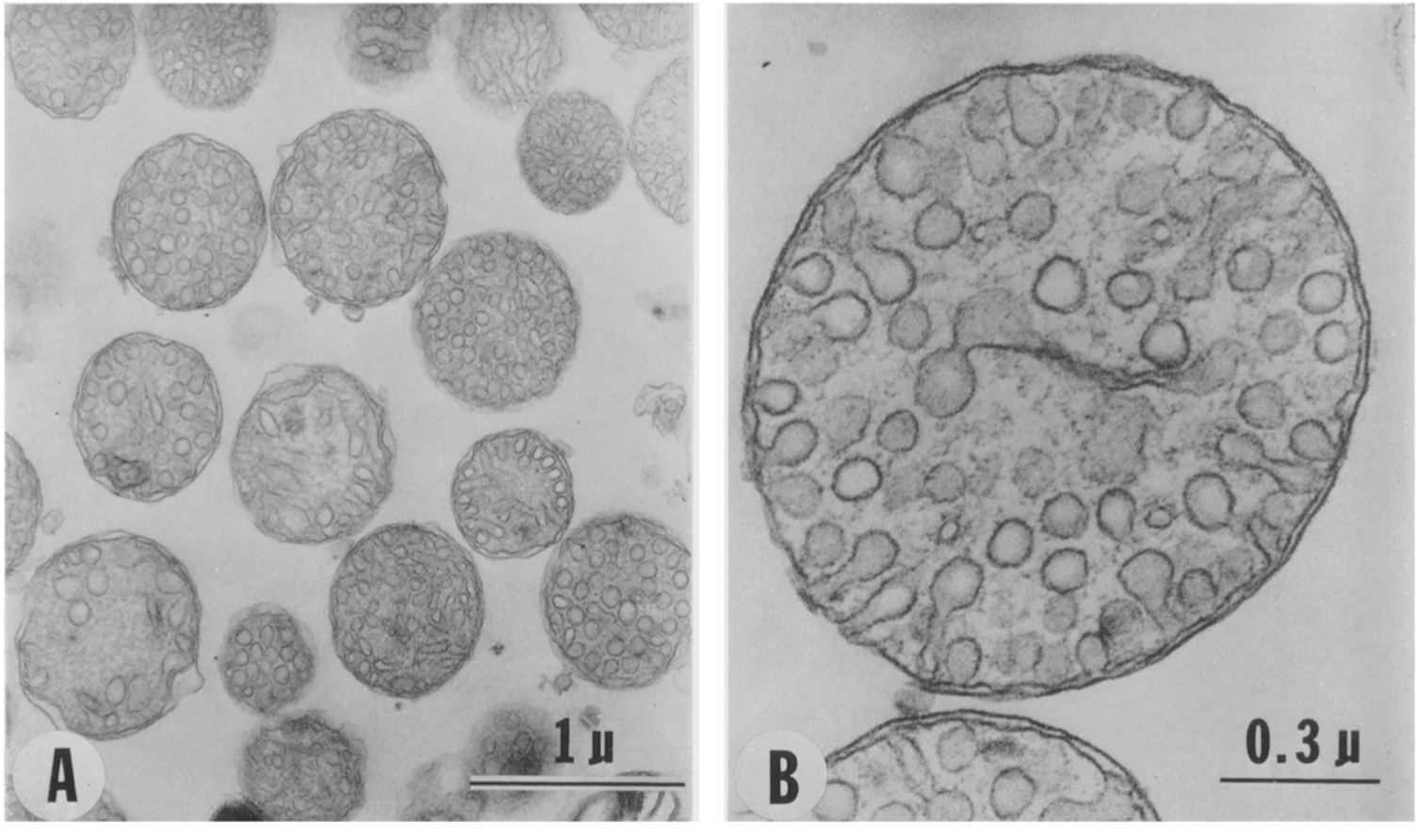


Figure 1. Electron micrographs of adrenal cortex mitochondria (isolated in a medium 0·25 M in sucrose and 10 mM in Tris-Cl, pH 7·6 (5°). (38911, 55074)

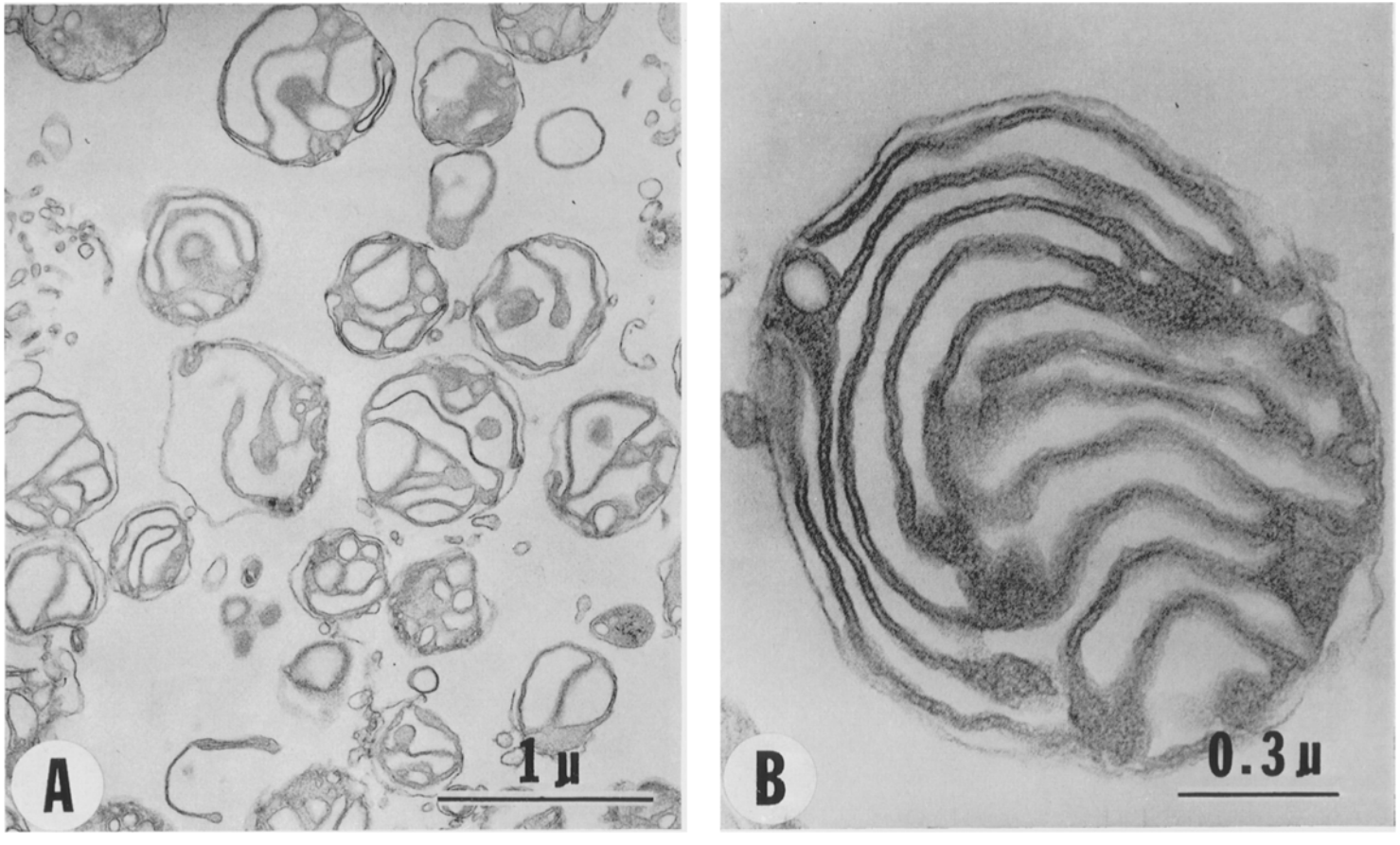


Figure 2. Electron micrographs of adrenal cortex mitochondria isolated in a medium 0·25 M in sucrose, 10 mM in TrisCl, pH 7·8, and 0·1 mM in EDTA (STE). (33547, 34027)

of 0·1 mM EDTA, at least 95% of the mitochondria were in the aggregated configuration (see Fig. 2). Furthermore, when mitochondria were isolated in the sucrose–TrisCl medium after it had been rendered calcium-free [ST (Ca^{2+}-free)] by treatment with

