Bioenergetics (1970) **1**, 247–271

Control of Mitochondrial Swelling by Mg^{2+}—The Relation of Ion Transport to Structural Changes

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Abstract

Mitochondria from rat heart, liver, and skeletal muscle, when isolated in a medium containing 10 mM EDTA, showed poor respiratory control and large volume changes in the presence of ATP or oxidizable substrates. Swelling required the presence of alkali metal cations and permeant anions and probably reflects ion transport. As shown previously for skeletal muscle mitochondria, addition of Mg^{2+} prevents major volume changes and also imposes control of respiration by phosphorylation. Addition of Mg^{2+} after ATP-energized swelling caused shrinkage of the mitochondria back to the original state. This reversal of swelling is energized by ATP, but not by substrate (when ATP synthesis is inhibited by oligomycin). Electron micrographs showed that the matrix space of mitochondria from all three sources expands during the swelling process. In addition, cristal membranes showed several three-dimensional arrangements. In the contracted state which existed in presence of ATP + Mg^{2+}, cristae of muscle mitochondria appeared "sheetlike", whereas in presence of substrates many of these cristae assumed a "tubular" appearance. The structural changes in liver mitochondria under these conditions appeared similar to those of muscle mitochondria, but because of the greater proportion of matrix in liver mitochondria, the terms "sheetlike" and "tubular" are less descriptive of the situation. A transition from tubular to sheetlike appearance occurred upon addition of either ATP or ADP to respiring mitochondria in presence of added substrates and phosphate. This transition required Mg^{2+} and may involve the same mechanism that brings about reversal of swelling. The implications of these studies are discussed in relation to proposed mechanisms of odixative phosphorylation.

Introduction

Raaflaub, in 1953, observed a decrease in absorbance at 400 mμ (mostly due to light scattering) by mitochondrial suspensions when the suspending medium was reduced to ½ isotonicity or less.[1] He attributed this change to osmotically-induced swelling of the mitochondria. Other workers have since confirmed his interpretation of light-scattering data using a variety of other methods and have also shown that ion accumulation by mitochondria causes swelling.[2–9] Ion-induced swelling is due, at least in part, to osmotic imbalance. The matrix space, i.e. the volume enclosed by the mitochondrial inner membrane, is the osmotically active space involved.[3,6]

Raaflaub, in the study referred to above, noticed that addition of various substances, including ADP, phosphate, and succinate, caused changes in scattering, even in isotonic

media. Chance and Packer, in 1958, found that a suspension of respiring mitochondria, in the presence of phosphate, underwent an increase in light-scattering upon addition of ADP.[10] These early observations represent the birth of an idea that has captivated a number of investigators in recent years, namely, that major changes in mitochondrial structure are closely associated with energy transfer processes.[11–18] Carrying the concept to its extreme, some believe that configurational changes observable with electron microscopy are actually a form of potential energy which can then be utilized to form ATP.[15, 18]

Mitchell has presented a rather strong case for the primary involvement of ion translocation in energy coupling.[19] Since it is clear that transport phenomena can cause mitochondrial structural changes, the strength of any hypothesis seeking to show a direct relationship between structural rearrangements and energy conservation depends on the manner in which transport phenomena have been excluded or controlled. Complete monitoring or control of transport is not easily accomplished since many substances likely to be involved in transport (e.g. metal ions, nucleotides, phosphate, substrates, H^+, and OH^-) are initially present endogenously, if not also in the suspending medium.

Results reported by several investigators suggest that Mg^{2+} plays a significant role in the permeability of mitochondrial membranes.[20–24] Although mitochondria ordinarily have only limited permeability to monovalent cations,[25, 26] Azzone and Azzi have found that treatment of liver mitochondria with EDTA produces permeability changes similar to those induced by valinomycin and gramicidin.[20]

Dow has reported several properties of rat skeletal muscle mitochondria isolated in the presence of 10 mM EDTA and heparin.[24] She found rapid, substrate-energized swelling of these mitochondria supported equally by Na^+ or K^+ in a medium containing a permeant anion such as acetate or phosphate. She also found an ATPase and ATP-supported swelling which were stimulated by Na^+ but not by K^+. The swelling could be prevented by Mg^{2+} or reversed after extensive swelling by the addition of ATP + Mg^{2+}. Reversal of swelling did not occur with substrate when oligomycin was present. Such EDTA-treated mitochondria also showed poor respiratory control. However, addition of Mg^{2+} restored full control of respiration by phosphorylation. Mg^{2+}, indeed, does appear to exert control over certain aspects of mitochondrial membrane permeability and transport.

The present investigation was undertaken: (1) to correlate electron micrographic studies of morphology with changes in light-scattering and estimated volume from Coulter-Counter measurements; (2) to see if the properties observed for skeletal muscle mitochondria are also found in mitochondria from other tissues; (3) to determine if these properties depend on the use of heparin in the isolation medium; and (4) to study morphological changes that appear closely associated with oxidative phosphorylation in mitochondria. In the present report, the role of Mg^{2+} in the reversible control of mitochondrial swelling and configurational changes suggests involvement of ion transport in both of these processes.

Materials and Methods

Reagents

All solutions employed in these studies were prepared with deionized water and showed no heavy metal contamination, as tested with 8-hydroxyquinoline.[27]

Isolation of Mitochondria

Liver, heart, and skeletal muscle mitochondria were isolated from 140 to 200-g rats in the cold. Mitochondria were isolated and suspended finally in a solution containing mannitol, 0·21 M; sucrose, 0·07 M; EDTA, 0·01 M; and heparin (330 units/ml-medium, approximately 0·5% crystalline sodium heparin, General Biochemicals). Heparin was employed in the isolation medium because it appears to preserve the functional integrity of mitochondria.[28] Mitochondria were also isolated in the absence of heparin for comparative purposes. The isolation procedure for skeletal muscle mitochondria and the high respiratory and energy-coupling activities observed for such mitochondria are outlined in a previous publication.[28] Heart mitochondria were isolated from five to eight rat hearts per preparation. Hearts were first chopped finely with the McIlwain tissue slicer, then homogenized in Tri-R, ground-glass-Teflon homogenizers (three passes each with pestles allowing 0·015 and 0·006 inch clearance used sequentially and rotated at 800–1000 rpm) in ten volumes of the isolation medium described above. Centrifugation for 10 min at 650 × *g* sedimented nuclei and cellular debris. The supernate was decanted through five layers of cheese-cloth and centrifuged at 12,000 × *g* for 10 min. A "light-heavy split" was made by adding a few milliliters of isolation medium to centrifuge tubes containing the pellets and gently swirling until the upper layer lifted off. Heavy mitochondria were resuspended in 10 ml of isolation medium and sedimented at 10,000 × *g* for 10 min. Any remaining light mitochondria were washed away and pellets were resuspended in approximately 2 ml of isolation medium. The method used for liver mitochondria was almost the same as for heart. A modified isolation medium omitting heparin and with 1 mM instead of 10 mM EDTA was used until the fraction containing the cellular debris had been discarded, since the presence of 10 mM EDTA during homogenization resulted in liver homogenates of high viscosity. Two livers were diced with scissors, washed several times in the modified medium and homogenized in an ordinary Potter-Elvehjem homogenizer with a Teflon pestle (three passes each with pestles allowing 0·016 and 0·006 inch clearance used sequentially and rotated at 800–1000 rpm). The remainder of the procedure was the same as for heart. High respiratory control index and good control of swelling usually persisted in these mitochondria for a period of 4–8 h.

Determination of Protein Content of Mitochondria

The protein content of the mitochondrial suspension was determined by the method of Lowry *et al.*[29] The protein concentration curve was run with mitochondria which had been calibrated against BSA by the biuret procedure.[30]

Polarographic Determination of Respiratory Activity and Control

Oxidation rates and respiratory control indices were measured polarographically at 37° employing a Clark Oxygen electrode. Polarographic determinations were performed on all mitochondrial samples used for swelling and electron microscope studies to ensure that all samples showed high respiratory activity and tight respiratory control. Oxygen utilization and respiratory control indices were measured routinely in the following Mg^{2+}-containing medium: potassium glutamate or pyruvate, 10 mM; potassium malate, 10 mM; potassium malonate, 10 mM; cytochrome *c*, 40 μM; $MgCl_2$, 5 mM; EDTA, 0·5 mM; KCl, 15 mM; $K.PO_4$, pH 7·4, 10 mM; Tris·HCl, pH 7·4, 2·5 mM; bovine

serum albumin, 0·2%; mannitol, to bring to isotonicity (320 mosM); mitochondrial protein, 0·17–0·22 mg protein/ml. A volume of 0·04 ml of 10 mM ADP was added after the basal respiratory rate had been determined. The final basal rate was determined after the addition of oligomycin, 5μg/ml, to the above suspension. Oxygen utilization was also measured in the absence of Mg^{2+}.

Measurement of Change in Absorbance

Changes in optical density at 520 mμ, resulting mainly from light-scattering changes, were used as a qualitative measure of change in mitochondrial volume. A correlation had been established previously between such absorbance measurements and the more direct measurement of volume change given by the Coulter Electronic Particle Counter.[24] Absorbance measurements at 520 mμ in a Gilford recording spectrophotometer were made at room temperature in isotonic salt media at pH 7·4, as outlined beneath the various figures. Approximately 0·2 mg of muscle or liver mitochondrial protein was used in 1·2 ml of medium to give a contracted state absorbance of 0·5 to 0·6. At zero time an aliquot of the mitochondrial suspension was added to the isotonic salt medium containing substrate or ATP as the energy source and the recording of absorbance was continued until a plateau was reached. The effects of various metabolic inhibitors were tested by adding them to the media prior to the addition of the mitochondria.

Electron Microscopy

Mitochondrial suspensions were fixed by addition of 10% glutaraldehyde in 0·1 M sodium cacodylate buffer, pH 7·4, to give a final glutaraldehyde concentration of 0·5%. Glutaraldehyde has been found to rapidly fix structural states of mitochondria.[7] Glutaraldehyde seems to work by reacting with free amino groups of proteins causing cross-linking and polymerization.[31] After 15 min in glutaraldehyde at room temperature, suspensions were centrifuged and pellets briefly washed with 0·1 M sodium cacodylate buffer, pH 7·4, with mannitol-sucrose added to give a final osmolarity of 0·32. Pellets were postfixed overnight at 4° with 1% OsO_4 in sodium Veronal, 0·02 M; sodium acetate, 0·03 M; NaCl, 0·11 M; pH adjusted to 7·2–7·4 with HCl. Before dehydration, pellets were washed with 0·1 M Veronal-acetate buffer, pH 7·4, containing 0·06 M NaCl, and block stained with 0·5% uranyl acetate contained in an identical Veronal-acetate buffer solution except that the pH was 6·0. After dehydration in a series of increasing ethanol concentrations, ethanol was replaced with propylene oxide and samples were embedded in Araldite for sectioning. Thin sections were prepared using an LKB Ultrotome. The sections were counterstained with 1% uranyl acetate followed by 1% $Pb(OH)_2$ in a solution 0·125% in NaOH and 0·1% in sodium-potassium-tartrate, and finally examined with an Hitachi HU-11B electron microscope.

The configurational states observed using the above methods were also obtained after fixation with either OsO_4 or glutaraldehyde alone. However, dual fixation was found to give the best structural preservation and the best contrast.

Results

Absorbance Changes Associated with Ion-supported Mitochondrial Swelling

Earlier studies by Dow[24] have shown that skeletal muscle mitochondria isolated in presence of 10 mM EDTA contain a Na^+-stimulated ATPase activity. In the absence

of added Mg^{2+}, the rate of sodium acetate-supported swelling is directly related to the rate of Na^+-stimulated ATPase. Potassium acetate is a poor, if not completely ineffective, substitute for sodium acetate in ATP-stimulated swelling. Substrate oxidation also facilitates swelling in the absence of Mg^{2+}, but this swelling occurs equally as well with K^+ as with Na^+.

