ULTRASTRUCTURAL BASES FOR METABOLICALLY LINKED MECHANICAL ACTIVITY IN MITOCHONDRIA

I. Reversible Ultrastructural Changes with Change in Metabolic Steady State in Isolated Liver Mitochondria

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ABSTRACT

By means of a new "quick-sampling" method, micropellets of mouse liver mitochondria were rapidly prepared for electron microscopy during the recording of steady state metabolism. Reversible ultrastructural changes were found to accompany change in metabolic steady states. The most dramatic reversible ultrastructural change occurs when ADP is added to systems in which only phosphate acceptor is deficient, i.e., during the State IV to State III transition as defined by Chance and Williams. After 15 min in State IV, mitochondria display an "orthodox" ultrastructural appearance as is usually observed after fixation within intact tissue. On transition to State III, a dramatic change in the manner of folding of the inner membrane takes place. In addition, the electron opacity of the matrix increases as the volume of the matrix decreases, but total mitochondrial volume does not appear to change during this transition. This conformation is called "condensed." Isolated mitochondria were found to oscillate between the orthodox and condensed conformations during reversible transitions between State III and State IV. Various significant ultrastructural changes in mitochondria also occur during transitions in other functional states, e.g., when substrate or substrate and acceptor is made limiting. Internal structural flexibility is discussed with respect to structural and functional integrity of isolated mitochondria. Reversible changes in the manner of folding of the inner membrane and in the manner of packing of small granules in the matrix as respiration is activated by ADP represent an ultrastructural basis for metabolically linked mechanical activity in tightly coupled mitochondria.

INTRODUCTION

Low-amplitude, nonspecific, optical density, and light-scattering changes are known to accompany change in the state of electron transport and oxidative phosphorylation in isolated mitochondria. Such low-amplitude changes have been interpreted structurally as small changes in total mitochondrial size, resulting from energy-linked swelling-contraction phenomena (1–8). It has been

suggested, however, that low-amplitude light absorbance changes under these conditions may actually reflect energy-linked geometrical rearrangements of optically refractive internal structural components of mitochondria without change in total mitochondrial size (9). Any structural or volumetric change in mitochondria during change in functional state can be considered important in the compartmentation of essential metabolites between intramitochondrial and extramitochondrial systems and, therefore, important in the control of cellular metabolism.

Current basic ideas suggest that structural and volumetric flexibilities in metabolically active mitochondria are functions of an energy-linked mechanochemical process (9–12), the basis of which may reside in a multienzyme respiratory assembly which carries out electron transport and oxidative phosphorylation. Because the respiratory assembly is generally believed to be located in or on the inner membrane component of mitochondria, it seemed appropriate to investigate the internal structure of coupled mitochondria in various states of respiratory activity with the electron microscope.

This paper describes characteristically distinct internal conformations for each of the metabolic steady states defined by Chance and Williams (13, 14). These functional states of mitochondria are characterized not only by differences in respiratory rate but also by differences in the relative oxidation-reduction level of carriers in the electron transport chain. Evidence is presented which demonstrates that reversible internal structural transformations take place during change in metabolic steady states. The results represent an ultrastructural basis for metabolically linked mechanical activity in tightly coupled mitochondria.

MATERIALS AND METHODS

Mitochondria were isolated from the livers of C57 black male mice in 0.25 M sucrose at 0°C according to Kielley and Kielley's modification (15) of Schneider's method (16). The final mitochondrial stock solution in 0.25 M sucrose was adjusted so that 100 μl (1.7 mg protein) diluted into 2.0 ml of 0.25 M sucrose at 30°C gave an optical density (OD_{520}) reading of 0.80. This method of dilution was found to give a consistent mitochondrial concentration from isolation to isolation. Mitochondrial protein was determined by a biuret method (17).

The Clark oxygen electrode (18) determined oxygen consumption, essentially according to the method of Kielley and Bronk (19). ADP:O[1] and ACR were calculated from oxygen electrode tracings (20). A G-11A Varian recorder with 10 mv potentiometric input for full scale deflection recorded the electrode signal.

The fluorescence of reduced intramitochondrial pyridine nucleotides during change in respiratory state was recorded by a Turner fluorometer, according to the procedure of Estabrook (21). A Corning 5840 (7–60) filter restricted the transmission of the exciting light to the mercury vapor line of 366 mμ. Fluorescence emission was selected with a Wratten 8 (K-2) barrier filter. The fluorescence signal was recorded on the linear channel of a Photovolt linear/log varicord Model 43 Recorder with 1 v input for full scale deflection. The fluorometer cuvette holder was modified so that a Clark oxygen electrode could be lowered into the cuvette. The reaction system was maintained at a constant temperature and at a constant stirring speed (Fig. 1). Fluorescence and oxygen consumption were recorded synchronously (Fig. 2).

Optical density changes were recorded from a Beckman/Spinco 151 Spectro-colorimeter at 520 mμ, where absorption bands of specific electron carriers do not interfere. Changes in signal were recorded on the logarithmic channel of a Photovolt linear/log recorder with 10 mv input for full scale deflection. The Beckman cuvette holder was modified to accept a round cuvette into which a Clark oxygen electrode could be lowered by a rack and pinion. The reaction system was maintained at a constant temperature and at a constant stirring speed (Fig. 1). Optical density and oxygen consumption were recorded synchronously. All experiments were carried out at 30°C. Succinate was used in most of the experiments. Glutamate was used in the fluorescence studies. Sucrose was Baker Analyzed reagent grade. Substrates were obtained from Calbiochem (Los Angeles, California) and adenine nucleotides from Sigma Chemical Co. (St. Louis, Missouri). Water was glass-distilled.