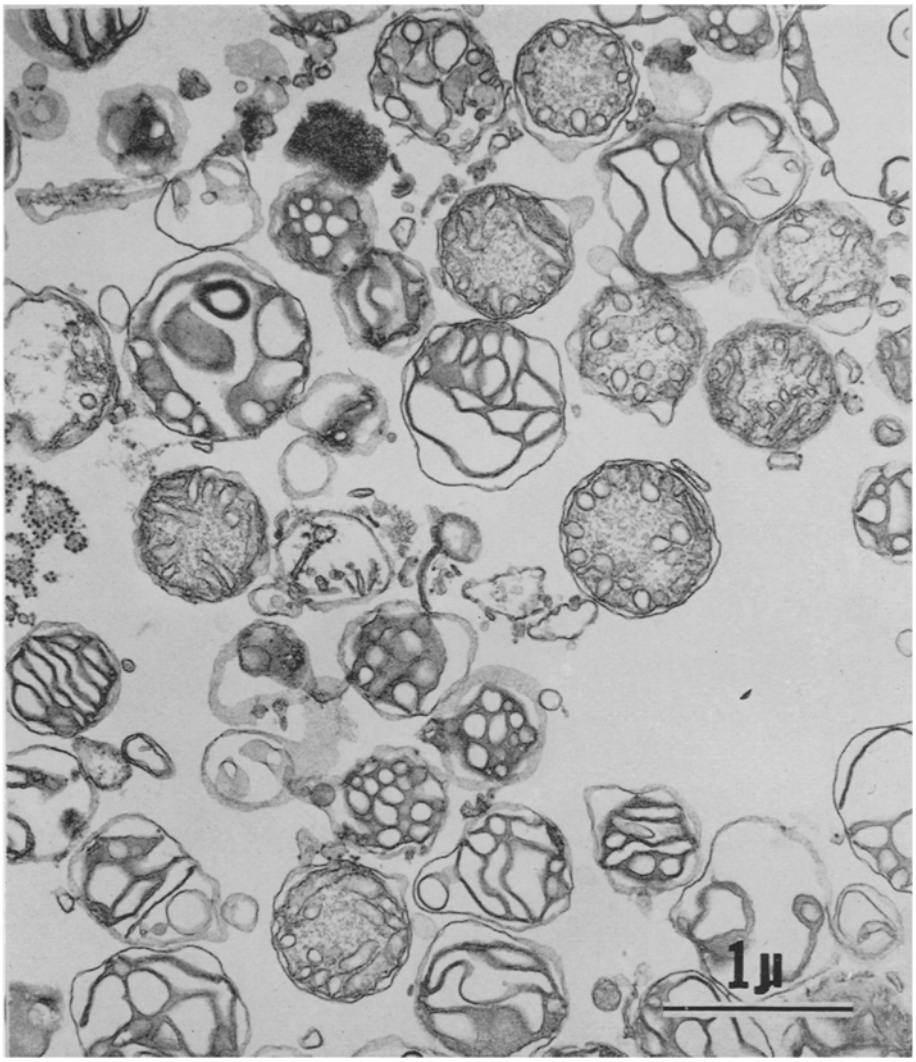


Figure 3. Electron micrograph of adrenal cortex mitochondria isolated in a medium 0·25 M in sucrose (Ca^{2+}-free) and 10 mM in TrisCl, pH 7·8. (38945)

Amberlite, about half of the population were in the aggregated configuration and half in the orthodox configuration (see Fig. 3). Thus, by selection of the appropriate medium the mitochondria can be isolated either predominantly in the orthodox configuration or predominantly in the aggregated configuration or mixed with respect to configurational state (roughly 1 : 1).

The Calcium Content as the Determinant of the Configuration of the Cristae

In Table I are summarized the Ca^{2+} and Mg^{2+} contents of the mitochondria isolated in each of the three media specified above. The Mg^{2+} content is barely affected by the presence of EDTA in the isolation medium but the Ca^{2+} content is drastically reduced. Thus, a relatively high calcium content of the mitochondria preserves the orthodox configuration, whereas a relatively low calcium content allows the formation of the aggregated configuration of the cristae. Mitochondria isolated in a medium containing Ca^{2+}-free sucrose were intermediate in calcium content and the population was mixed with respect to cristal configurations. It should be emphasized that the ST medium which preserves the orthodox configuration contains considerable amounts of calcium (8 mμmoles/μmole of sucrose). When the sucrose is rendered calcium free, the calcium level of the mitochondria falls below the level at which all of the mitochondria can be maintained in the orthodox configuration in 0·25 M sucrose. Clearly, the binding of calcium is readily reversible as evidenced by the removal of calcium during extensive washing of

TABLE I. Ca^{2+} and Mg^{2+} content of adrenal cortex mitochondria isolated in different media

Type of medium*	Configuration	Ca^{2+} (mμmoles/mg protein)	Mg^{2+}
STE	Aggregated	2·4	18·0
ST	Orthodox	22·5	21·8
ST (Ca^{2+}-free)	Mixed	13·0	19·3

* The composition of the media was as follows: STE—0·25 M sucrose, 0·01 M TrisCl, pH 7·8, and 0·1 mM EDTA. ST—0·25 M sucrose, 0·01 M TrisCl, pH 7·8. ST (Ca^{2+}-free)—0·25 M sucrose (Ca^{2+}-free) and 0·01 M TrisCl, pH 7·8.

the mitochondria in the presence of 0·1 mM EDTA. The concentration of calcium in the fluid of the adrenal cortex is relatively high (1 mM)—in fact, sufficient to maintain the mitochondria in the orthodox configuration.

Conversion of Mitochondria from the Aggregated to the Orthodox Configuration by Addition of Ca^{2+}

Isolated mitochondria with cristae initially in the aggregated configuration can be transformed quantitatively into the orthodox configuration by addition of Ca^{2+} (100–500 mμmoles/mg protein) (see Fig. 4). This conversion is not an energy-dependent process. It proceeds in presence of reagents which inhibit electron transfer and hydrolysis of ATP. The conversion proceeds equally well at 0° as at 35° and does not require the presence of phosphate, substrates, or nucleotides. There appears to be no specificity toward the anion, since Cl^- can be replaced by acetate, phosphate, lactate, tartrate, carbonate, or sulfate.

The induction, by added Ca^{2+}, of the orthodox configuration can be correlated with the uptake of Ca^{2+} by the mitochondrion. The extent of Ca^{2+} binding as a function of the concentration of added Ca^{2+} is plotted in Fig. 5. The maximum amount of calcium bound was about 25–30 mμmoles/mg protein. The uptake of calcium was unaffected by the presence of inhibitors of electron transfer or of ATP hydrolysis. Moreover, the rate of