Figure 1 shows the percentage of contracted-state absorbance plotted against time for swelling at room temperature in isotonic sodium acetate media at pH 7·4. The above-reported observations of energy-dependent swelling and its control in EDTA-treated skeletal muscle mitochondria is confirmed in Fig. 1. The same swelling and control properties appear to be present in EDTA-treated heart and liver mitochondria. As had been shown previously for skeletal muscle mitochondria, the ATP-supported absorbance changes were oligomycin-sensitive, and swelling supported by glutamate + malate + malonate or by succinate + rotenone, was inhibited by rotenone or cyanide respectively. The swelling supported by substrate at room temperature was less rapid than the swelling supported by ATP. Na^+-ATPase activity is relatively independent of temperature in contrast to oxidation rate.[24] Presumably the rate of swelling supported by substrate oxidation would approach the ATP-supported rate at a higher incubation temperature. As shown previously, swelling supported either by substrate oxidation or by ATP was almost completely inhibited by Mg^{2+}; swelling supported by ATP in the absence of Mg^{2+} was reversed by the subsequent addition of Mg^{2+} to the medium. Mitochondria from all three tissues behaved similarly (Fig. 1).

Mg^{2+}-imposed Control of Respiration by Phosphorylation

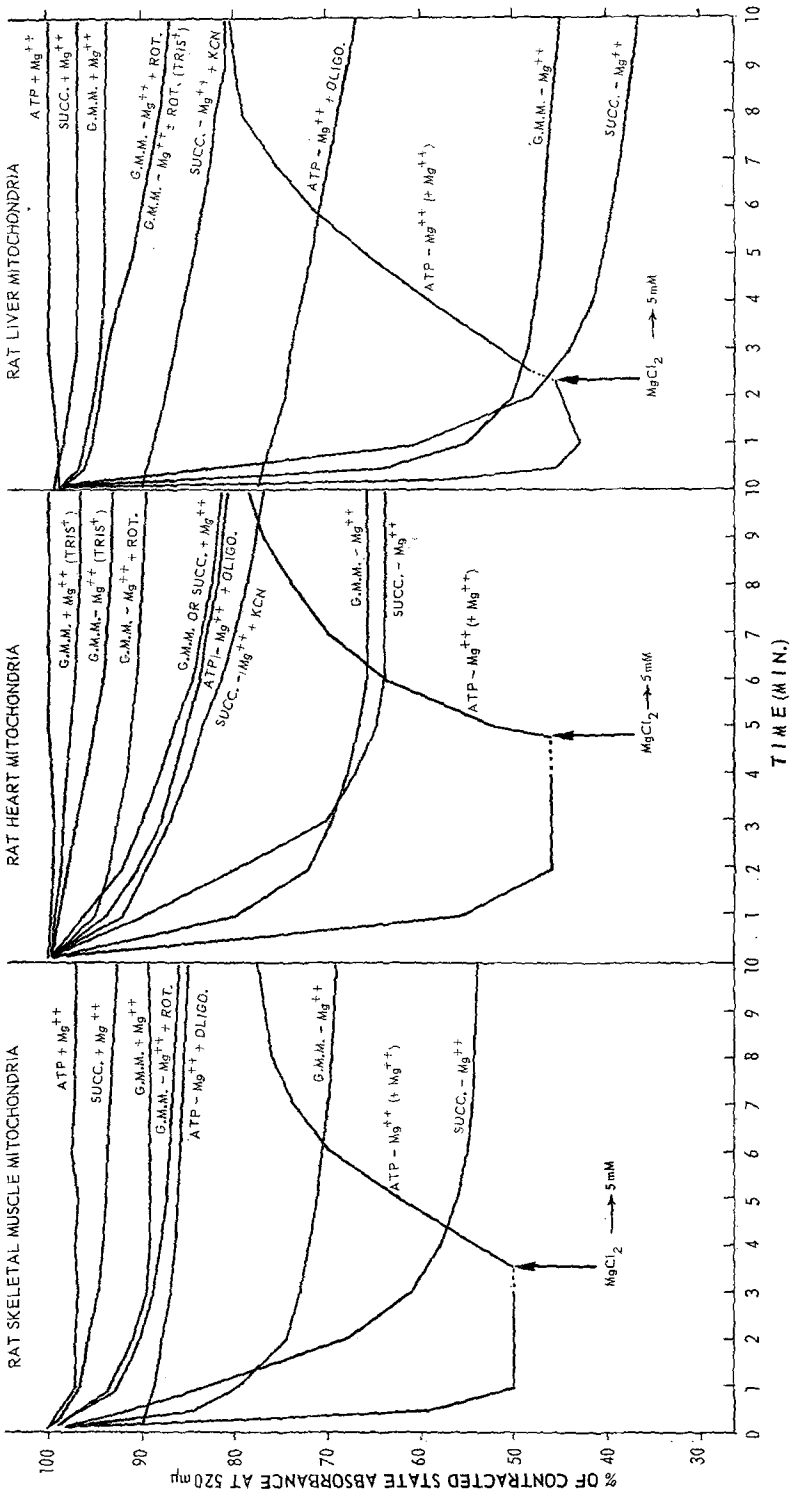
As had been illustrated in earlier communications,[24, 32] addition of Mg^{2+} to EDTA-treated mitochondria increased control of respiration by phosphorylation several fold. This was true regardless of whether the experiments were carried out in a medium which supported massive swelling (e.g. sodium acetate) or in a medium where swelling was limited (e.g. Tris acetate or NaCl). Respiratory activity and respiratory control were not affected by substituting Na^+ for K^+ in the medium.

Effects of Omitting Heparin From Isolation and Resuspension Media

In earlier studies[24, 28] addition of heparin to isolation media, and to the final mitochondrial suspension, preserved the functional integrity of skeletal muscle mitochondria. In the present study, mitochondria isolated and finally suspended in the absence of heparin showed good respiratory, swelling, and swelling-control properties but these activities deteriorated more rapidly than when heparin was used.

Ultrastructural Changes Associated with Energized Swelling

Electron micrographs are presented for mitochondria incubated under conditions which permit energized swelling and under conditions which inhibit or reverse swelling. In most cases, mitochondria were allowed to reach a "steady-state" of swelling or contraction before fixation. All experiments were carried out on mitochondria which had been exposed to 10 mM EDTA. Unless noted otherwise, mitochondria were fixed with 0·5% glutaraldehyde after incubation for 5 min at room temperature in a neutral, isotonic sodium acetate medium. Other additions will be specified for each experiment.


RAT SKELETAL MUSCLE MITOCHONDRIA
RAT HEART MITOCHONDRIA
RAT LIVER MITOCHONDRIA
% OF CONTRACTED STATE ABSORBANCE AT 520mμ
TIME (MIN.)
ATP + Mg++
SUCC. + Mg++
G.M.M. + Mg++
G.M.M. - Mg++ + ROT.
ATP - Mg++ + OLIGO.
G.M.M. - Mg++
SUCC. - Mg++
ATP - Mg++ (+ Mg++)
MgCl2 → 5mM
G.M.M. + Mg++ (TRIS+)
G.M.M. - Mg++ (TRIS+)
G.M.M. - Mg++ + ROT.
G.M.M. OR SUCC. + Mg++
ATP - Mg++ + OLIGO.
SUCC. - Mg++ + KCN
G.M.M. - Mg++ + ROT. (TRIS+)
SUCC. - Mg++ + KCN

Mitochondria as Isolated

Mitochondria as isolated, fixed after a 5-min incubation in the mannitol-sucrose-EDTA-heparin isolation medium, showed a contracted or condensed structure with the inner membrane adhering tightly to a darkly-staining matrix (Figs. 2A–C). Most of the cristae appeared sheetlike. Cristae occasionally showed a zig-zag or wrinkled character, and, in some cases, discontinuities suggested tabular forms (Fig. 2A). We shall see later that predominance of one appearance over another in the population was controlled by the conditions of the experiment.

Mitochondria from all three tissues exhibited similar characteristics, the main difference being a more voluminous and more darkly staining matrix in liver mitochondria than in mitochondria from either muscle tissue.

Swelling Energized by ATP

In the absence of Mg^{2+}, the presence of ATP during incubation in swelling medium containing sodium acetate resulted in mitochondria with markedly expanded and less electron-opaque matrix (see Figs. 3A–C). In many cases the cristae appeared discontinuous and vesicular or tubular in form. Swelling occurred to various degrees throughout the population, in some cases resulting in lysis of the mitochondria. These changes occurred under the same conditions that promoted a pronounced decrease in absorbance (Fig. 1). The presence of oligomycin in the swelling medium + ATP greatly reduced the changes observed by electron microscopy (see Fig. 3D) as well as the decrease in absorbance (Fig. 1). Presumably, the fact that some swelling was observed even in presence of oligomycin was due to the design of this experiment, i.e., the reaction was started by adding mitochondria to swelling medium containing ATP + oligomycin; the effect of oligomycin was thus somewhat delayed. The heterogeneity of the population indicates that, under these conditions, mitochondria were not captured in a steady state. Nevertheless, oligomycin was clearly inhibitory to swelling in presence of ATP, indicating that the action of ATP in supporting swelling is related to an oligomycin-sensitive ATPase. Similar results were obtained for all three types of mitochondria.

Prevention and Reversal of ATP-energized Swelling by Mg^{2+}

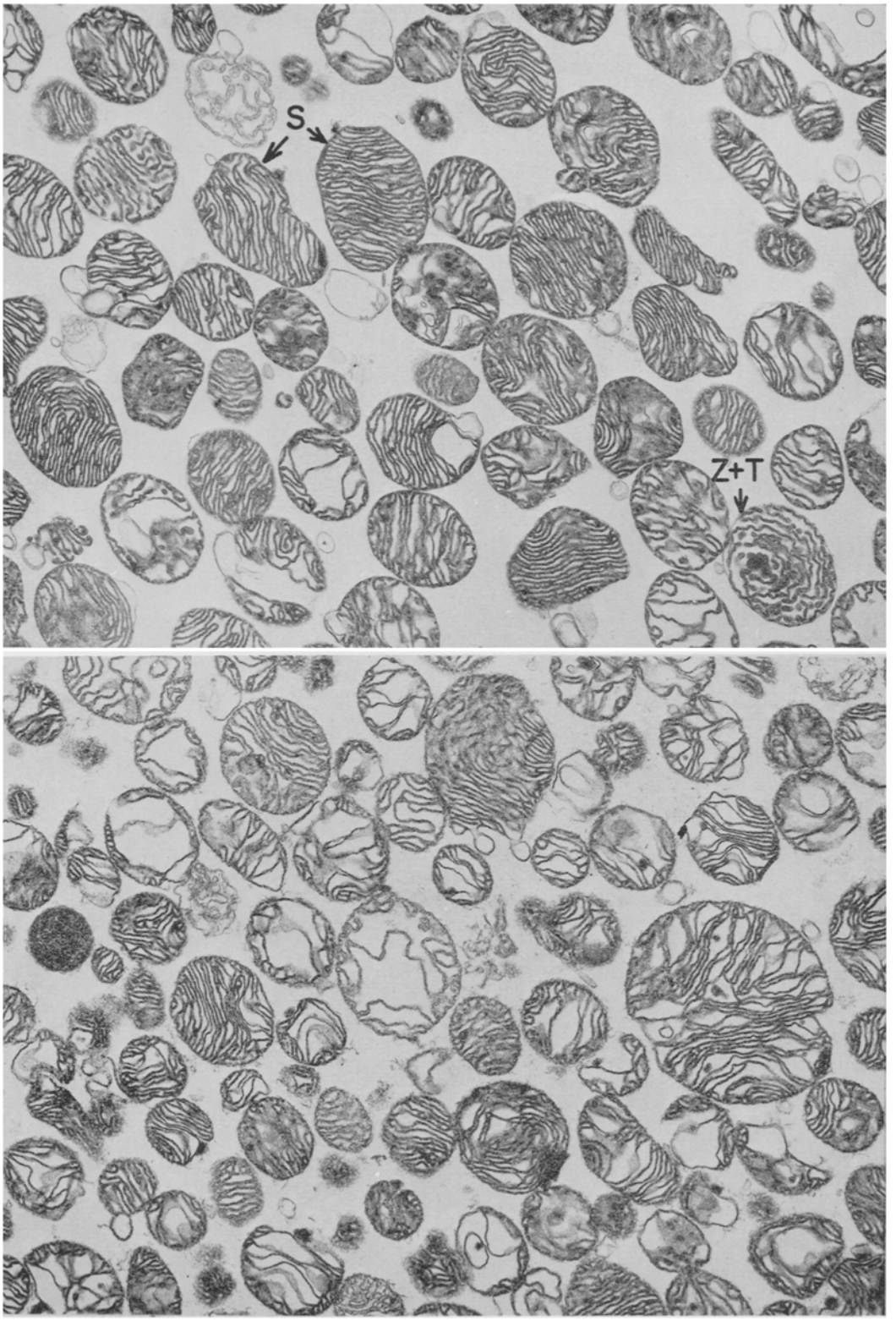
The addition of Mg^{2+} to mitochondria in swelling medium + ATP completely prevented the structural changes that occurred in absence of added Mg^{2+} (Figs. 4A, B).