A "quick-sampling" method was developed for preparing micropellets for electron microscopy. With the aid of a system of light baffles to eliminate stray light, 100 μl samples could be taken from the reaction cuvette with a Carlsberg micropipette without the recording of optical density and oxygen consumption being interrupted. The samples were immediately transferred to polyethylene 200 μl microcentrifuge tubes and centrifuged for 30 sec at approximately 15,000 g (Fig. 1). A Beckman/Spinco 152 Microfuge, which accelerates and decelerates within 2 sec, was

[1] Abbreviations used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; ADP:O, adenosine diphosphate to oxygen ratio(s); ACR, acceptor control ratio(s).

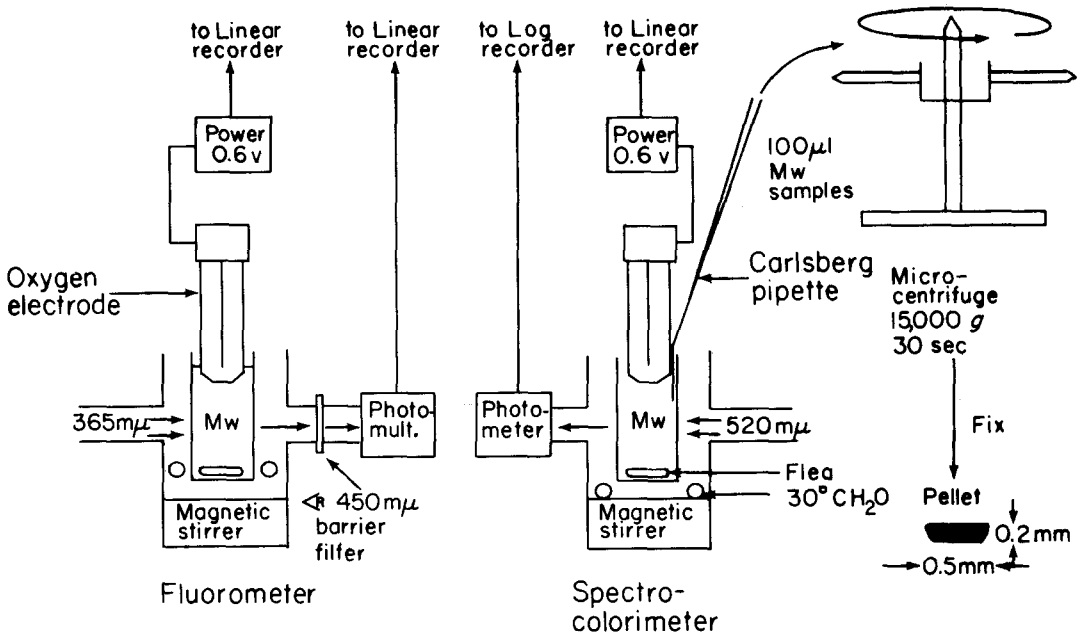


FIGURE 1 Block diagram of instrumentation for measuring oxygen consumption, optical density, and pyridine nucleotide fluorescence and for simultaneous sampling for electron microscopy. 100 μl samples were removed from the reaction cuvette and centrifuged for 30 sec to yield mitochondrial micropellets measuring approximately 0.2 x 0.5 mm. Mw, stock mitochondria.

controlled by a variable transformer to give the desired speed (ca. 140 v). After centrifugation, the supernatant was quickly replaced with fixative. The final micropellets, consisting of mitochondria obtained from 100 μl of reaction medium (0.085 mg protein), measured approximately 0.5 × 0.2 mm. The time interval between removal of the sample from the reaction cuvette and fixation was 35 sec.

The fixative was 2% osmium tetroxide in a sodium phosphate buffer. The final phosphate buffer was, as in the total phosphorylation medium, 10 mM and pH 7.0. Sucrose was added to adjust the final osmium tetroxide–phosphate-sucrose osmolarity to be isosmotic with respect to the sucrose isolation medium (0.25 osmolar) and to adjust the final phosphate-sucrose osmolarity to be isosmotic with respect to the total phosphorylation medium (0.16 osmolar). Fixation was at 0°C for 1 to 4 hr with dehydration in ice cold ethanol. Pellets were embedded in an epoxy resin (22) and preoriented in BEEM polyethylene capsules so that complete cross-sections, parallel to their top-to-bottom axes, could be obtained. Cross-sections were picked up on uncoated 300-mesh (LKB) copper grids. Sections were stained for 15 min in a 40°C 1.0% sodium borate solution saturated with uranyl acetate, followed by Karnovsky's mixture A lead hydroxide (23) 1/50 dilution for 5 min.

Electron micrographs were taken on Kodak 3¼ × 4 inch high-contrast lantern slides at initial magnifications of 3000 and 7100 with an RCA 3C electron microscope at 50 kv.

Total mitochondrial volume was determined by averaging the volumes, based on diameters, of 15% of the largest and 15% of the smallest mitochondria in each micrograph (which contains approximately 80 mitochondria). Only those mitochondria which are intact and which contain sharp limiting membranes, i.e. are approximately sectioned close to center, should be counted. A qualitative change in total mitochondrial volume is indicated by agreement in the direction of the volume change by the largest and smallest mitochondria. Quantitative changes are reflected by agreement in amplitude of the volume change by the largest and smallest mitochondria. Measurements of membrane thickness and particle sizes were made from electron micrographs of 50,000 to 110,000 magnification with a Bausch and Lomb 7 × magnifier containing a measuring disc enscribed with 0.1 mm divisions.