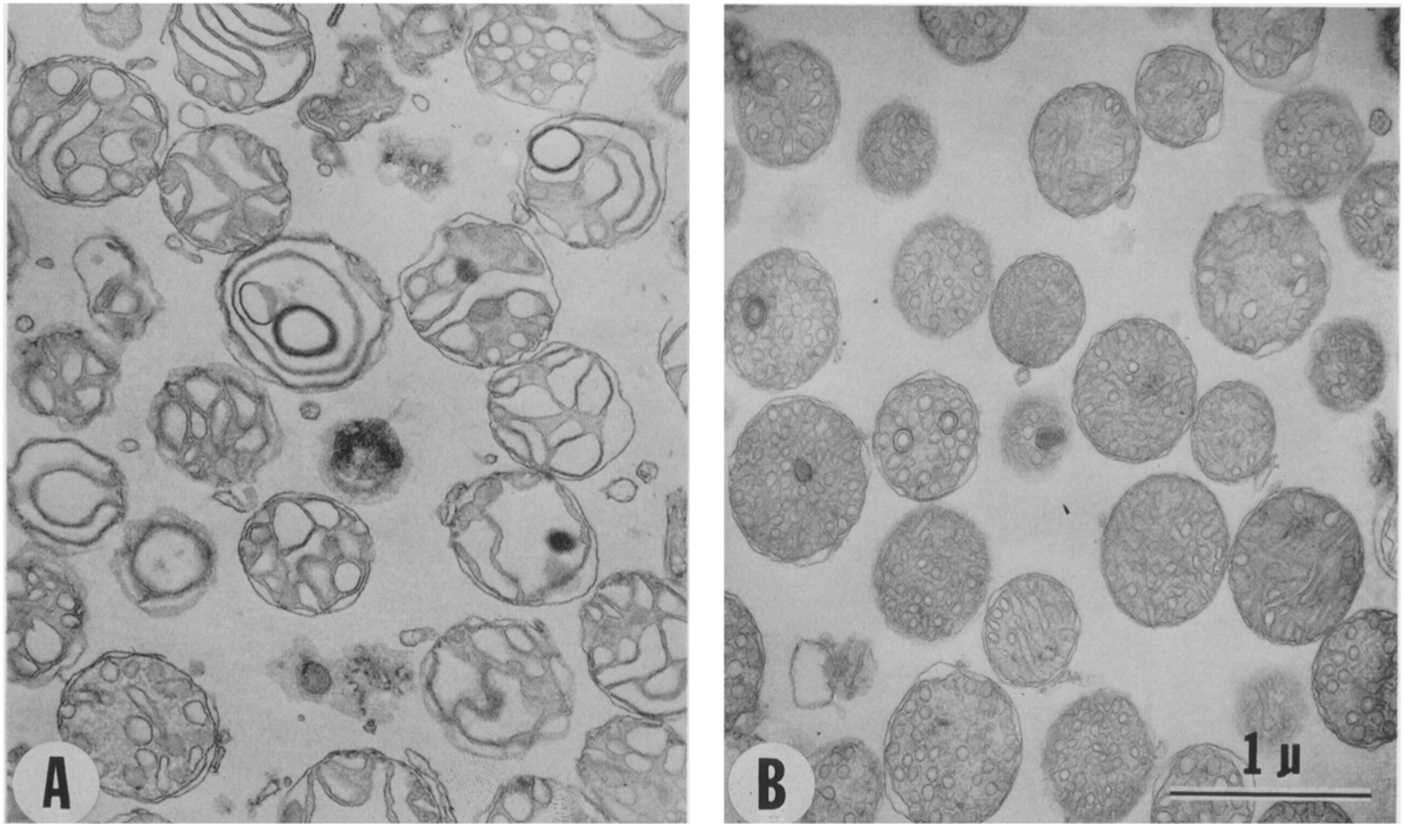


Figure 4. Electron micrographs of adrenal cortex mitochondria isolated in STE and incubated at 25° in ST (Ca^{2+}-free) at a protein concentration of 1 mg/ml. A (38490): typical field of mitochondria in the aggregated configuration before addition of $CaCl_2$; B (38949): a typical field of mitochondria 5 min after the addition of 155 $m\mu$moles of $CaCl_2$/mg protein.

calcium uptake (see Table II) was too rapid to be measured directly (complete in less than 15 sec).

The amount of Ca^{2+} binding required to effect the transformation of the crista from the aggregated to the orthodox configuration is directly proportional to the osmotic pressure of the suspending medium. The outer boundary membrane of adrenal cortex mitochondria, as is the case for the corresponding membrane of heart mitochondria,[15] is permeable to sucrose whereas the inner membrane is essentially impermeable. The sucrose concentration of the external medium is thus a direct measure of the osmotic pressure exerted on the matrix space. When adrenal cortex mitochondria (in the aggregated configuration) are exposed to Ca^{2+}-free 0·25 M sucrose, the binding of 20–25 mμmoles of Ca^{2+}/mg protein is required to induce the orthodox configuration. However, when the sucrose concentration was lowered to 0·150 M the induction of the orthodox configuration required the binding of only 8–15 mμmoles of Ca^{2+}/mg protein. When the sucrose concentration of the medium was greater than 0·25 M, more than 30 mμmoles of Ca^{2+} had to be bound/mg protein to induce the orthodox configuration. The influence of the osmotic pressure of the medium on the orthodox to aggregated transition can also be observed by isolating the mitochondria in the orthodox configuration in ST, then exposing these mitochondria to higher concentrations of sucrose (Ca^{2+}-free). Under these conditions, cristae in the orthodox configuration undergo transition to the aggregated configuration.

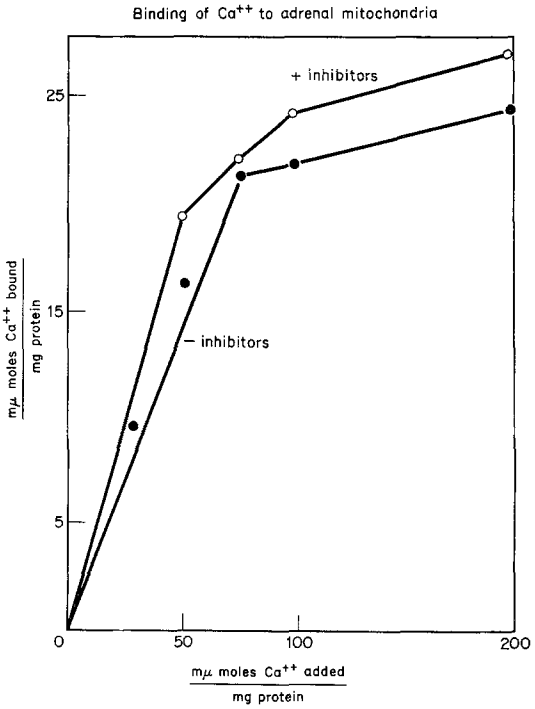


Figure 5. The extent of $^{45}Ca^{2+}$ binding as a function of the concentration of $^{45}CaCl_2$ added to mitochondria initially in the aggregated configuration. The binding of $^{45}Ca^{2+}$ was assayed in the absence and presence of 2 μg rutamycin, 2 μg rotenone, and 2 μg antimycin/mg protein.

The effect of osmotic pressure exerted by relatively impermeable solutes such as sucrose on the configuration of the crista can also be observed in mitochondria *in situ*. Crane[17] observed that the cristae of heart mitochondria *in situ* could be transformed from the orthodox configuration into the aggregated configuration by exposing the tissue to 0·25 M sucrose. Recently Williams *et al.*[8] have observed a similar transition in the cristae of liver mitochondria *in situ*, from the orthodox to aggregated configuration by the addition of 0·25 M sucrose. The electron micrographs in Fig. 6A and B illustrate the effect of the composition of the medium on the configurational state of adrenal cortex mitochondria *in situ*. The micrograph in Fig. 6A is a section of a specimen of adrenal cortex incubated in Ca^{2+}-free Krebs-Ringer-Phosphate (0·304 osM) while the micrograph in Fig. 6B is a section of a specimen of adrenal cortex incubated in 0·25 M sucrose (0·205

TABLE II. The rate of binding of Ca^{2+} to adrenal cortex mitochondria in the aggregated configuration

Time in sec	mμmoles Ca^{2+}/mg protein
15	24·6
30	25·7
60	28·2
120	26·6
240	26·0

Adrenal cortex mitochondria (1 mg/ml) isolated in STE were incubated in 5 ml of ST (Ca^{2+}-free) supplemented with 100 mμmoles Ca^{2+} per mg protein at 25° with ^{45}Ca as a tracer. After the specified time, aliquots (0·5 ml) were removed and the bound ^{45}Ca determined as described in the methods.

osM). The rationale for the induction of the aggregated configuration by 0·25 M sucrose is that the outer boundary membrane is permeable to sucrose, whereas the inner membrane is impermeable. Thus, sucrose can enter only the intracristal space of the mitochondria. The osmotic gradient induces a water movement from the matrix space into the intracristal space, and concomitant generation of the aggregated configuration. The inability of Krebs-Ringer-Phosphate medium to induce the aggregated configuration despite its higher osmolarity must be due to the penetrability to salts of both the intracristal and matrix spaces of the mitochondria. This penetrability prevents the formation of an osmotic gradient and movement of water. From these observations we may conclude that the transition from the orthodox to aggregated configuration is determined both the extent of Ca^{2+} binding and by the osmotic gradient between the matrix and intracristal spaces.