Figure 1. Absorbance Changes. Mitochondria were incubated at 23° in the media described below. The energy source, inhibitors, and presence or absence of Mg^{2+} are indicated on the separate curves. Where "TRIS$^+$" appears, Tris salts were substituted for sodium salts in the medium. The reaction was initiated by addition of mitochondria to various media at 23°: skeletal muscle mitochondria, 0·10 mg protein/ml; heart mitochondria, 0·15 mg protein/ml; liver mitochondria, 0·15 mg protein/ml. Abbreviations: G.M.M., glutamate, malate, malonate; SUCC., succinate; ROT., rotenone; OLIGO., oligomycin. Composition of media as follows:

Medium for aTP-supported absorbance changes. Sodium acetate, 20 mM; NaCl, 110 mM; Tris·ATP, 5 mM; Tris·HCl, pH 7·4, 2·5 mM; bovine serum albumin, 0·2%, $\pm MgCl_2$, 5 mM; mannitol, to bring to isotonicity (0·32 OsM); ±oligomycin, 5 μg/ml; EDTA, 0·5 mM; heparin, from isolation medium, 0·01%.

Medium for absorbance changes supported by uncoupled respiration of DPN-linked substrates. Sodium acetate, 20 mM; NaCl, 70 mM; sodium glutamate, 10 mM; sodium malate, 10 mM; sodium malonate, 10 mM; oligomycin, 5 μg/ml; cytochrome *c*, 5 μM; Tris·HCl, pH 7·4, 2·0 mM; bovine serum albumin, 0·2%; $\pm MgCl_2$, 5 mM; EDTA, 0·5 mM; mannitol, to bring to isotonicity; heparin, from isolation medium, 0·01%; ±rotenone, 5×10^{-7} M. Where indicated, Tris salts were substituted for sodium acetate, NaCl, sodium glutamate, sodium malate and sodium malonate.

Medium for absorbance changes supported by uncoupled respiration of succinate + rotenone. Sodium acetate, 20 mM; NaCl, 90 mM; sodium succinate, 20 mM; rotenone, 5×10^{-7} M; oligomycin, 5 μg/ml; cytochrome *c*, 5 μM; Tris·HCl, pH 7·4, 2·0 mM; bovine serum albumin, 0·2% $\pm MgCl_2$, 5m M; EDTA, 0·5 mM; mannitol, to bring to isotonicity; heparin, from isolation medium, 0·01%; ± KCN, 0·8 mM.


S
Z+T

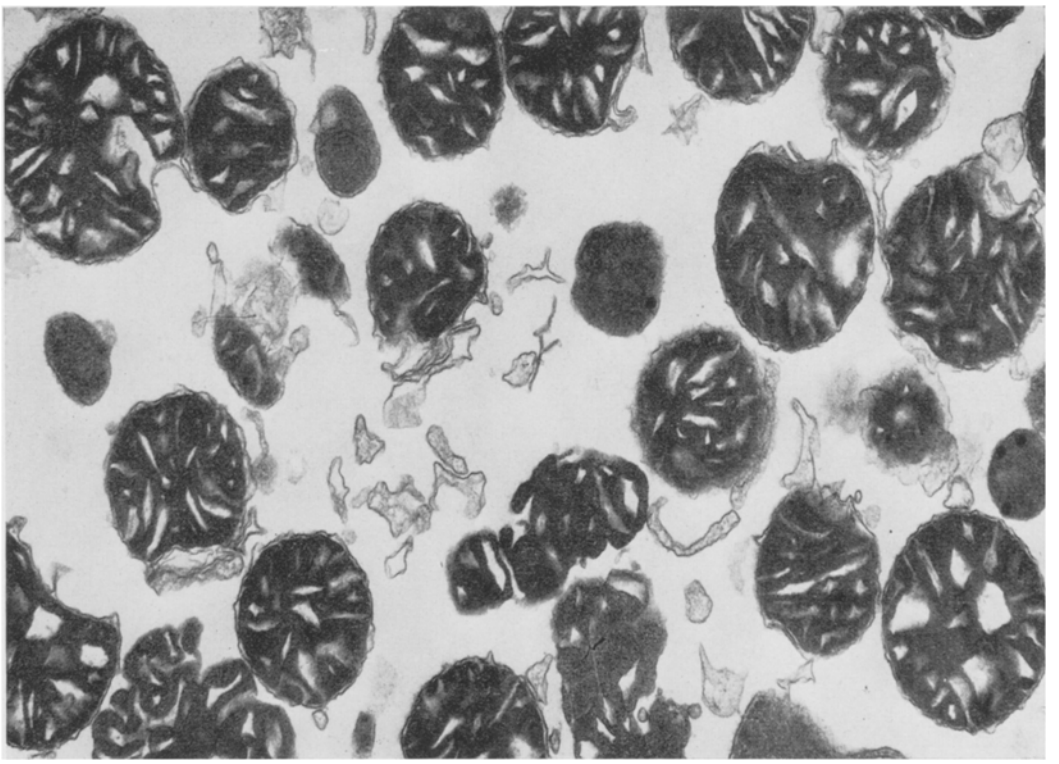
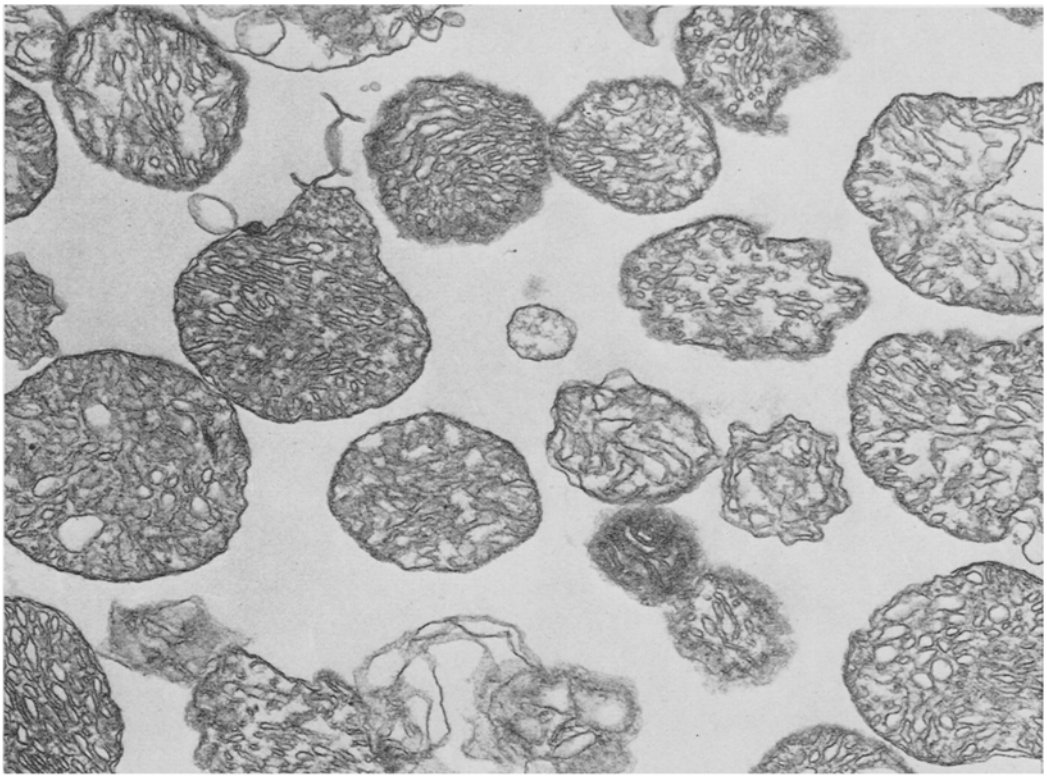


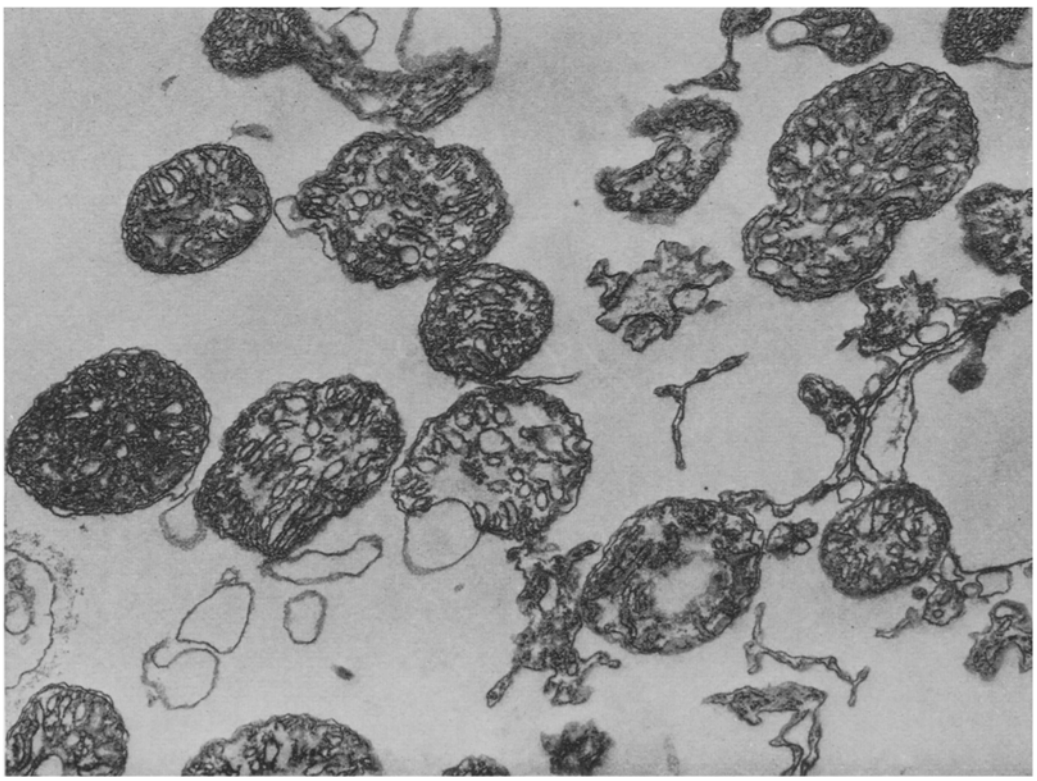
Figure 2. Mitochondria as isolated. (A) Heart mitochondria, 0·17 mg protein/ml. Arrows point to: S, "sheet-like"; Z + T, "zig-zag" and "tubular" cristal arrangements (×17,500). (B) Skeletal muscle mitochondria 0·12 mg protein/ml (×17,500). (C) Liver mitochondria, 0·20 mg protein/ml (×35,000). Medium contained mannitol, 0·21 M; sucrose, 0·07 M; Na_2K_2 EDTA, 10 mM; Tris·HCl, 10 mM, pH 7·4; heparin, 0·5%; and mitochondria.