RESULTS

Identification of Metabolic States

Fig. 2 shows a typical recording of changes in oxygen consumption and fluorescence of intramitochondrial pyridine nucleotides which reflect changes in the metabolic states of mitochondria.

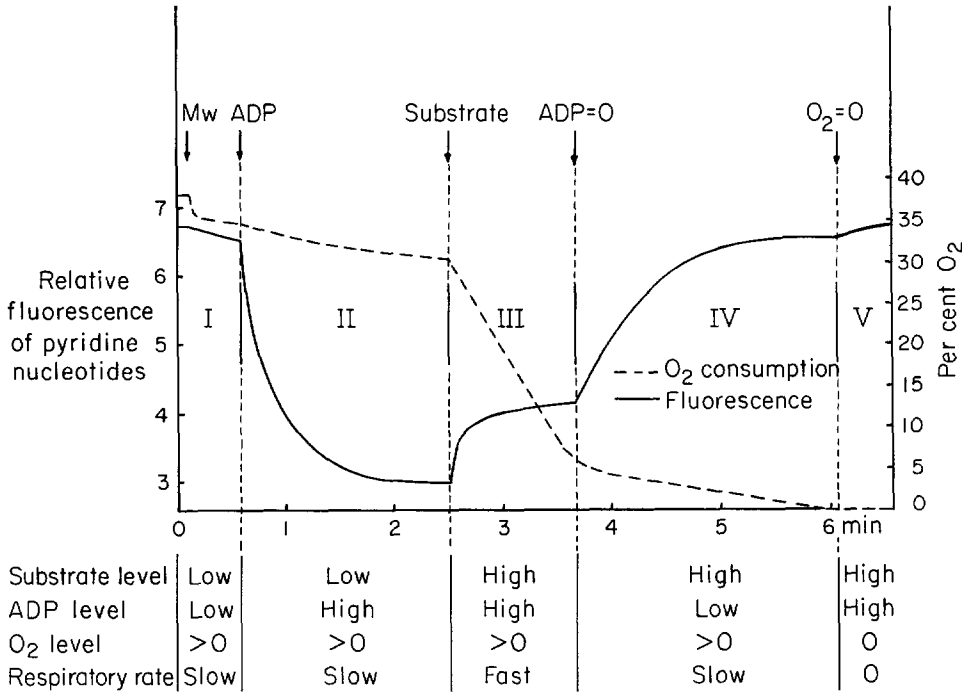


FIGURE 2 The curves show the changes in rate of oxygen consumption and in fluorescence of pyridine nucleotides which identify changes in metabolic steady states in mitochondria. Terminology and characteristics after Chance and Williams (13). Roman numerals represent five metabolic steady states. The reaction system contained sucrose (0.113 M), PO_4 buffer (0.01 M, pH 7.0), $MgCl_2$ (5.0 mM), glutamate (2.5 mM), ADP (250 μM), and Mw (3.4 mg protein). Total volume, 4.0 ml.

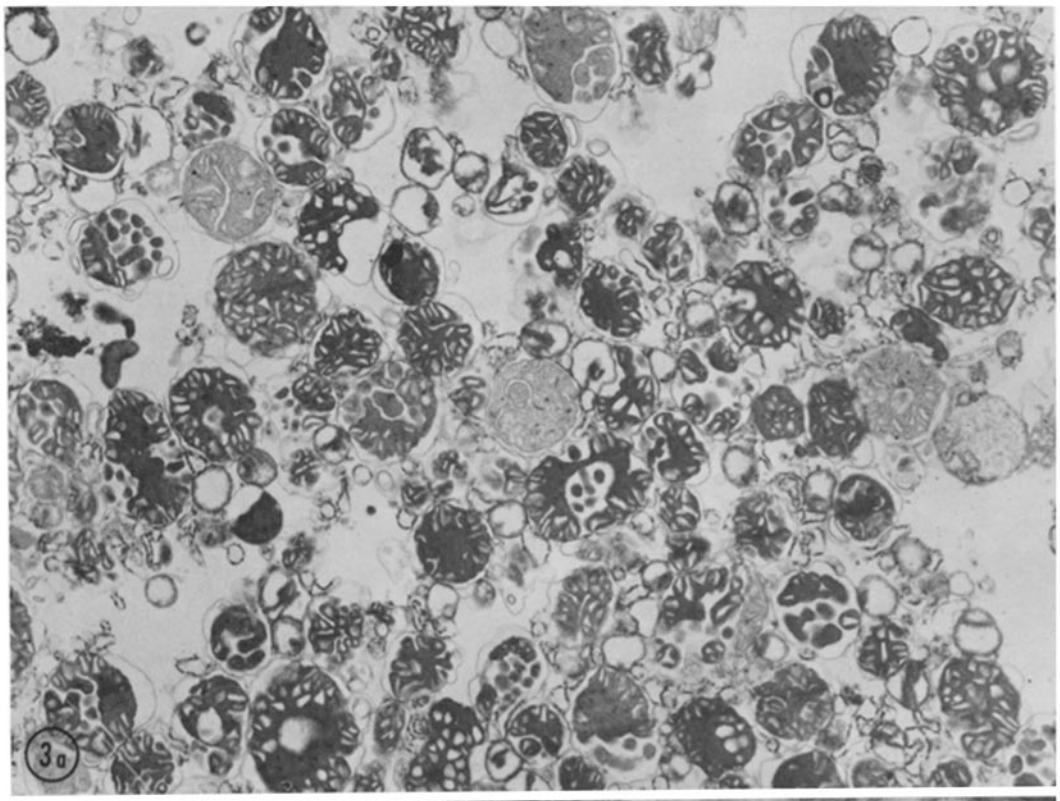
The characteristics of each state are listed, according to the terminology of Chance and Williams (13). The level of fluorescence, which is a function of the relative per cent of reduced pyridine nucleotides present (21), and the rate of oxygen consumption are apparently determined by the availability of substrate, ADP, phosphate, and oxygen.