Physical Changes which Accompany the Aggregated to Orthodox Transition

The calcium-induced transition of mitochondria from the aggregated to orthodox configuration can be followed by a decrease in 90° light scattering (see Figs. 7 and 8), by a decrease in optical density at 520 mμ (see Fig. 9), by a release of protons (see Fig. 10), and finally by electron microscopic examination (see Figs. 3 and 11). The curve in Fig. 6 shows the change in the 90° light scattering induced by addition of 155 mμmoles of $CaCl_2$/mg protein. The observed response is relatively slow, requiring 3–5 min for completion. This decrease in light scattering is independent of temperature from 10–35°; does not require the addition of phosphate, substrate, or nucleotides; and is not affected by inhibitors either of respiration or of ATP hydrolysis (rotenone, antimycin, and rutamycin). The magnitude of the light scattering as a function of the Ca^{2+} concentration is plotted in Fig. 8. When 30 mμmoles of $CaCl_2$/mg protein were added to the medium the light scattering change was 50% of the maximum. The change in configuration could also be monitored by measuring changes in the optical density at 520 mμ. Figure 9 shows that there is a decrease in light absorption at 520 mμ following the addition of $CaCl_2$. The decrease in optical density induced by addition of Ca^{2+} to adrenal mitochondria had been previously observed but this effect was interpreted in terms of the swelling of the

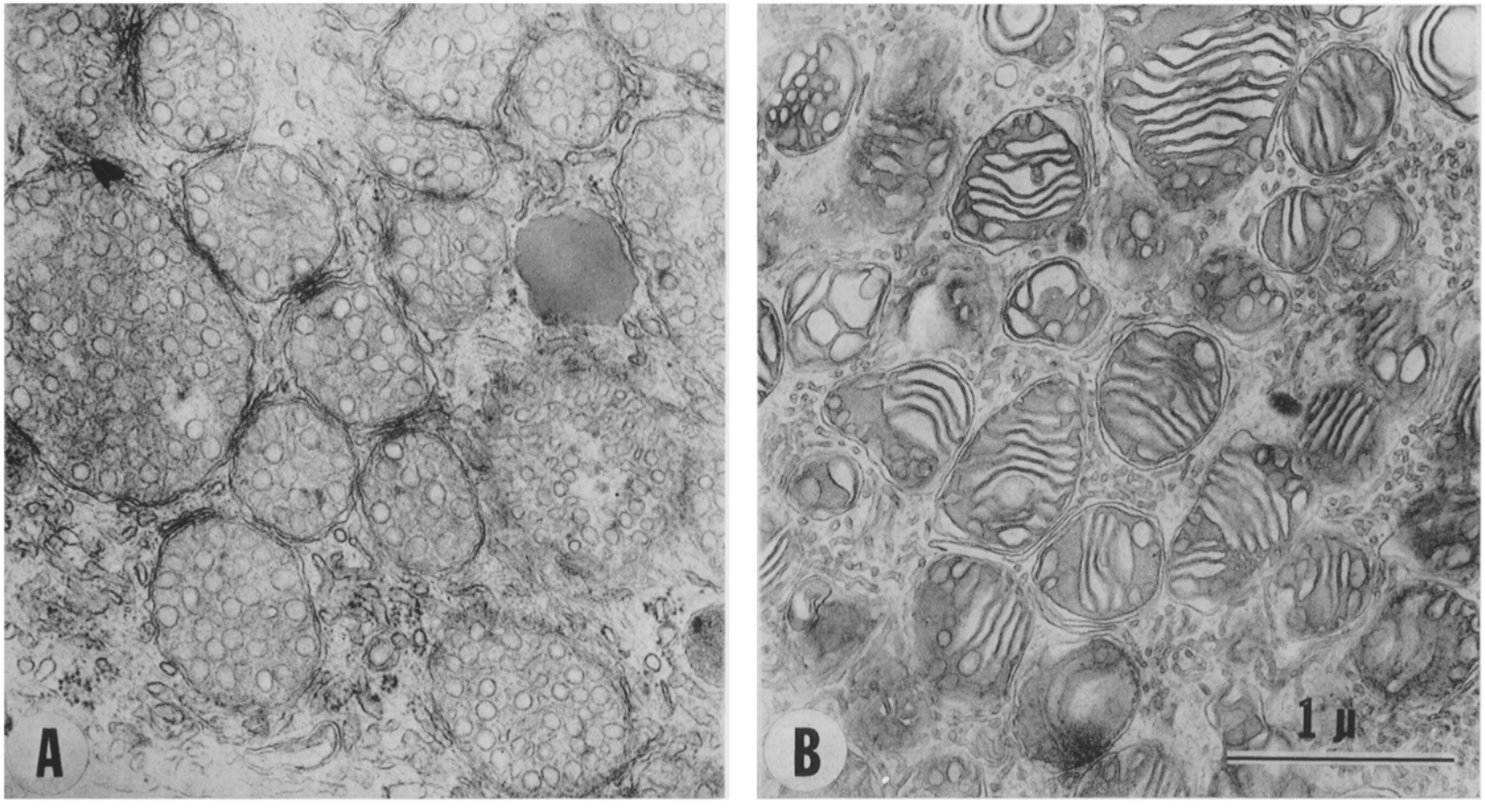


Figure 6. Electron micrographs of adrenal cortex mitochondria *in situ*. A (24300) : a section of a slice of adrenal cortex incubated for 20 min at room temperature in Ca^{2+}-free Krebs-Ringer-Phosphate buffer prior to fixation with glutaraldehyde. B (37149) : a section of a slice of adrenal cortex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

mitochondria.[18–20] As shown in Table III, the water content of the mitochondria after removal from the medium by centrifugation was only slightly increased after exposure of the mitochondria to Ca^{2+}; this small change in water content could hardly account for the extensive light scattering change. This conclusion is in accord with the results of electron microscopy which fail to show any marked swelling of the mitochondria during the aggregated-to-orthodox transition. The light scattering and optical density changes reflect an internal rearrangement of the mitochondria by transfer of water from the intracristal space to the matrix space.

The addition of $CaCl_2$ to a suspension of adrenal cortex mitochondria under anaerobic conditions and in presence of the following inhibitors (antimycin, rotenone, and rutamycin) induces a rapid release of protons (the protons being released within 3 sec) (see Fig. 10). The binding of 20–25 mμmoles of Ca^{2+}/mg protein is accompanied by the release of 28–30 mμmoles of protons/mg protein. Thus, about 1·4 mμmoles of protons are released/mμmole Ca^{2+} bound.