This prevention of swelling was also reflected in the negligible absorbance change under these conditions (Fig. 1). Furthermore, addition of Mg^{2+} after ATP-energized swelling reversed the direction of the change in absorbance (cf. Fig. 1). Electron micrographs from such an experiment (fixation at 5 min after addition of Mg^{2+}) showed that the majority of mitochondria had a contracted appearance similar to that of non-swollen mitochondria in the presence of ATP and Mg^{2+} (Fig. 4C). In some mitochondria there was no evidence of swelling reversal. However, all mitochondria that failed to exhibit swelling reversal showed severe damage, probably due to lysis during the incubation under swelling conditions, i.e., before Mg^{2+} was added. Note that the cristae of some mitochondria appeared in a transitional state between tubular and sheetlike arrangement (see arrow in Fig. 4C). Mg^{2+} had similar effects in the three types of mitochondria studied.

Substrate-energized Swelling

Figures 5A and B show heart and liver mitochondria respectively, which were incubated in the absence of Mg^{2+} and presence of glutamate + malate + malonate. Oligomycin was included to prevent ATP formation or utilization. A medium containing succinate + rotenone + oligomycin also supported swelling (Fig. 5E). Addition of rotenone prevented swelling in presence of glutamate + malate + malonate (Figs. 5C, D), while addition





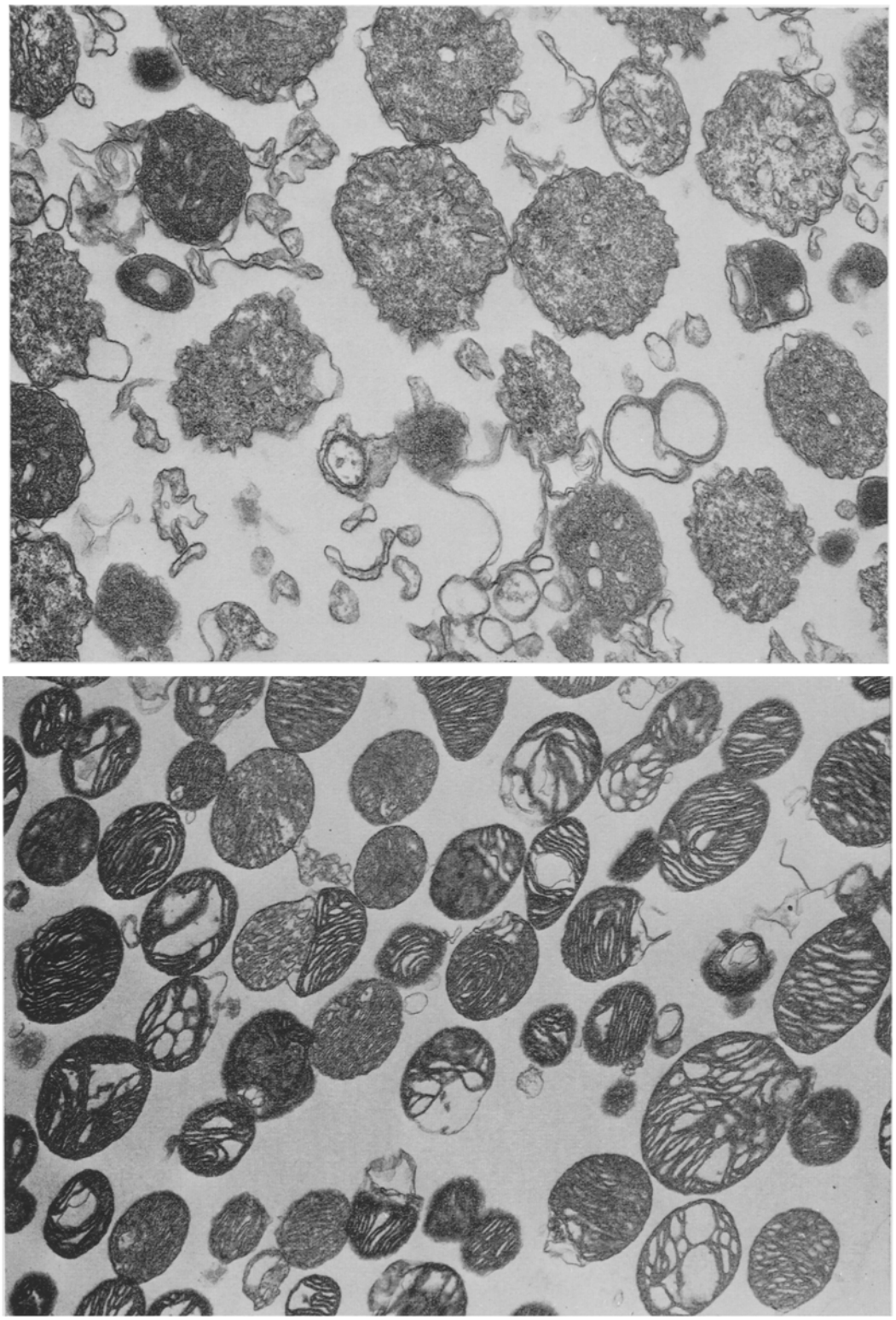
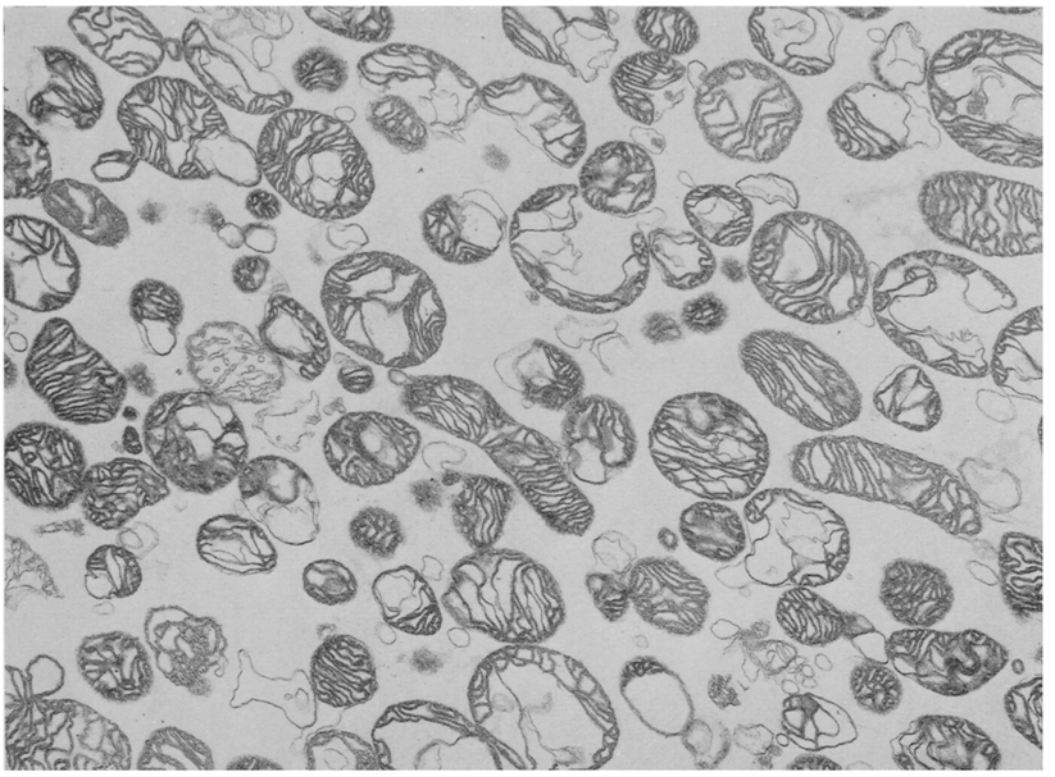
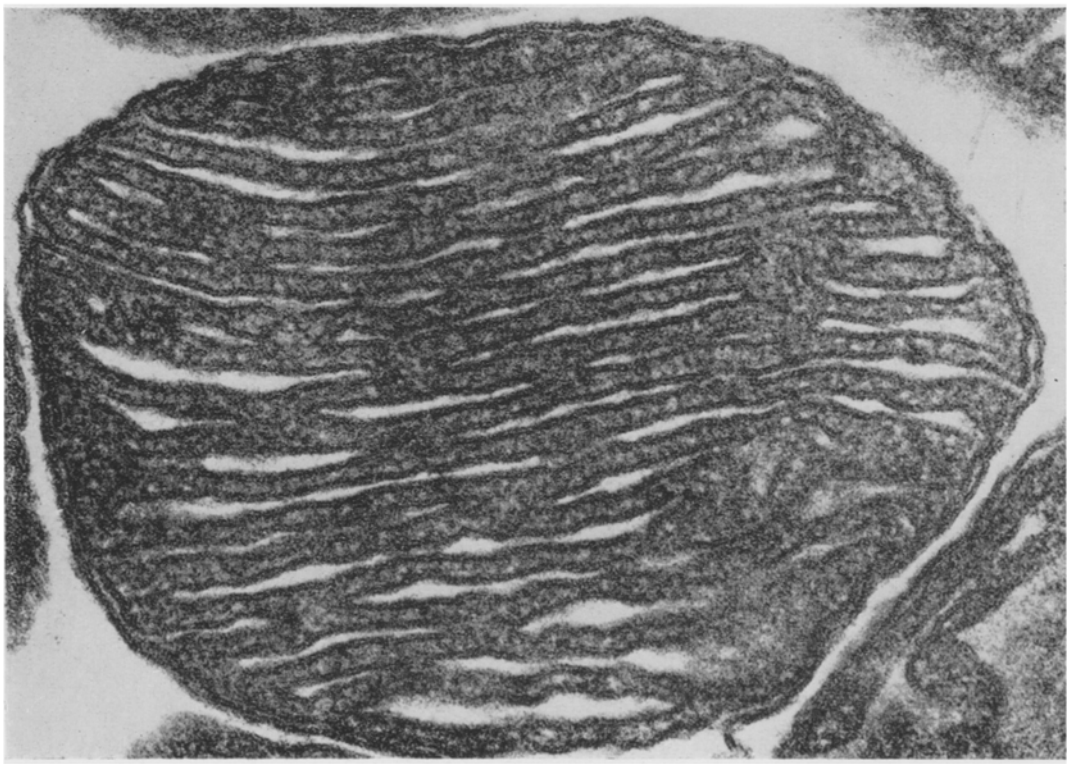


Figure 3. Swelling energized by ATP. (A) Heart mitochondria, 0·17 mg protein/ml. (B) Skeletal muscle mitochondria, 0·18 mg protein/ml. (C) Liver mitochondria, 0·22 mg protein/ml. (D) Heart mitochondria with conditions as in (A) except for addition of 6 μg oligomycin/ml. [(A–C) × 35,000; (D) × 17,500]. Medium as for ATP-supported absorbance changes in the absence of Mg^{2+} (cf. Fig. 1).