In State I, mitochondria display a slow respiratory rate, presumably due to the metabolism of endogenous substrate and nucleotide. State II,

FIGURE 3 Mitochondria incubated in 0.25 M sucrose at 30°C for 1.5 min.

FIGURE 3 *a* These mitochondria display a condensed conformation and are essentially identical in structure to stock mitochondria in 0.25 M sucrose at 0°C. Note that, compared to orthodox mitochondrial structure, as observed in situ, the matrix of intact mitochondria is polymorphic, condensed, and smaller in volume. There is a relative volume increase in outer compartment and intracristal spaces. × 10,700.

FIGURE 3 *b* Higher magnification shows the condensed matrix to be composed of granules 60 to 100 A in diameter. The mitochondrion in the upper left has a highly electron-opaque matrix with the small matrical granules in very close packing. In the upper right a mitochondrion contains a less electron-opaque matrix and the granules are not so closely packed. The inner membrane (*IM*) is irregularly folded. Limiting membrane, *LM*; outer compartment, *OC*; intracristal space, *IS*. × 50,000.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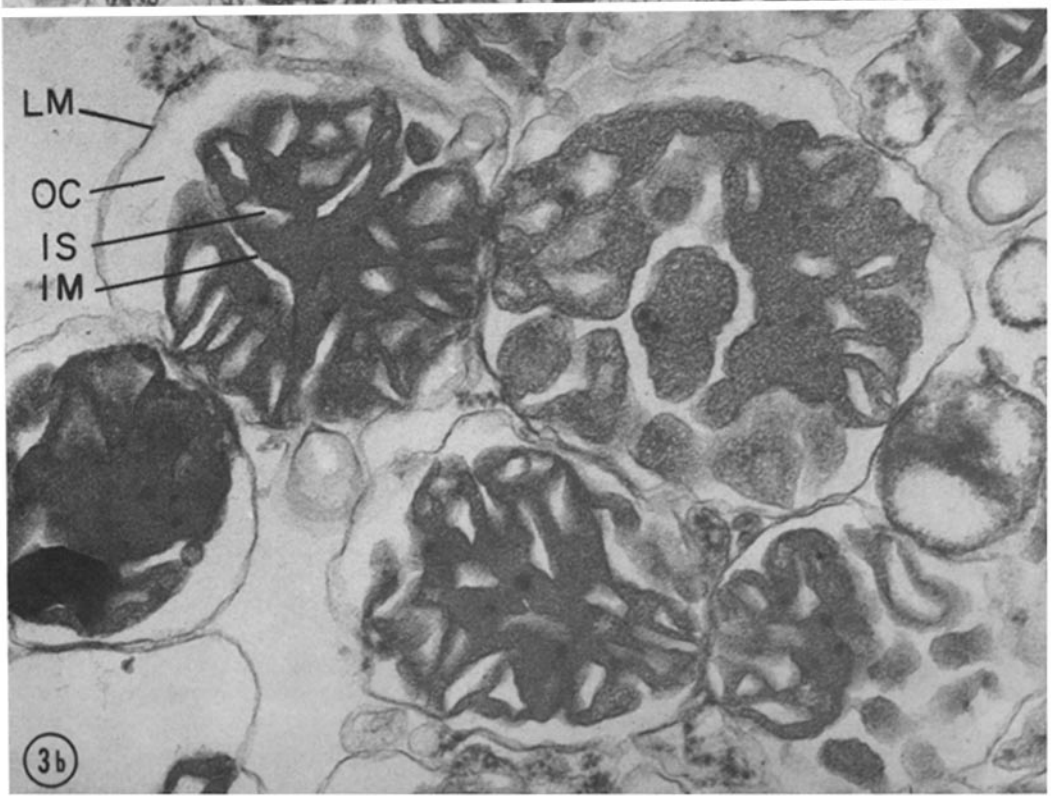
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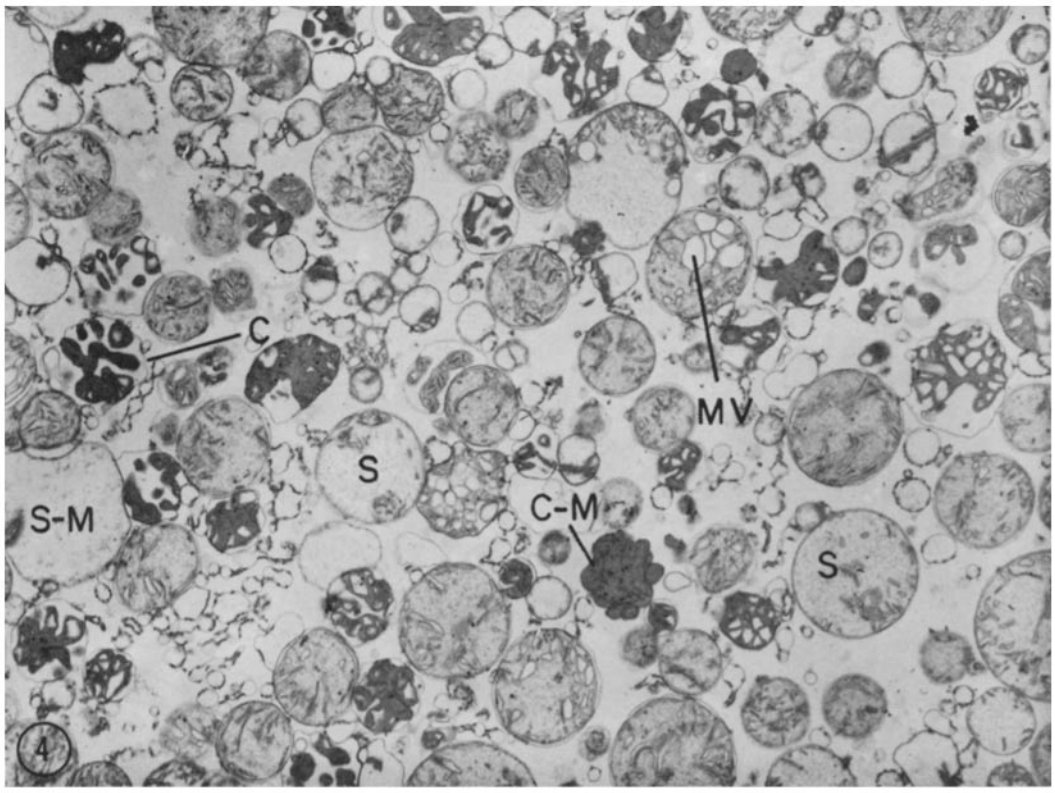