The Speed of the Aggregated-Orthodox Transition

The transition of cristae from the aggregated configuration to the orthodox configuration can be separated into two steps: (a) the binding of Ca^{2+} and release of protons, which is a rapid process; and (b) the configurational change, as measured either by decrease in light scattering or by decrease of optical density at 520 mμ, which is a relatively slow change. The Ca^{2+} binding was complete within seconds (see Table II). Although the shortest time period tested was 15 sec, we have reason to believe that the binding of Ca^{2+} is as fast as the release of protons, i.e., in 1–3 sec. However, in this short time period no light

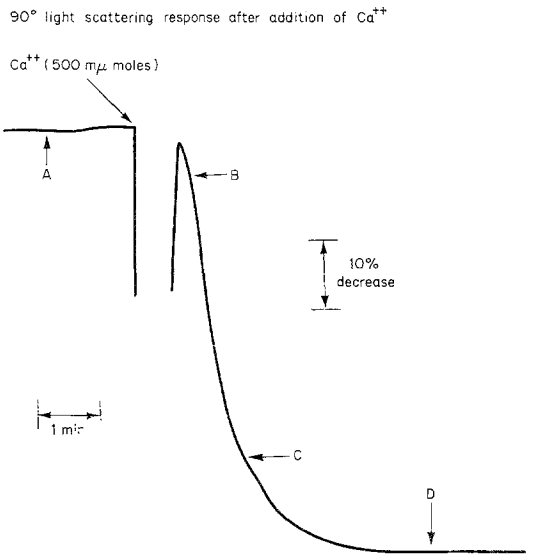


Figure 7. Effect of added $CaCl_2$ on the 90° light scattering response of mitochondria in the aggregated configuration. Mitochondria initially in the aggregated configuration were incubated at 25° in ST (Ca^{2+}-free) at a protein concentration of 1 mg/ml. 500 mμmoles of $CaCl_2$/3 ml were added to induce the decrease in light scattering. The letters *A*, *B*, *C*, and *D* indicate times at which aliquots were fixed for electron microscopy as referred to in Fig. 11.

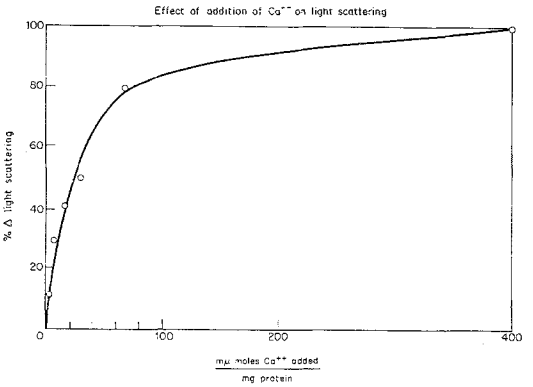


Figure 8. Effect of increasing concentrations of $CaCl_2$ on the 90° light scattering response of mitochondria initially in the aggregated configuration. The data are expressed as a percentage of the total light scattering excursion 6 min after the addition of 400 mμmoles of $CaCl_2$/mg protein. A similar curve was obtained when the data were expressed as the initial rate of light scattering change as a percentage of the rate obtaining when 400 mμmoles of $CaCl_2$/mg protein were added. The assay conditions were identical with those described in the legend of Fig. 7.

scattering change was observed (see Fig. 7) and no change in the ultrastructure (see Fig. 11A and B). As the extent of the light scattering change increased, an increasing proportion of the mitochondrial population was found in the orthodox configuration (see Fig. 11C and D), until finally at 5 min when the light scattering change was completed, the mitochondria were all in the orthodox configuration.

Specificity of Ca^{2+} for Induction of the Orthodox Configuration

Zn^{2+} was the only divalent metal ion among a group of seven tested that could duplicate the effect of Ca^{2+} (see Fig. 12). Mg^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+}, and Ni^{2+} were inactive.

Prevention of the Ca^{2+}-Induced Transition to the Orthodox Configuration by Other Divalent Metals

When mitochondria with cristae in the aggregated configuration are pretreated with any one of the following set of divalent cations (Mg^{2+}, Ba^{2+}, Mn^{2+}, or Sr^{2+}) before addition of Ca^{2+}, relatively little transition in configuration is induced by Ca^{2+}. For example, the addition of $MnCl_2$ or $MgCl_2$ at a concentration of 100 mμmoles per mg protein will reduce by 90% the light scattering change induced by Ca^{2+} at a concentration of 100 mμmoles/mg. This is not a question of competition for binding sites (see Fig. 13). The binding of Ca^{2+} is relatively unaffected by the presence of Mn^{2+}. The neutralization by other divalent metal ions of the induction by calcium must, therefore, involve a facet other than or additional to binding. Added Mg^{2+} or Mn^{2+} suppresses not only the light scattering change induced by Ca^{2+}, but also the release of protons. This result indicates that the proton release induced by Ca^{2+}

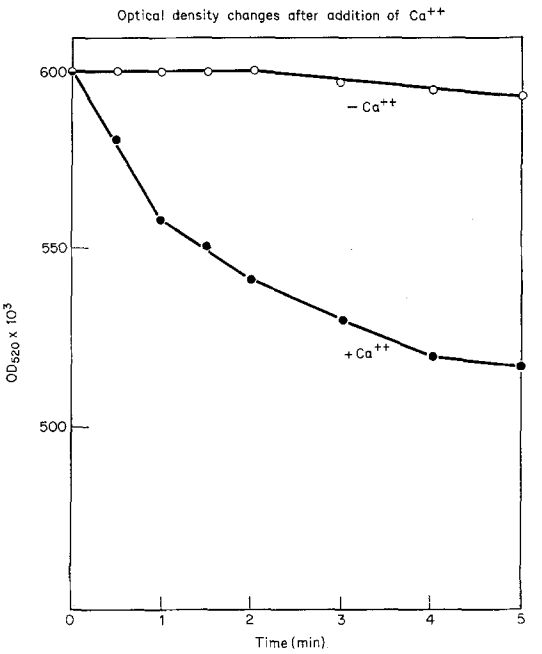


Figure 9. Effect of $CaCl_2$ on optical density at 520 mμ of a suspension of mitochondria initially in the aggregated configuration. Mitochondria were suspended in a medium 0·25 M in sucrose (Ca^{2+}-free) and 10 mM in TrisCl, pH 7·5, at a protein concentration of 0·5 mg/3 ml. Incubation was carried out at 25°. Changes in optical density were initiated by addition of 300 mμmoles Ca^{2+}/mg protein.

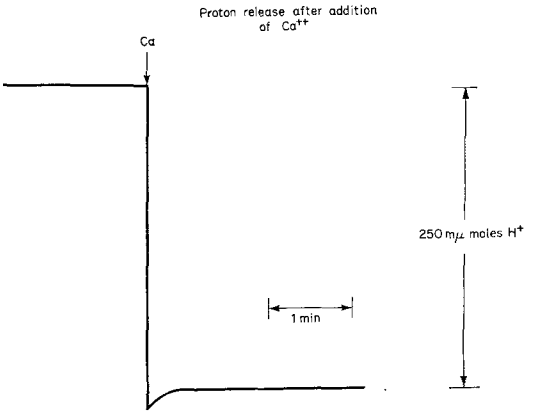


Figure 10, The release of protons induced by addition of Ca^{2+} to mitochondria in the aggregated configuration. The mitochondria (10 mg protein) were incubated in 5 ml of 0·25 M sucrose (Ca^{2+}-free) containing 20 μg rotenone, 20 μg antimycin, and 20 μg rutamycin. To initiate the proton release 150 mμmoles of $CaCl_2$/mg protein were added. The H^+ equivalence of the excursion of the pen in the recording system was determined by addition of a known quantity of standard HCl.

TABLE III. Total water content of adrenal cortex mitochondria in the aggregated and orthodox configurations

mμmoles $CaCl_2$ added/mg protein	mg water/mg protein
0	5·20
93	5·73
370	5·80

Adrenal cortex mitochondria isolated in STE were incubated at 25° in 5 ml of ST (Ca^{2+}-free) supplemented with varying amounts of $CaCl_2$. The final concentration of mitochondria was 1 mg/ml. After 5 min the samples were centrifuged for 10 min at 100,000 × *g* in a SW No. 65 rotor. The pellets were weighed before and after drying overnight at 100° in a vacuum oven. The difference in weight was taken as a measure of the water content. This value includes both the extraparticulate water and the intramitochondrial water.

cannot reflect merely the binding of Ca^{2+} to the mitochondrion since it can disappear even when binding is taking place.