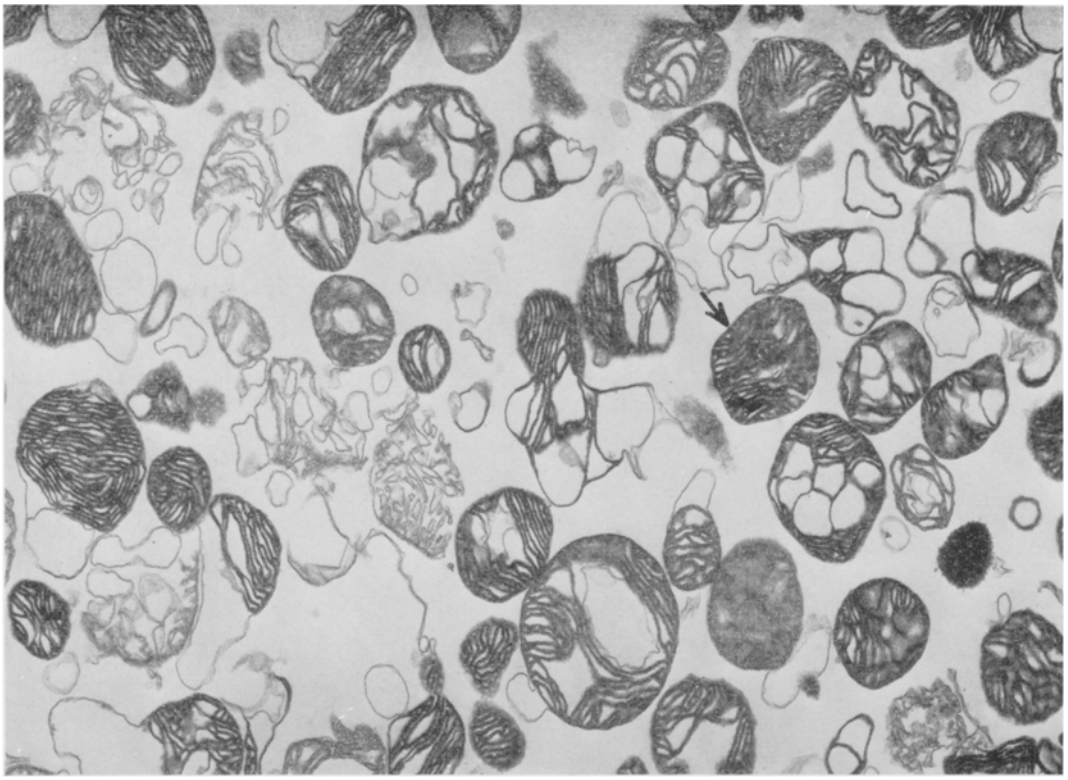
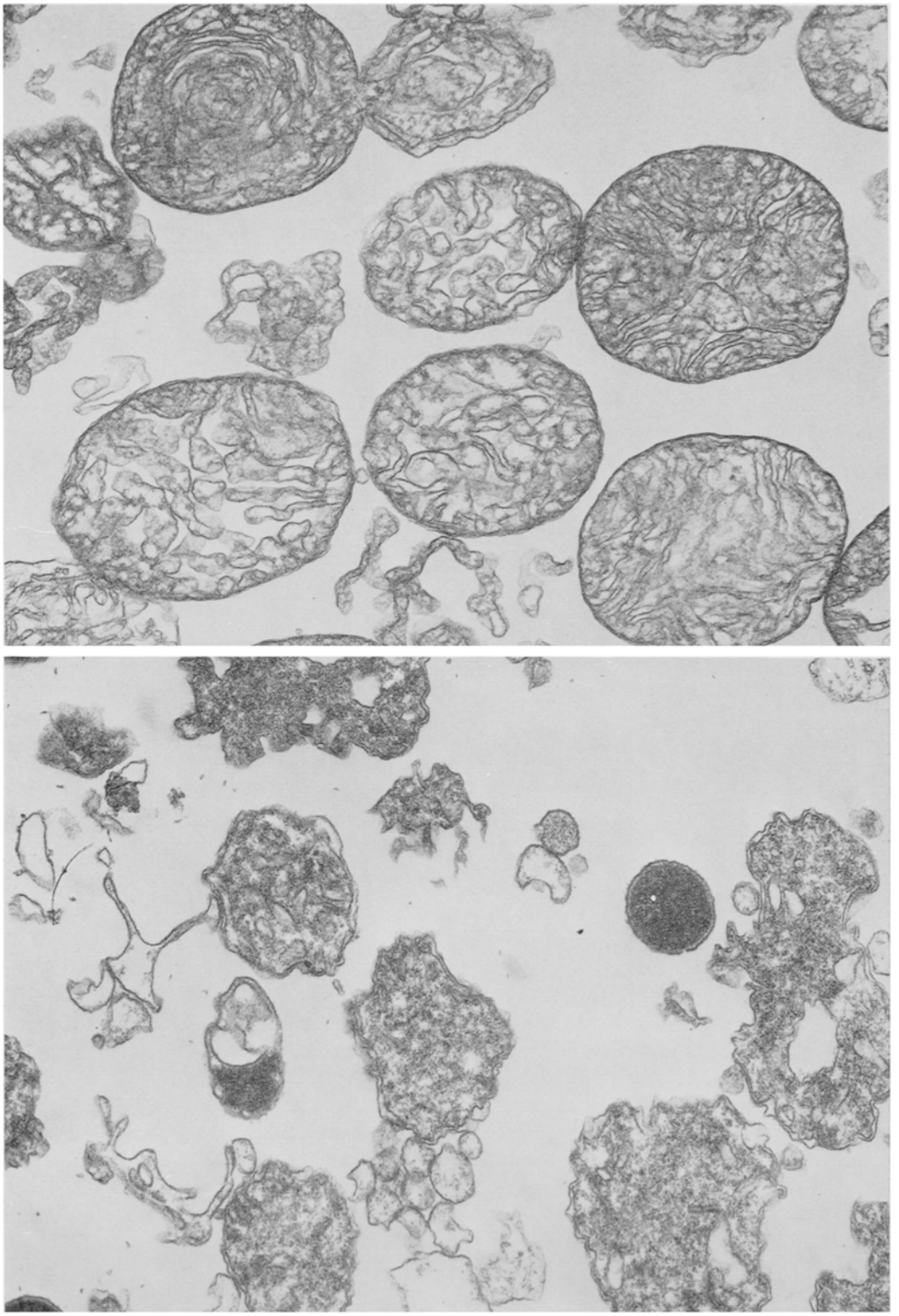


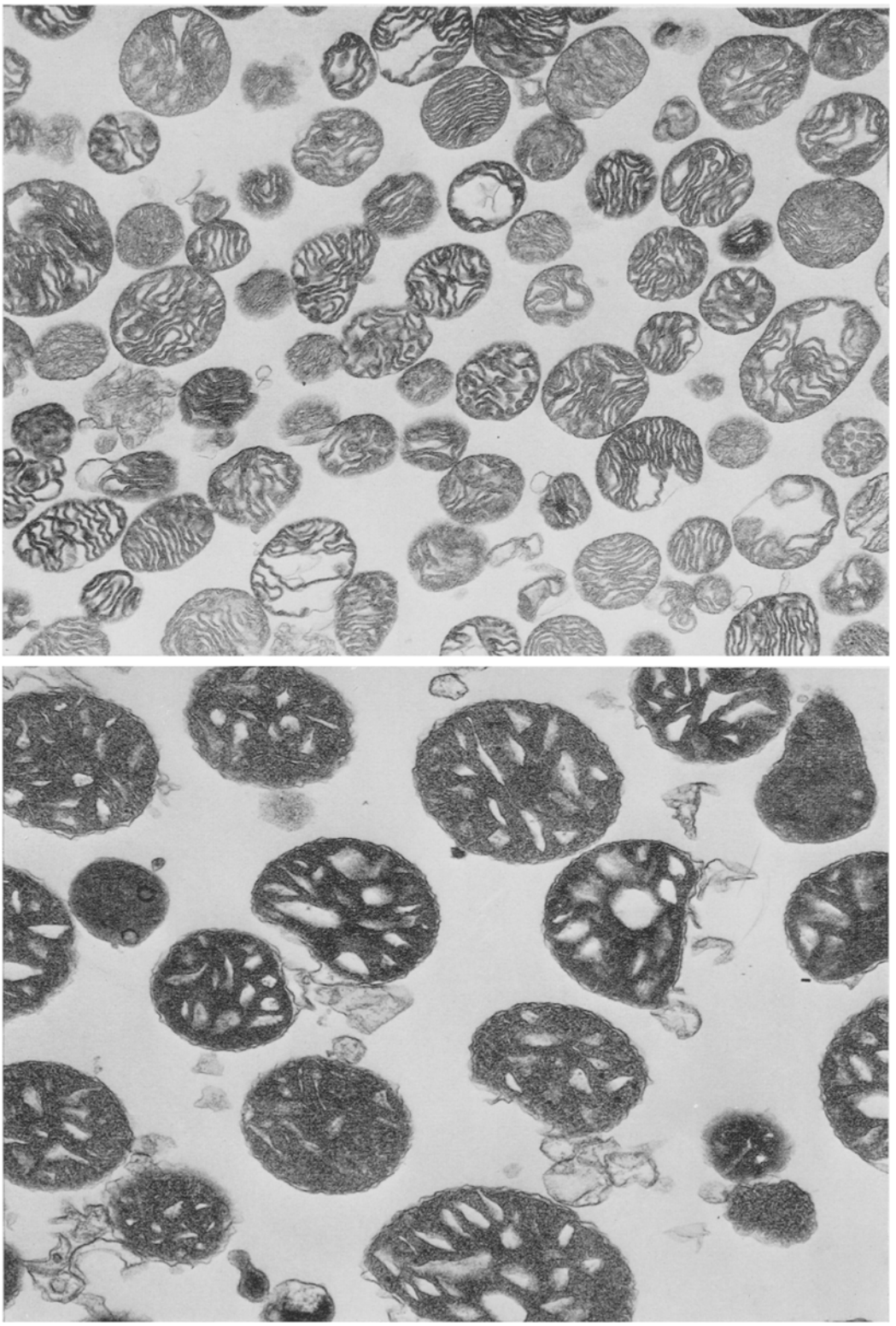
Figure 4. Prevention and reversal of ATP-energized swelling by Mg^{2+}. (A) Skeletal muscle mitochondria, 0·17 mg protein/ml (×17,500). (B) Heart mitochondrion, 0·18 mg protein/ml (×175,000). (C) Heart mitochondria, 0·18 mg protein/ml, Mg^{2+} added after 5 min under energized-swelling conditions and sample fixed 5 min later. Arrow points to "transitional" appearance of cristae (×17,500). Medium as for ATP-supported absorbance changes + Mg^{2+} (cf. Fig. 1).

of KCN prevented succinate-supported swelling (Fig. 5F). These swelling changes corresponded closely to what was predicted on the basis of the absorption data (Fig. 1). Mitochondria from all three tissues showed similar behaviour.

Effects of Mg^{2+} on substrate-supported swelling

Presence of Mg^{2+} during incubation with DPN-linked substrates + oligomycin or succinate + oligomycin prevented the extensive swelling seen in the absence of Mg^{2+} (Figs. 6A–D). Addition of Mg^{2+} following swelling supported by DPN-linked substrates caused slow reversal of swelling in absence of oligomycin, but no reversal in presence of oligomycin. This is consistent with the observed reversal of swelling by ATP + Mg^{2+}. However, there was a striking difference in appearance between mitochondria + substrate + Mg^{2+} and mitochondria + ATP + Mg^{2+} (compare Fig. 6 with Fig. 4). In the presence of ATP + Mg^{2+} the cristae appeared predominately as smooth sheets, whereas with substrate + Mg^{2+} many of the cristae assumed either a zig-zagged or a tubular appearance (Figs. 6A–D). Substrate-supplemented mitochondria appeared to maintain some tubular character during swelling (Figs. 5A, E).