FIGURE 4 Mitochondria incubated in 0.25 M sucrose at 30°C for 15 min. These incubations lead to a mixture of morphological forms which are described in detail in the text. The major forms are: condensed, *C*, apparently unchanged from 1.5 min sucrose incubations; swollen, *S*; condensed matrix with no limiting (outer) membrane, *C–M*; swollen matrix with no limiting membrane, *S–M*; dilated intracristal spaces appearing as matrical vesiculations, *MV* (cf. Fig. 3 *a*). × 10,700.

initiated by addition of ADP to State I mitochondria, results in minimal fluorescence of pyridine nucleotides and a slight initial increase in oxygen consumption due to increased oxidation of endogenous substrate. The addition of substrate to State II mitochondria activates oxidative phosphorylation which is characteristic of State III. Increased electron flux in the respiratory chain during State III metabolism is indicated by a temporary increase in the rate of oxygen consumption and a shift in reduction of pyridine nucleotides to a higher steady state level. State IV is established when a further reduction of pyridine nucleotides and a deceleration of respiration occur synchronously and spontaneously, signifying the complete phosphorylation of available ADP. The low respiratory rate of State IV continues until all available oxygen in the reaction system is consumed, at which time the mitochondria enter the anaerobic State V. In State V the lack of oxygen prevents electron transport, the respiratory chain becomes electron-saturated, and, consequently, reduced pyridine nucleotides display maximal fluorescence. It should be noted that the addition of substrate, rather than ADP, to State I mitochondria, would result in a transition to State IV.

Controls: Ultrastructure of Mitochondria Incubated in Sucrose

Fig. 3 shows the typical results which occur when mitochondria are incubated for 1.5 min in 0.25 M sucrose at 30°C. The basic structure observed is essentially identical to the structure of stock mitochondria (Mw) freshly isolated in 0.25 M sucrose at 0°C. Except for approximately 5% of the mitochondria, which possess incomplete membranes, the mitochondria of this control incubation exhibit a condensed, polymorphic matrix, and an

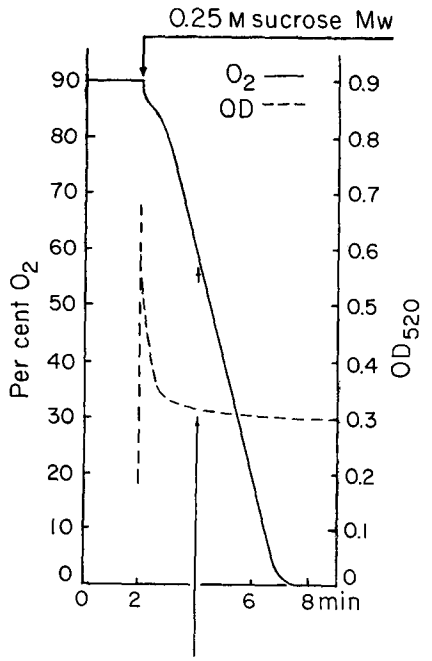

FIGURE 5 Oxygen consumption and optical density of mitochondria placed into a swelling, nucleotide-free medium. Loose coupling develops during high-amplitude decrease in absorbancy. Mitochondria isolated in 0.25 M sucrose, which are characterized by a condensed conformation (Fig. 3), were placed into the cuvette at 2 min (upper arrow); samples removed and fixed for electron microscopy 2 min later (lower arrow) are shown in Fig. 6. The reaction system consisted of sucrose (0.013 M), PO_4 buffer (0.01 M, pH 7.0), succinate (0.01 M), and Mw (1.7 mg protein). Total volume, 2 ml.

extensive outer compartment and intracristal space. The matrices within some mitochondria appear as discontinuous fragments of membrane-bounded matrix. However, these "fragments" may actually represent cross-sections of branches which are continuous with the main matrical mass in another plane. Many of the large intracristal spaces are continuous with a large outer compartment. The inner membrane component adheres tightly to the matrix and there is very little suggestion of its folding into cristae. The limiting membrane appears relaxed and independent of the inner membrane. The largest intact mitochondria measure about 1.6 μ in diameter.