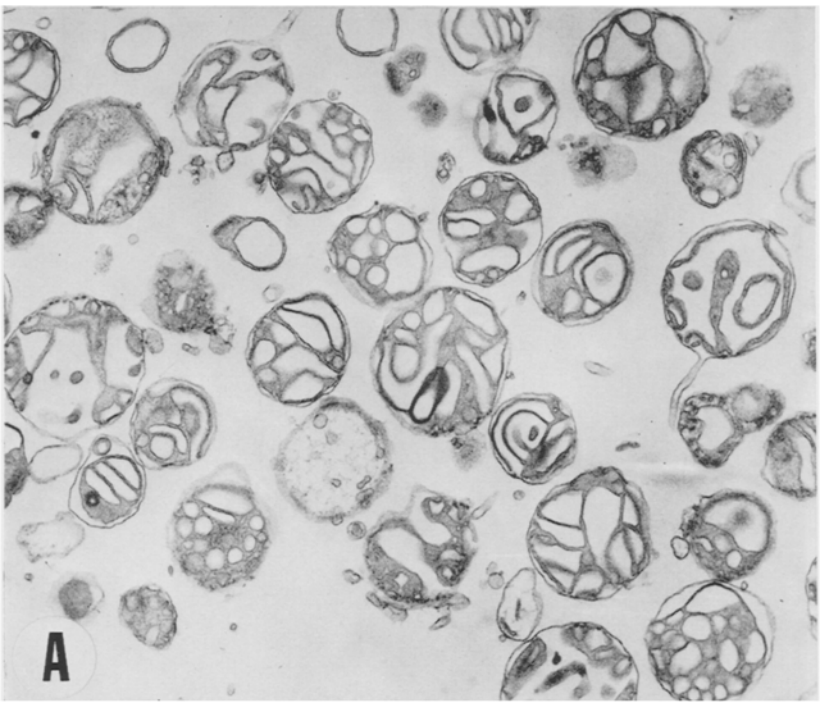
Transition to the Aggregated Configuration Induced by Mg^{2+} and Mn^{2+}

When mitochondria in the orthodox configuration were exposed to Mn^{2+} (1 mM) in the presence of inorganic phosphate (5 mM), both binding of Mn^{2+} (60–80 mμmoles/mg protein) and light scattering changes were observed; these changes were preceded in turn by an uptake of protons. Electron microscopic examination of the mitochondria thus exposed to Mn^{2+} in presence of inorganic phosphate showed that >50% had undergone the transition from the orthodox to the aggregated configuration (see Fig. 14). The level of bound Ca^{2+}, was not affected by the configurational transition.

A mixture of Mn^{2+} and Mg^{2+} or even Mg^{2+} alone can induce the aggregated configuration. When both Mg^{2+} and Mn^{2+} are added, the total amount of ions bound is about the same as for Mn^{2+} alone, but the amount of Mn^{2+} bound is reduced since both Mg^{2+} and Mn^{2+} compete for the same binding site.

The transition of the cristae from the aggregated to the orthodox configuration leads to a proton uptake just as the transition of the crista from the orthodox to the aggregated configuration leads to a proton release. This proton uptake is not a reflection of Mn^{2+} binding because conditions can be imposed in which Mn^{2+} will be bound as usual without accompanying proton uptake or configurational change. This deadlock in respect to configurational change is accomplished by balancing the binding of Ca^{2+} and Mn^{2+}.

The proton jump would appear to be a measure of the conformational change attending the orthodox-aggregated transition. Since the conformational change from the orthodox to aggregated is the reverse of that from the aggregated to orthodox, the proton jump for the first transition is equal in magnitude and opposite in direction to that of the reverse transition. The proton jump is too rapid to be measured (complete within seconds) whereas the light scattering changes are considerably slower (3–5 min required for completion). The rapid proton jump is a token for the speed of conformational change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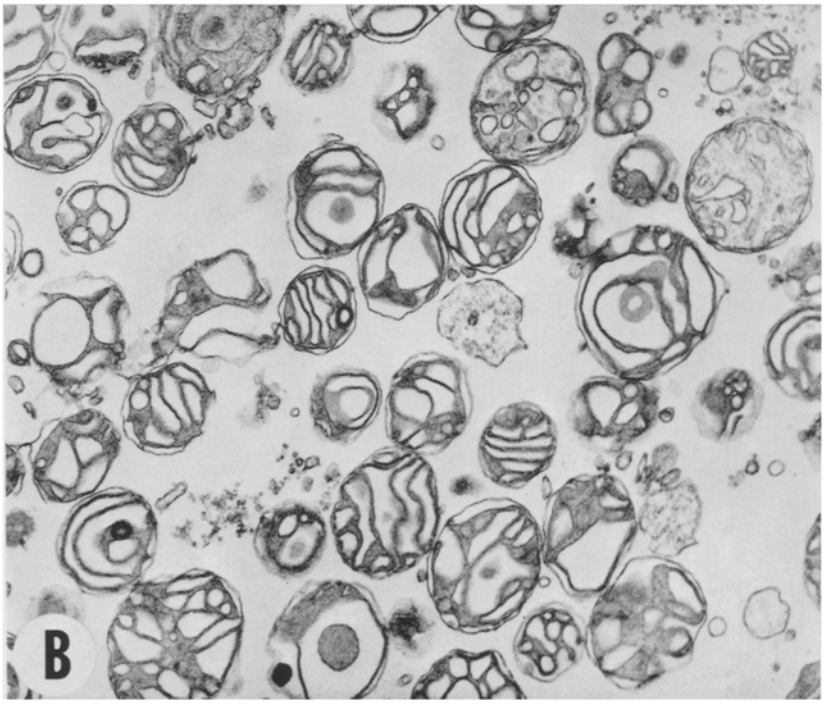
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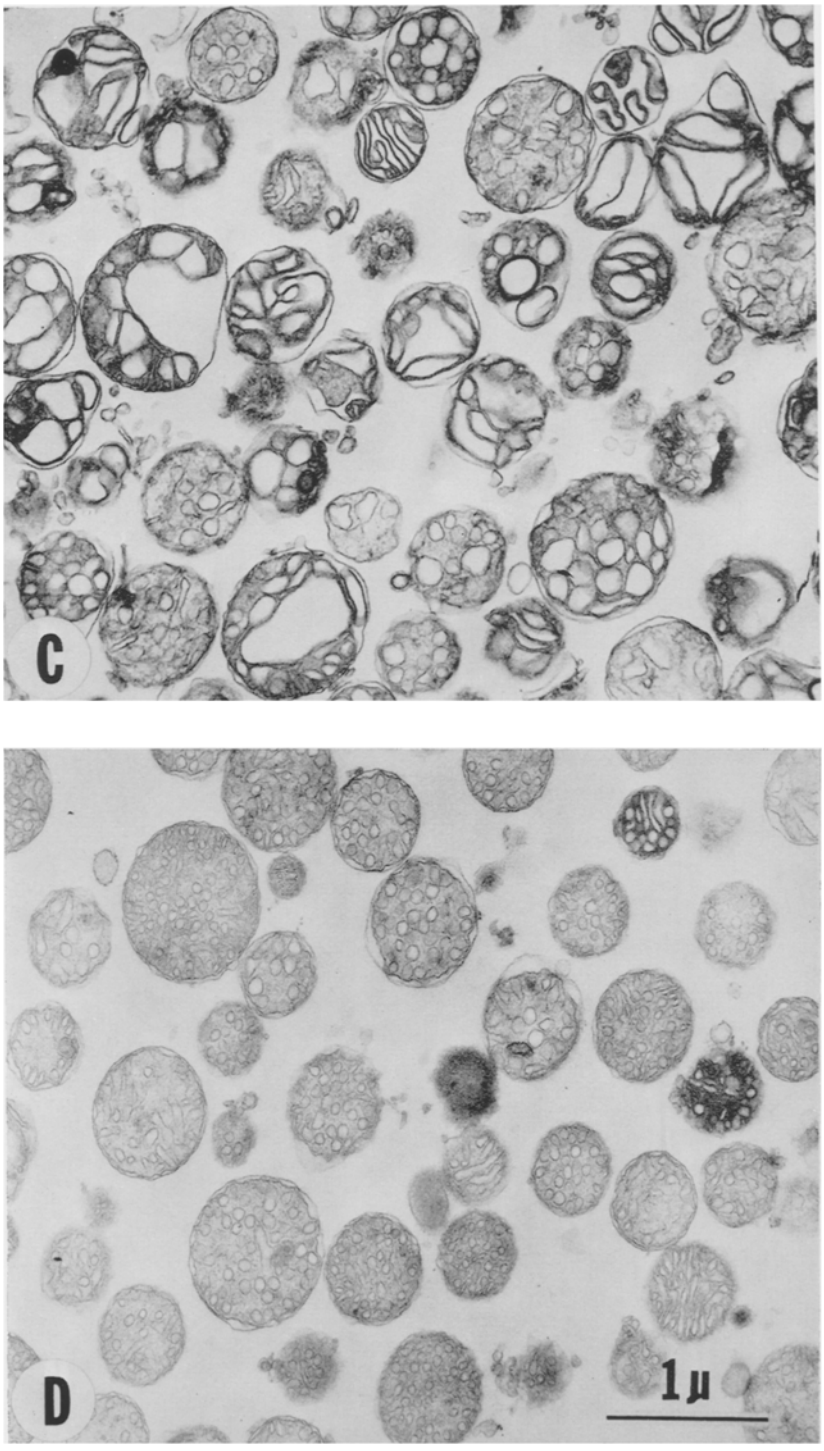