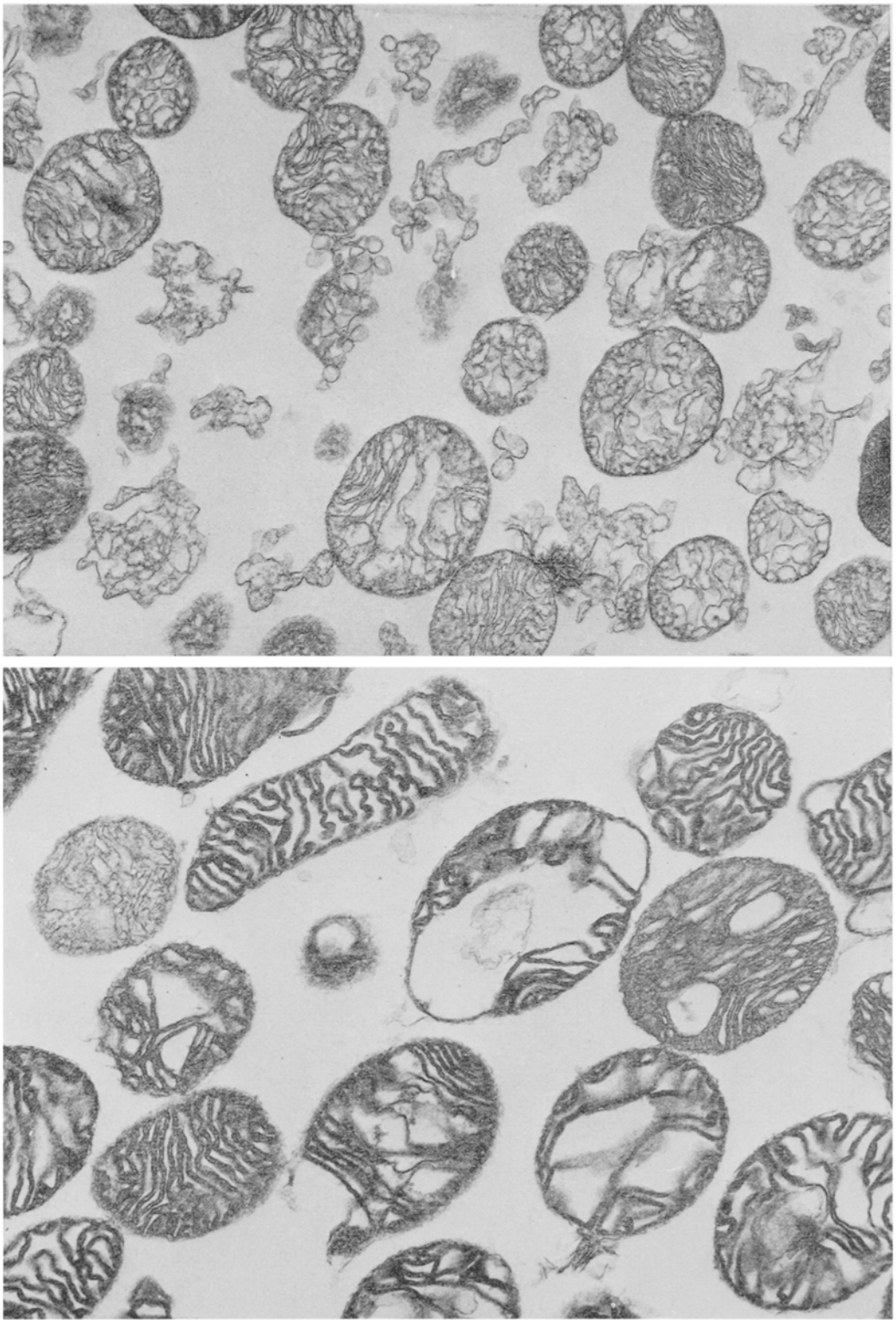
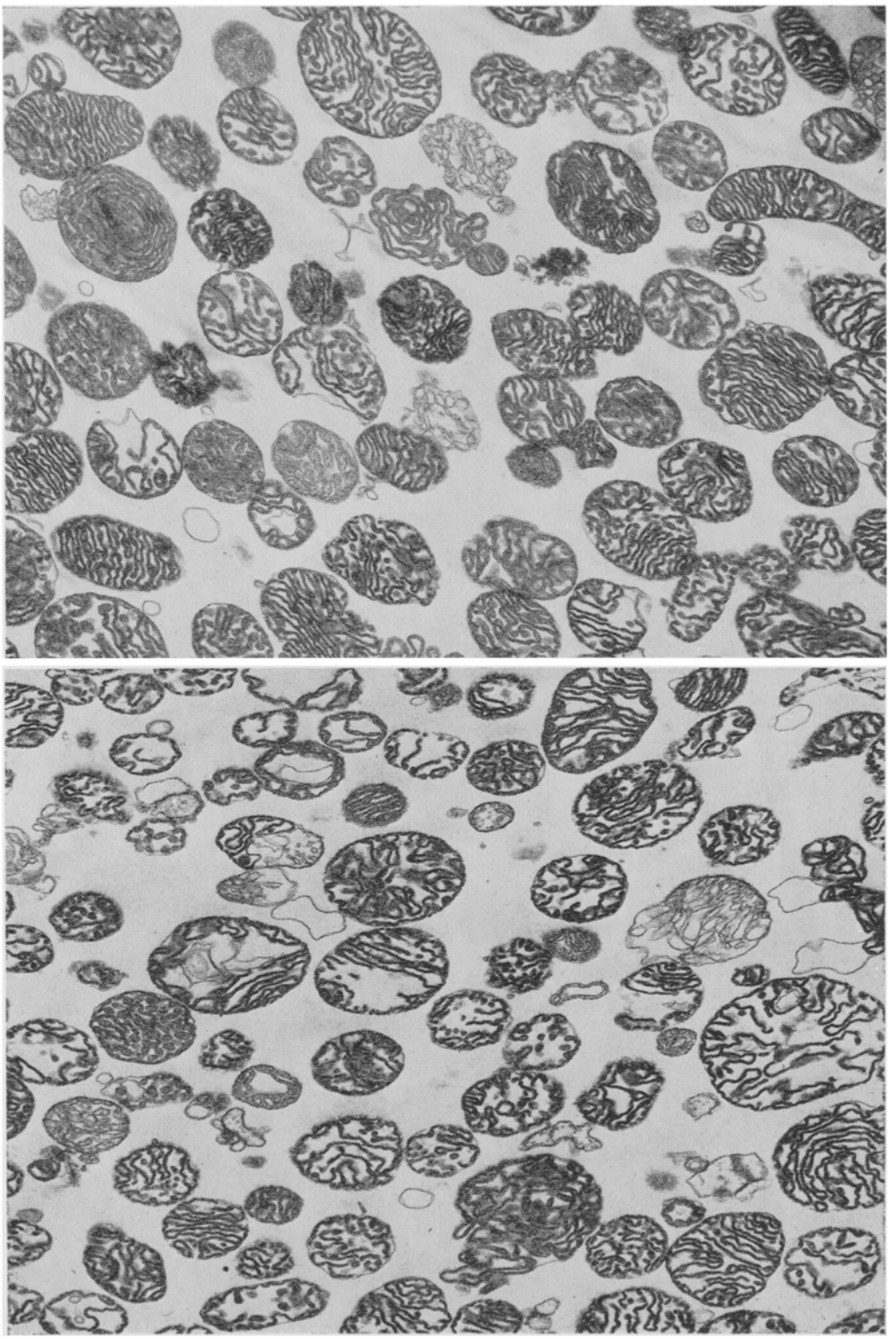


Figure 5. Substrate-energized swelling. (A) Heart mitochondria, 0·18 mg protein/ml, +glutamate, malate, malonate (G.M.M.), +oligomycin (×35,000). (B) Liver mitochondria, 0·25 mg protein/ml, +G.M.M. + oligomycin (×35,000). (C) and (D): Same as (A) and (B) respectively, except that 5×10^{-7} M rotenone was included. (E) Heart mitochondria, 0·18 mg protein/ml, +succinate, +rotenone, +oligomycin, (×17,500). (F) Same as (E) except that 0·8 mM KCN was included, (×35,000). Medium as for DPN-linked substrates or succinate (cf. Legend Fig. 1) with no Mg^{2+} added.



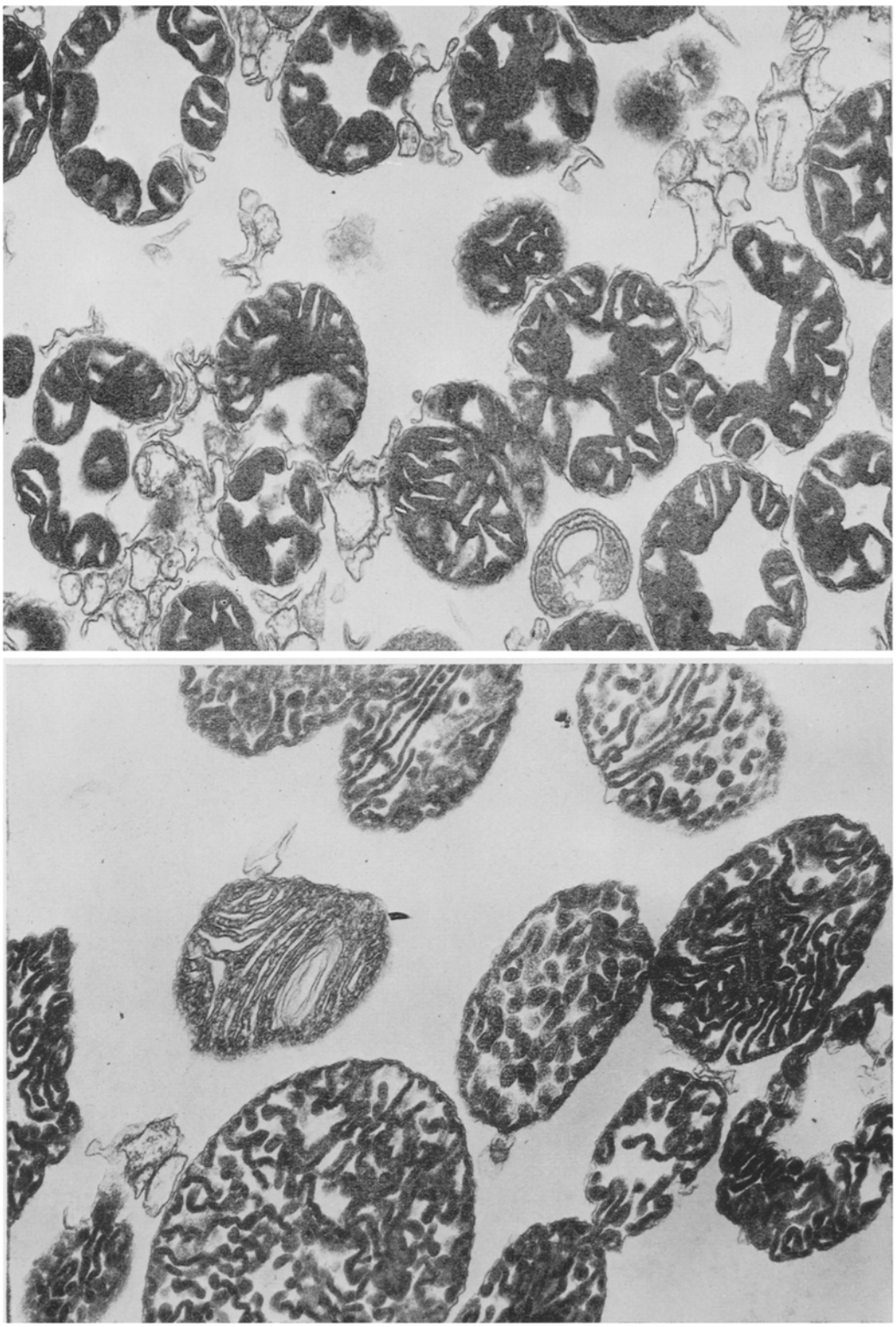


Figure 6. Effects of Mg^{2+} on substrate-supported swelling. (A) Heart mitochondria, 0·18 mg protein/ml, +glutamate, malate, malonate, +oligomycin, (×17,500). (B) Skeletal muscle mitochondria, 0·15 mg protein/ml, +G.M.M., +oligomycin, (×17,500). (C) Liver mitochondria, 0·25 mg protein/ml, +G.M.M., +oligomycin, (×35,000). (D) Heart mitochondria, 0·17 mg protein/ml, +succinate, +rotenone, +oligomycin (×35,000). Medium as for DPN-linked substrates or succinate (cf. Legend Fig. 1) with the Mg^{2+} added.