On close examination (Fig. 3 *b*), the degree of electron opacity of the matrix is related to the manner in which small spherical and short filamentous granules, 60 to 100 A in diameter, are packed; in the denser matrices, these granules are packed more closely together. The typical large intramitochondrial granules of 240 to 400 A in diameter are also obvious in the matrix. Both the inner and limiting membrane components have a thickness of approximately 70 A.

The major points to be noted in this control of isolated mitochondria, in comparison to *orthodox* mitochondrial conformation (i.e. as observed after fixation within intact tissues), are the obvious decrease in matrical volume, the increase in matrical density, the irregular pattern of inner membrane folding, and the increase in volume in the outer compartment and intracristal space. In this study, these mitochondria are characterized as having a *condensed* conformation.

Mitochondria left to incubate in 0.25 M sucrose at 30°C develop a slow rate of oxygen consumption after 1.5 min, which lasts for approximately 10 min, a fact which suggests the utilization of endogenous substrate. Samples were fixed every 5 min (up to 30 min) for electron microscopy. Fig. 4 shows a sample from a 15 min incubation. While it is clear that a change in structural organization takes place in many mitochondria, this transition does not appear to be in any consistent direction. Many mitochondria maintain the condensed conformation of 1.5 min incubations. Some contain a generally expanded and dilute matrical mass, with a few folds of inner membrane suggestive of cristae; or the limiting membrane may be discontinuous and closely apposed to a largely unfolded inner membrane component. In this study, mitochondria having these features are designated *swollen*, regardless of their size. Other mitochondria possess a discontinuous limiting membrane, or occasionally none at all, with either a condensed or an excessively expanded matrix. Dilated intracristal spaces, appearing as matrical vesiculations, are very obvious in some of the mitochondria. The largest mitochondria measure 2 μ in diameter. In general, the appearance of this 15 min control incubation in 0.25 M sucrose is one of structural heterogeneity.

Controls: Ultrastructure of Mitochondria Swollen to High-Amplitude Levels

Distinct from the low-amplitude volume changes, which, according to Packer (3), take place during change in metabolic state, are the more widely studied high-amplitude volume

changes. The literature on this topic has been recently reviewed by Lehninger (9). Essentially, low-amplitude volume changes are represented photometrically by optical density changes of 0.01 to 0.05 which may be equivalent to a 20 to 40% volume change (3, 6), or less (9). High-amplitude changes, however, are characterized by optical density changes of approximately 0.5, or an order of magnitude higher, which represents a doubling or tripling of mitochondrial volume (9). In addition, high-amplitude swelling, unlike low-amplitude swelling, usually leads to irreversible organizational changes which result in loose coupling of respiration.

Fig. 5 shows oxygen consumption and kinetics of optical density of a sample of Mw placed into a nucleotide-free swelling medium. Loose coupling is observed to develop with the swelling process which is identified by a high-amplitude decrease in optical density. Oxygen is consumed at the rapid rate of 2.3×10^{-3} μatoms/sec.

Mitochondria fixed after 2 min in this medium are shown in Fig. 6. Morphologically, swelling is obvious, and in the majority of mitochondria either the inner or limiting membrane is discontinuous, but not both, at least in the same plane of sectioning. The small matrical granules are widely dispersed. The cristae appear to be tubular remnants, often continuous with the inner membrane component, and bear attached granules approximately 80 A in diameter (Fig. 6 *b*). The appearance of these cristae is reminiscent of those observed in negatively stained preparations (24, 25). The largest mitochondria measure approximately 3 μ in diameter, which represents more than a 6-fold volume increase over the volume of the largest mitochondria observed in the 1.5 min sucrose incubations.

This control represents mitochondria which should be contrasted with those undergoing low-amplitude changes. These mitochondria display high-amplitude levels of swelling photometrically, display loose coupling, and are ultrastructurally excessively swollen.

Ultrastructure of Mitochondria in State I

Fig. 7 illustrates changes in oxygen consumption and optical density of State I mitochondria incubated for 15 min followed by oxidative phosphorylation. A typical State I sample, fixed after 1.5 min' incubation, is shown in Fig. 8. It is obvious that the majority of the intact mitochondria (ca. 95%) contain a condensed conformation similar to that of the control mitochondria incubated for 1.5 min in sucrose. Some, however, possess matrices which occupy more of the total mitochondrial volume, and, therefore, have outer compartments of less volume. These mitochondria display a suggestion of cristal folding, and will be referred to as mitochondria of *intermediate* conformation (Fig. 8 *b*). The large intramitochondrial granules are more obvious, owing to the reduced electron opacity of the matrix (cf. Fig. 3 *b*). Following 1.5 min incubations, State I mitochondria routinely give classical ADP:O ratios of 1.9 to 2.0 for succinate, and ACR of 5 to 7. Samples of these mitochondria, which are fixed as phosphorylation ceases, show an increase toward the condensed conformation, while intermediate forms are less frequently observed.