Figure 11. The rate of transition of cristae from the aggregated configuration to the orthodox configuration following addition of $CaCl_2$. The conditions were identical with those described in the legend of Fig. 7. The mitochondria were fixed by addition of 10 mM glutaraldehyde at the times specified by the letters A (38548), B (38550), C (38762), and D (38953) in Fig. 7.

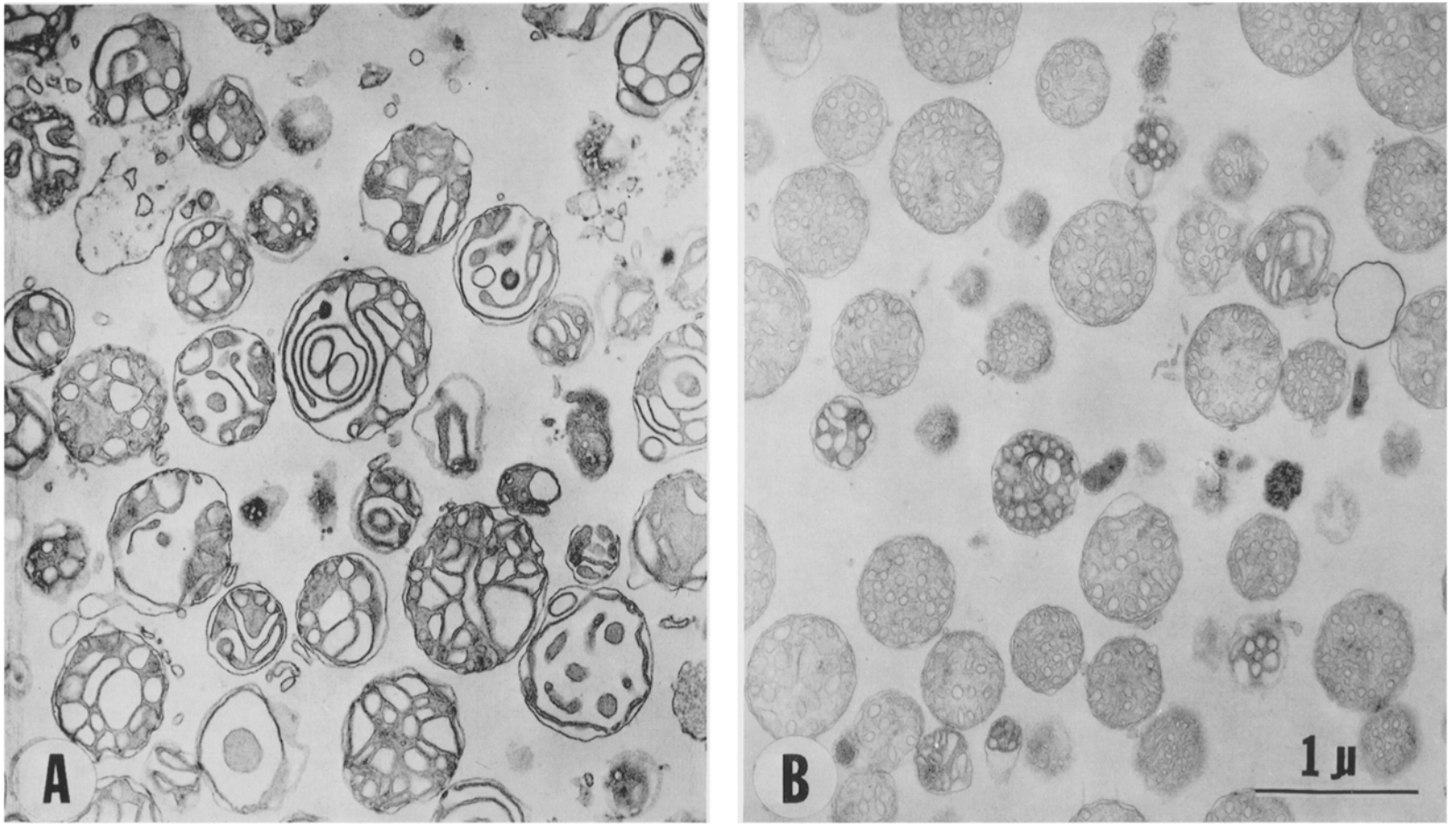

Figure 12. The transition of adrenal cortex mitochondria from the aggregated to the orthodox configuration induced by $ZnCl_2$. Mitochondria were suspended in a medium 0·25 M in sucrose (Ca^{2+}-free) and 10 mM TrisCl, pH 7·5, at 25°. The concentration of mitochondria was 1 mg protein/ml. A is an electron micrograph of mitochondria in the aggregated configuration prior to addition of $ZnCl_2$; B is an electron micrograph of mitochondria 5 min after addition of 200 mμmoles $ZnCl_2$/mg protein. (38551 and 38952)

while the relatively slow light scattering change is a token of the fact that the configurational change can be and in this case is, indeed, much slower than the conformational change.

Discussion

A key question to be evaluated is why the mitochondria of adrenal cortex can be isolated in 0·25 M sucrose in the orthodox configuration whereas all other mitochondria after isolation in the same medium are in the aggregated configuration.[8–12] The orthodox to aggregated configurational transition is determined by two variables—the Ca^{2+}:Mg^{2+} ratio in the mitochondrion, and the osmotic pressure of the medium given the impermeability of the inner membrane to the solute in question. Thus, the tendency of added or endogenous Ca^{2+} to induce the orthodox configuration can be repressed by increasing the osmotic pressure of the medium. Conversely, the tendency of 0·25 M sucrose to generate the aggregated configuration can be repressed by increasing the Ca^{2+}:Mg^{2+} ratio. Adrenal cortex mitochondria are unique in that the Ca^{2+}:Mg^{2+} ratio is a more critical determinant of the orthodox-aggregated transition than the osmotic pressure. Providing the Ca^{2+} level of the mitochondrion is higher than 20 mμmoles/mg protein, isolated adrenal cortex mitochondria will remain in the orthodox configuration even in 0·25 M sucrose.