Effects of Omitting Na^+ or Acetate from the Incubation Medium

If Na^+ was replaced by $Tris^+$ or $acetate^-$ by Cl^-, major swelling changes did not occur in presence of substrate, with or without Mg^{2+} (Figs. 7 and 1). The majority of the cristae assumed the wrinkled or tubular appearance whether or not Mg^{2+} was added. Occasionally concentric or spiral structures were observed under these as well as other conditions (Fig. 7). These observations were consistent for the three types of

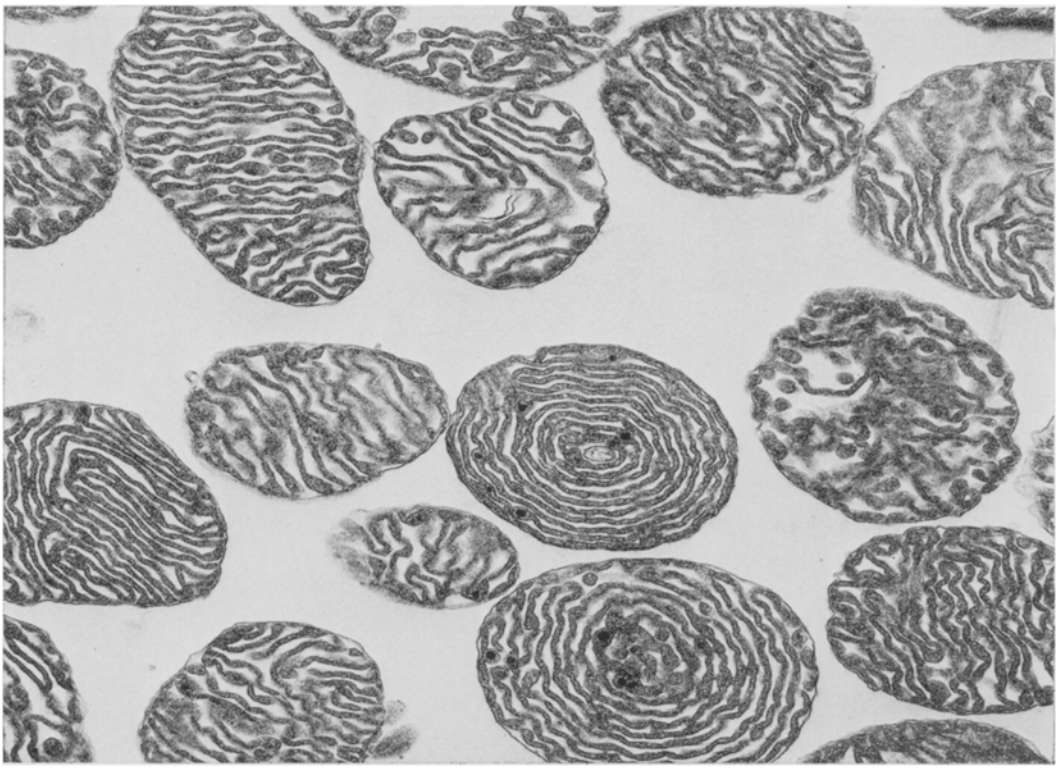


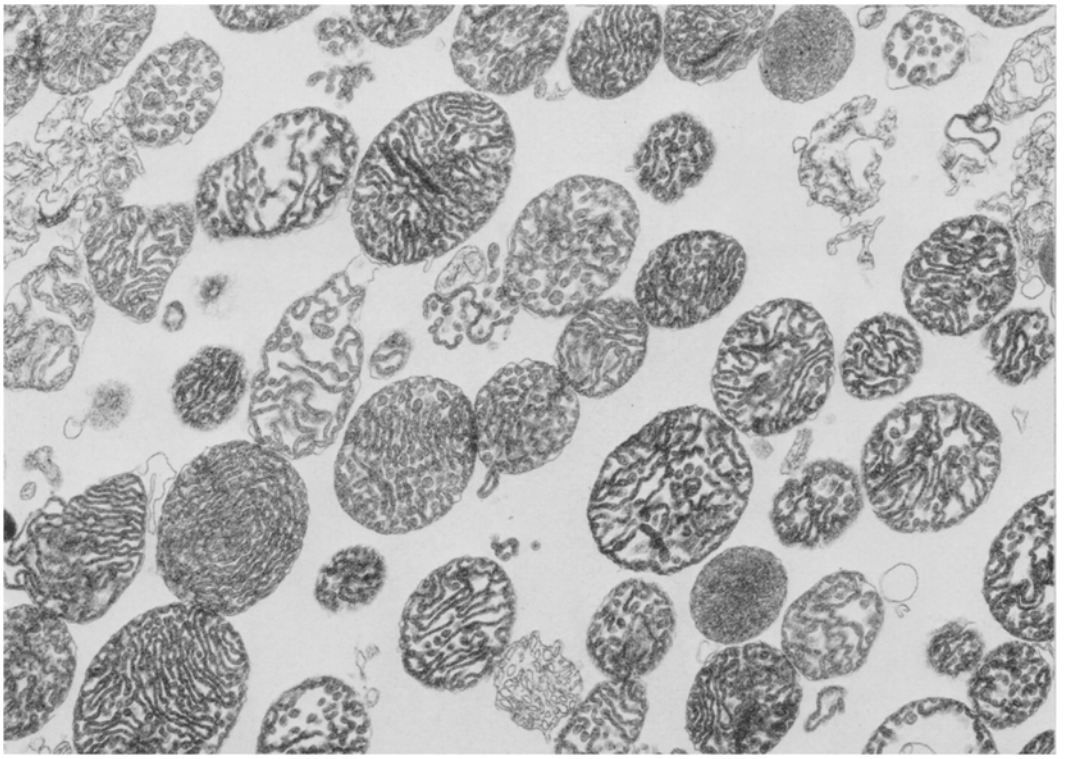
Figure 7. Effects of omitting acetate from the medium. Heart mitochondria, 0·17 mg protein/ml, +G.M.M., +oligomycin, (×35,000). Medium as for DPN-linked substrates (cf. Legend Fig. 1) except that NaCl replaced sodium acetate and without Mg^{2+} or rotenone.

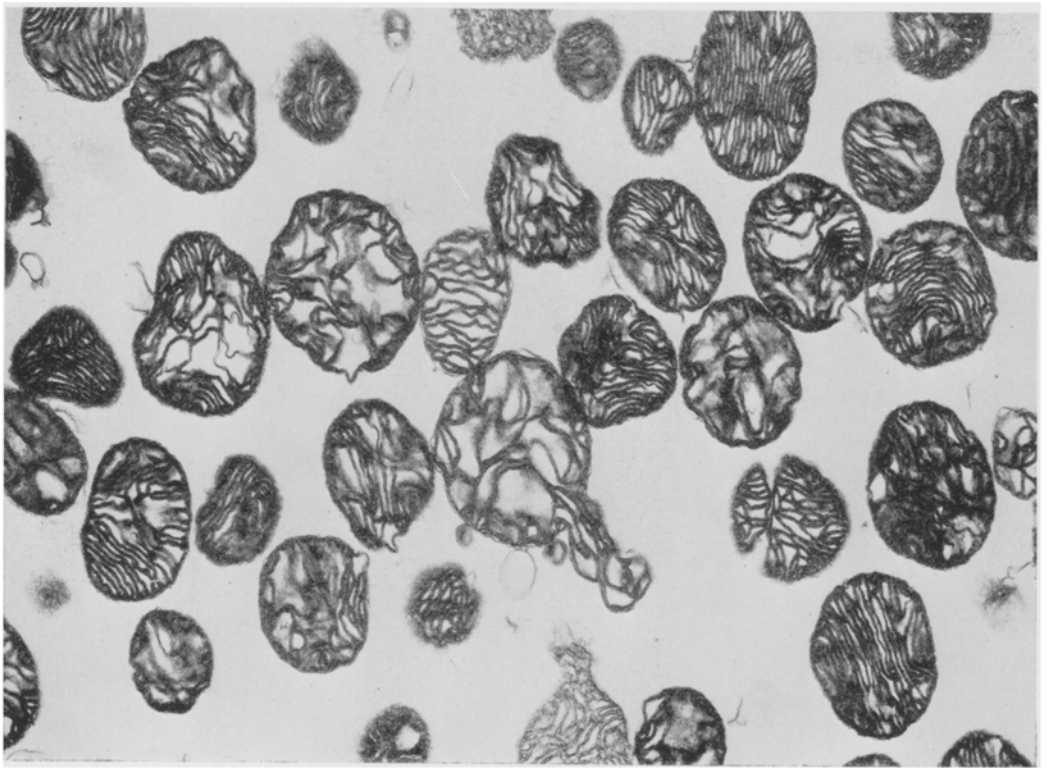
mitochondria tested. It was apparent, therefore, that presence of added Mg^{2+} was not responsible for the transformation to the tubular structure

Effects of ADP and ATP on Mitochondria Preincubated in Presence of Substrates and P_i

These experiments were carried out in mannitol-sucrose medium without acetate, with Na^+ present only as sodium phosphate and the sodium salts of glutamate, malate and malonate. Mitochondria were added to the incubation medium + Mg^{2+} at room temperature.

In the experiment represented by Fig. 8A, glutaraldehyde was added after 1·5 min at room temperature. Note that the majority of cristae exhibited either the tubular or the wrinkled appearance. Fig. 8B represents an experiment in which ADP was added after 1·5 min incubation and glutaraldehyde was added 15 sec later. Figure 8C