When State I mitochondria are left to incubate, a slow initial rate of oxygen consumption approaches zero uptake after approximately 12 min, and a slow but sustained drop in optical density follows a rapid initial drop (Fig. 7). Samples for electron microscopy were fixed at 5-min intervals

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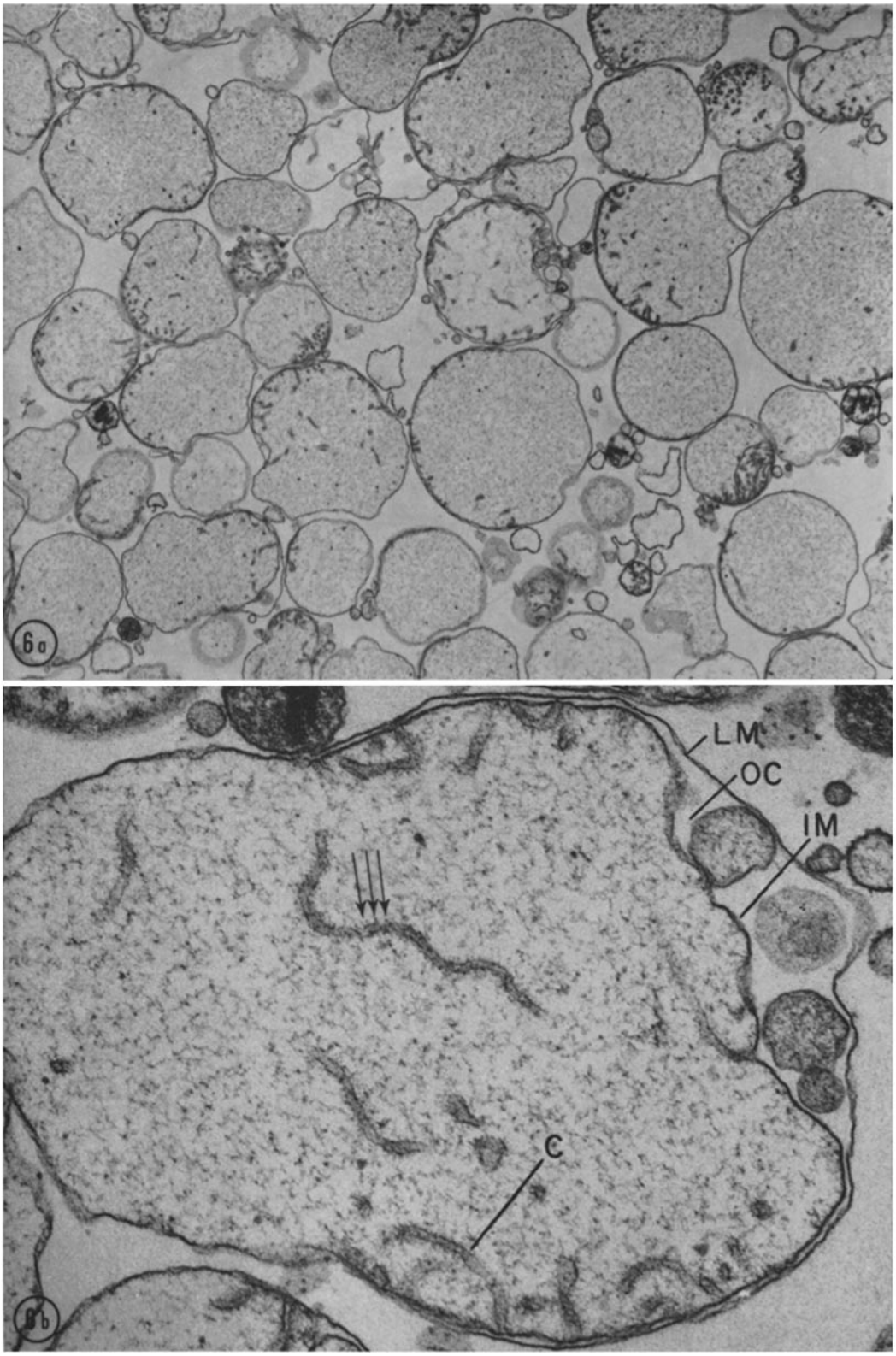
FIGURE 6 Mitochondria were fixed after 2 min' incubation in a swelling medium during which a high-amplitude absorbance decrease suggestive of swelling was reached (Fig. 5).

FIGURE 6 *a* The utrastructural evidence for swelling, which justifies the interpretation of the absorbance decrease, is clear; the matrix is expanded and dilute, the cristae are residual, and a majority of mitochondria are volumetrically 3 to 4 times larger than preswollen controls (cf. Fig. 3 *a*). × 10,700.