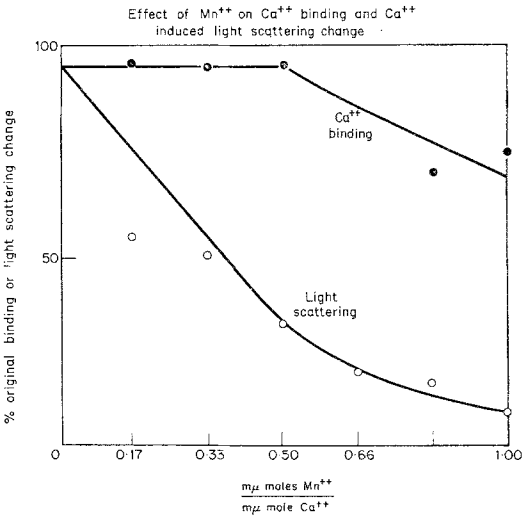

Figure 13. Effect of prior addition of $MnCl_2$ to mitochondria in the aggregated configuration on both the binding of Ca^{2+} and the light scattering response induced by addition of $CaCl_2$. The mitochondria were incubated at a protein concentration of 1 mg/ml in a medium 0·25 M in sucrose (Ca^{2+}-free) and 10 mM in TrisCl, pH 7·5, at 25°. Various concentrations of $MnCl_2$ were added to the suspension. The light scattering changes were induced by the addition of 100 mμmoles of $CaCl_2$/mg protein. The light scattering data are expressed as percentages of the excursion 6 min after addition of $CaCl_2$, in the absence of $MnCl_2$.

The orthodox to aggregated transition involves a configurational change triggered either by an osmotic change induced by addition of a nonpermeant solute to the medium or by addition of a particular divalent metal ion (Ca^{2+} or Mg^{2+}). While it is not possible at the present time to specify the exact sequence of events, what is clear is that there are two alternative ways of triggering the configurational transition and that coincident with this transition is a proton change. Although we are observing by electron microscopy only configurational changes, it is obvious, if only from the extreme changes in geometry of the cristal membrane, that conformational changes in the repeating units underlie the gross configurational changes in the membrane. The tentative conclusions to be drawn from these correlations are that a conformational change can be induced by osmotic means or by a change in charge density of the membrane mediated by addition of the appropriate ions and that the conformational change in turn leads to a whole series of other sequellae—ejection or uptake of protons, configurational changes, further changes in charge density, etc. The orthodox to aggregated transition is admittedly not an

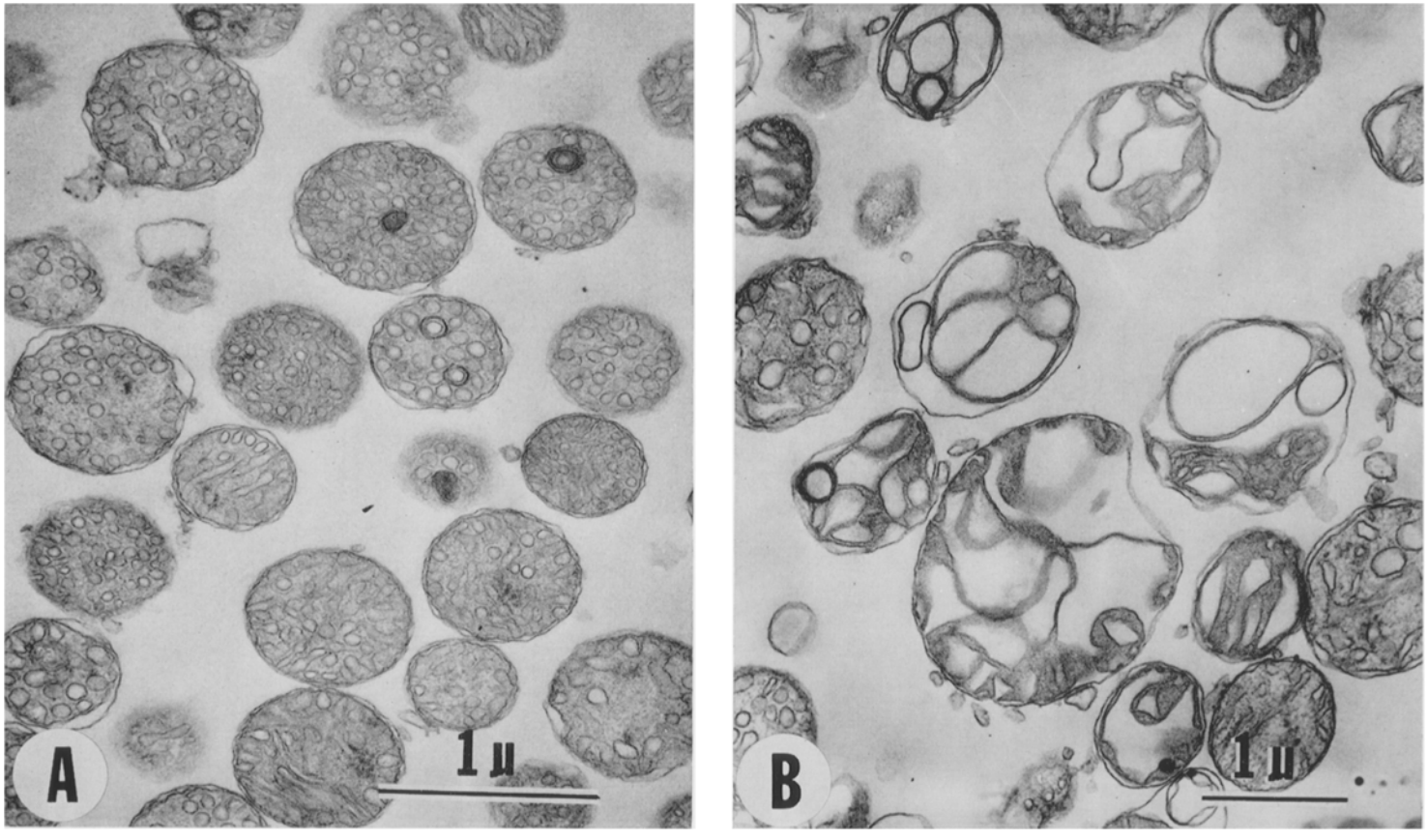

Figure 14. $MnCl_2$-induced transformation of adrenal cortex mitochondria from the orthodox to the aggregated configuration. Mitochondria in the orthodox configuration were incubated at a protein concentration of 1 mg/ml in a medium 0·25 M in sucrose (Ca^{2+}-free), 10 mM in TrisCl and 10 mM in phosphate, pH 7·5, at 25°. A (38949): a micrograph of the orthodox mitochondria prior to the addition of $MnCl_2$; B (38766): a micrograph 5 min after addition of $MnCl_2$ (1 mM).

energized process, but nonetheless it provides a model for energized conformational transitions. When a conformational change is triggered by electron transfer or ATP hydrolysis, the sequellae (osmotic changes, changes in charge density, configurational changes, etc.) are the same as those established for the orthodox to aggregated transition. Elsewhere G. Vanderkooi and J. Young will be developing a rigorous thermodynamic treatment of the interrelationships between conformational change and the relevant variables. The present study provides the experimental basis for the validity of such interrelationships.

The orthodox to aggregated configurational transition is emerging as one of the critical control processes in the cell and this process is modulated by the ratio of Ca^{2+} to Mg^{2+}, by the osmotic pressure of the medium and by a variety of charged molecules such as thyroxine, free fatty acids, anaesthetics, ionophores, and polyanions (silicomolybdate). In other communications of this series, data bearing on the action of the above modulators will be presented.

Acknowledgements

We gratefully express our thanks to Mrs. A. Abrahamson, Miss G. Ghatala, and Mr. D. Silver for expert technical assistance.

This work was supported in part by the U.S.A. Public Health Service Program Project Grant GM-12,847 from the National Institute of General Medical Sciences and by a training grant GM-88 from the same source.

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