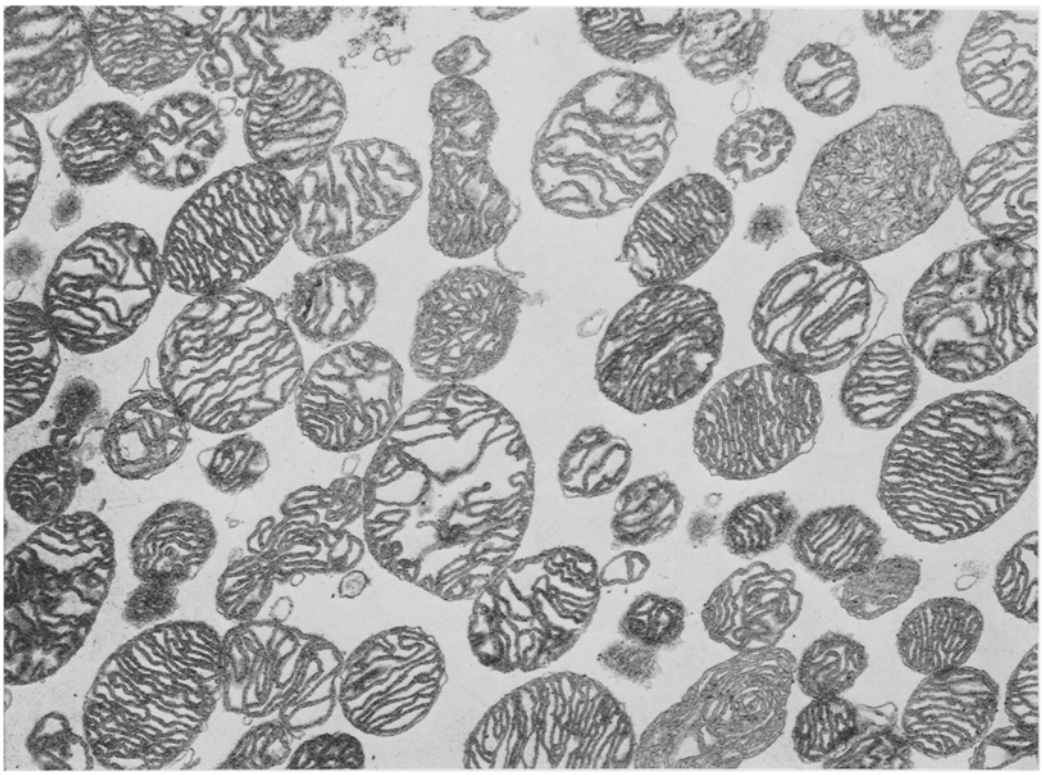


Figure 8. Effects of ADP and ATP on mitochondria preincubated in presence of substrates and P_i. Incubation was at room temperature in the following medium: 0·14 M mannitol, 0·05 M sucrose, 10 mM each of sodium glutamate, sodium malate, sodium malonate, 5 μM cytochrome *c*, 5 mM $MgCl_2$, 0·5 mM EDTA, 10 mM $Na \cdot PO_4$ buffer, pH 7·4, 2 mM Tris·HCl buffer, pH 7·4, and 0·2% bovine serum albumin; rat heart mitochondria, 0·33 mg protein/ml. Heparin was omitted from the preparation procedure (×17,500). (A) 1·5 min after addition of mitochondria to incubation medium at room temperature, glutaraldehyde was added to initiate fixation; (B). ADP to give 0·5 mM was added 1·5 min after addition of mitochondria to incubation medium and glutaraldehyde was added after 15 sec further incubation; (C) as in (B) except ATP was substituted for ADP.

represents an identical experiment except that ATP was substituted for ADP. In both cases the cristae reverted to the sheetlike structure observed in the presence of ATP + Mg^{2+}, the conditions shown above to reverse swelling (cf. Fig. 4). The same results were obtained when nucleotides were present for only 5 sec before fixation. However, when only one-tenth the adenine nucleotide concentration was used, little if any structural transition was observed when mitochondria were fixed either 5 or 10 sec after addition of ATP or ADP. The mitochondria used in these experiments showed good control of respiration by phosphorylation (respiratory control index of 8·5 or greater in the same medium used here).

Discussion

We found that rat heart and liver mitochondria isolated in the presence of 10 mM EDTA showed the same energized swelling in presence of alkali metal cations and permeant anions as previously observed[24] for skeletal muscle mitochondria isolated in

presence of EDTA and heparin. As in EDTA-treated skeletal muscle mitochondria, EDTA-treated heart and liver mitochondria showed poor control of respiration by phosphorylation. In all three types of mitochondria addition of Mg^{2+} restored tight coupling and prevented or reversed swelling. The same isolation procedure, but without the use of heparin, gave mitochondria with the same properties described above for mitochondria isolated in the presence of heparin. Heparin thus appeared to be responsible for an increase in mitochondrial stability to storage at 0–4°, but was not responsible for the swelling properties. It appears that these are properties of mitochondria generally. Various swollen or contracted states, as indicated by absorbance and Coulter-Counter measurements,[24] were correlated with morphological changes as visualized by electron microscopy. Electron micrographs showed that the matrix space expands during swelling and that cristal membranes can assume several different three-dimensional arrangements.

The rapid swelling of EDTA-treated mitochondria requires permeant anions and an energy source as well as alkali metal cations.[24] Mitochondrial swelling in presence of ion-carrying antibiotics, especially gramicidin, has similar requirements.[34] In EDTA-treated mitochondria, as with mitochondria in the presence of gramicidin, oxidation is significantly uncoupled from phosphorylation.[34,35] Electron micrographs showed that swollen EDTA-treated mitochondria have an expanded matrix space (Figs. 3 and 5) closely resembling pictures of mitochondria swollen in presence of gramicidin.[3] Electron micrographs further showed that presence of Mg^{2+} prevented the major swelling changes, as predicted from the light-scattering data. Mitochondria that had undergone ATP-energized swelling in the absence of Mg^{2+} could be induced to contract by subsequent addition of Mg^{2+}. Mitochondria first swollen then recontracted had a "sheetlike" structure very similar to that observed when swelling was prevented by having Mg^{2+} + ATP present from the beginning. Some mitochondria were badly disrupted during swelling. Many, if not all, disrupted mitochondria failed to show reversal of swelling.

Gramicidin and other ion-carrying antibiotics, in effect, increase membrane permeability to various cations.[34,35] The high degree of uncoupling in presence of gramicidin may be due to increased proton permeability.[19,35–37] This would be predicted by the Mitchell hypothesis. The striking similarity between the swelling properties of gramicidin-treated mitochondria and EDTA-treated mitochondria appears compatible with earlier suggestions that Mg^{2+} can regulate ion permeability in mitochondria.[20–24] It is unlikely that a carrier-like function of EDTA in membranes is responsible since added Mg^{2+}, even in the presence of an excess of EDTA, still inhibits swelling.[24]

In addition to the major morphological changes associated with swelling, we also observed a variety of structural arrangements of the cristal membranes. Some of these forms resembled the configurations reported by Hackenbrock[13–15] and by Green *et al.*[16–18] and believed by them to be closely associated with oxidative phosphorylation. These investigators considered mitochondria in State IV (presence of substrates, O_2, and phosphate) to be in a higher energy state than mitochondria in State III (presence of substrate, O_2, phosphate, and ADP). Hackenbrock associates a "condensed" form of liver mitochondria with the lower energy level (State III) and an "orthodox" form with the higher energy level (State IV). The liver mitochondria in our studies were in the "condensed" form as isolated and remained in this form in presence of ATP or substrates + O_2 when Mg^{2+} was included to prevent swelling. The "orthodox" form

was observed only under conditions which promoted swelling. These results would appear to be in contradiction to those of Hackenbrock and suggest that the formation of the "orthodox" state depends on ion transport.

Structures like those reported by Green *et al.*,[18] were seen in electron micrographs of heart and skeletal muscle mitochondria. The form which we have called "tubular" resembles the "energized-twisted" form of Green *et al.* Though we found some of these structures in mitochondria as isolated, a greater proportion of mitochondria existed in this form in the presence of added substrates, Mg^{2+}, and O_2, conditions similar to those of Green *et al.* except that they also needed phosphate.[18] A "sheetlike" form was observed in presence of ATP + Mg^{2+} regardless of whether Mg^{2+} was added before or after swelling (Fig. 4). The "sheetlike" form may represent the "energized" configuration reported by Green *et al.* In the specific version of the mechanochemical hypothesis set forth by Green,[18] the tubular configuration ("energized-twisted") has higher energy than the sheetlike configuration ("energized"), and addition of ADP causes the tubular-to-sheetlike transition by accepting energy from the tubular form. We found that addition of ADP to mitochondria in State IV did result in a tubular-to-sheetlike transition observed 5 sec after addition of ADP. However, addition of ATP had the same effect. Since heart and skeletal muscle mitochondria always showed the sheetlike form in presence of ATP + Mg^{2+}, and since addition of ADP resulted in the structural transition only under conditions where ATP was quickly formed, the possibility arises that ATP might be responsible for the tubular-to-sheetlike transition. Further testing of this possibility would require means of controlling ATP and ADP interconversion.

The fact that 0·5 M sucrose (approximately twice isotonicity for mitochondria) prevents the formation of the tubular form in State IV[33] suggests that an osmotic gradient is involved in the formation of the tubular configuration. Asai *et al.*, studying mitochondrial swelling, have shown that contracted mitochondria with a "sheetlike" cristal arrangement transform to the "tubular" structure before major expansion of the matrix space.[4] Though our experiments were not designed to trap intermediate stages of swelling, the results appeared compatible with the above scheme (eg. see "transitional" forms in Fig. 4C and swollen "tubular" forms in 5A and E). We would, thus, not like to exclude the possibility that ion transport is involved in the formation of the "tubular" structures, and would further suggest that the tubular-to-sheetlike transition could reflect the reversal of swelling caused by ATP + Mg^{2+}. It should be noted that much lower concentrations of Mg^{2+} than were used here can still support swelling reversal[24] and that mitochondria not treated with EDTA probably have enough endogenous or bound Mg^{2+} to support reversal of swelling.

The mechanism by which the energy released during electron transport is coupled to the formation of ATP remains obscure. The hypothesis of high-energy, chemical intermediates has not been validated despite intensive efforts on the part of many investigators.

An alternate idea, the mechanochemical hypothesis, envisions an "energized state" in the form of conformational changes which can be discharged with the formation of ATP.[38] The version of this hypothesis presented by Green *et al.* relies on structural changes of a magnitude visible through electron microscopy.[18] Light-dependent, reversible changes in thickness and spacing of chloroplast membranes, possibly associated with photophosphorylation, have been reported by Murakami and Packer.[39] However,

the concept of mechanochemical coupling does not demand that the conformational changes be large enough to be detected by electron microscopy. Recently, Graham and Wallach have interpreted infra-red spectra of membranous vesicles to mean that there is an increase in β-conformation of the proteins upon uncoupling of phosphorylation.[40] Oxidation of respiratory chain components occurs within milliseconds after the addition of O_2 to an anaerobic mitochondrial suspension in presence of substrates. The primary coupling event probably also occurs in milliseconds. Thus, studies such as those above could be related more definitively to the primary step in energy coupling if the time correlation could be reduced from seconds to milliseconds. However, as it stands, conformational changes may be secondary to ion transport or other changes. In fact, structural changes and ion transport are not mutually exclusive and might occur simultaneously.

The chemiosmotic hypothesis, like the mechanochemical hypothesis, stresses the need for a highly organized, membranous structure.[19] According to the chemiosmotic hypothesis an electrochemical potential gradient across the membrane barrier drives oxidative phosphorylation. If EDTA-treatment causes selective changes in membrane permeability, such an electrochemical gradient might drive ion transport producing the large-magnitude swelling that we observed. The uncoupling observed in these mitochondria might represent a decrease of the potential gradient through increased proton permeability. Tupper and Tedechi raised serious doubts about the chemiosmotic hypothesis. They made direct measurements of the potential across the mitochondrial inner membrane.[41] In contrast to the strong negative potential predicted by the Mitchell hypothesis, a slight positive potential was observed. If these studies are not invalidated on technical grounds, then the Mitchell hypothesis would be ruled out, although other pump mechanisms such as the electrogenic cation pump postulated by Pressman would gain favor.[42]

In nerve conduction the action potential is propagated as a consequence of changes in permeability to Na^+ and K^+ which can be modulated by Ca^{2+}. In a similar way Mg^{2+} has been shown to have a profound effect on swelling as well as energy coupling in mitochondria. The precise relationship of permeability changes to energy coupling is as yet unclear, but it would seem that this relationship is basic to energy conservation.[43–46] The importance of a rapid and reversible modulation of permeability in membrane phenomena generally is worth further consideration.

Acknowledgements

The electron microscopy was carried out most ably by Mr. Akitsugu Saito. We should also like to thank Miss Ute Lhotka for valuable technical assistance.

This research was supported in part by USPH Grant GM 12831 and a Grant-in-Aid of the American Heart Association. This work was carried out while Dr. S. Fleischer was Established Investigator of the American Heart Association. This has been contribution No. 367 from the Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Canada.

A portion of this work was in partial fulfilment of the Ph.D. dissertation requirements for K. G. Walton, Vanderbilt Univ., 1970.

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