FIGURE 6 *b* As in negatively stained mitochondrial preparations, cristae (*C*) appear as tubular remnants, some of which are shown here to be continuous with the inner membrane (*IM*). The small matrical granules are very dispersed, but many appear to be attached to the cristae (arrows). Limiting membrane, *LM*; outer compartment, *OC*. × 50,000.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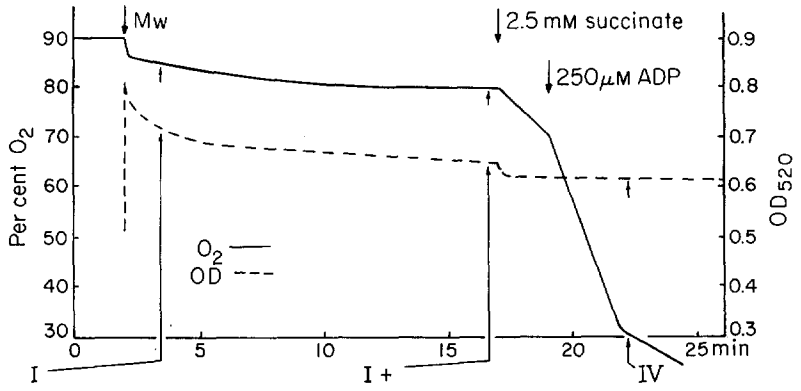


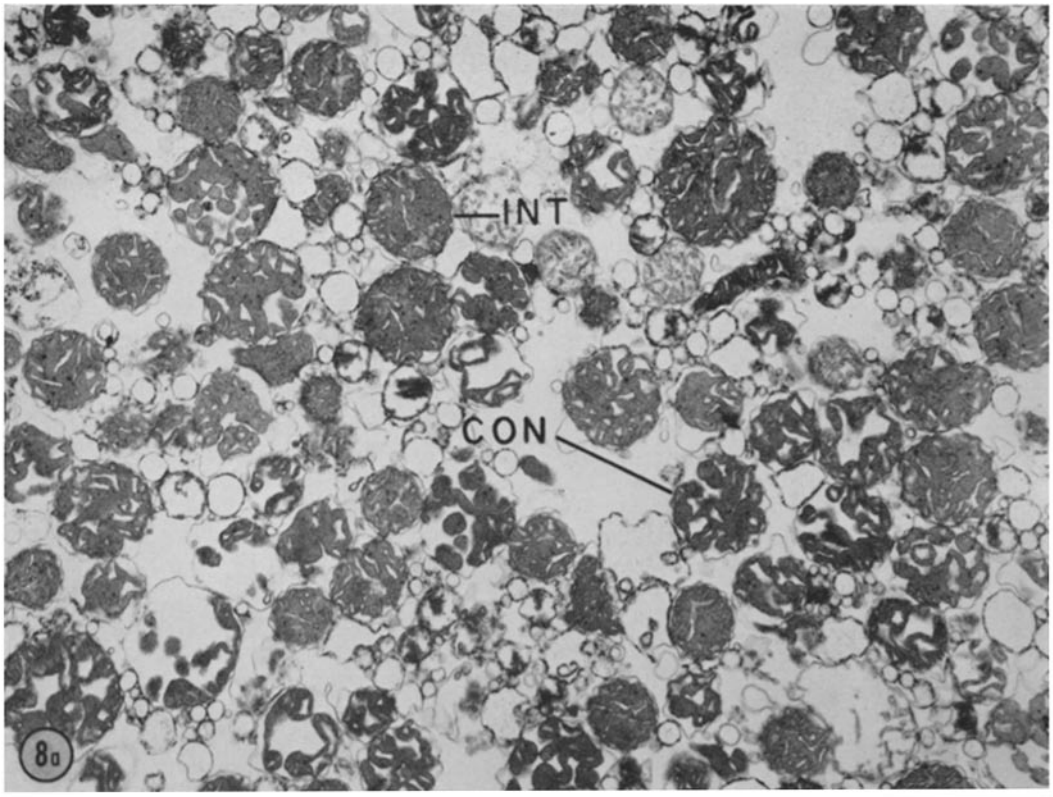
FIGURE 7 Traces show oxygen consumption and optical density changes of mitochondria incubated in State I followed by oxidative phosphorylation. Microsamples for electron microscopy were fixed consecutively in State I at 1.5 min (arrow I) (Fig. 8), and at 15 min (arrow I +) (Fig. 9). After addition of succinate and ADP, a sample was fixed as phosphorylation ceased (arrow IV) (Fig. 10). Note the low-amplitude absorbance decrease upon addition of substrate and the lack of absorbance change with ADP-induced phosphorylation. The reaction system contained sucrose (0.113 M), PO_4 buffer (0.01 M, pH 7.0), $MgCl_2$ (5.0 mM), succinate (2.5 mM), ADP (250 μM), and Mw (1.7 mg protein). Total volume 2.0 ml.

during State I incubations, and Fig. 9 illustrates a 15 min sample. The results show that mitochondria subjected to a State I condition for short periods of time undergo structural reorganization from the condensed to a basically *orthodox* conformation indicative of mitochondria fixed in situ. This structural reorganization takes place in all intact mitochondria within the first 7 min of incubation. In comparison to the mitochondrial structure observed in the 1.5 min control sucrose and 1.5 min State I incubation, the outer compartment and intracristal spaces, often continuous, are volumetrically decreased. The volume of the matrix is increased and the small matrical granules are more dispersed, resulting in a less electron-opaque inner compartment. When these structurally orthodox mitochondria carry out phosphorylation, the resulting ADP:O ratios are considerably lower than established values, i.e., 1.5 or less for succinate. It is noteworthy that the addition of succinate causes a low-amplitude decrease in optical density, but addition of nucleotide (of the same volume) does not result in any obvious optical density change, although it initiates some phosphorylation (Fig. 7). Samples fixed as phosphorylation ceases (Fig. 10) display no organizational change in structure compared with the orthodox conformation of 15 min incubated State I mitochondria. However, there are some subtle differences which may account for the low-ampli-

FIGURE 8 Mitochondria were fixed after 1.5 min' incubation in State I. They display a generally condensed conformation (*CON*).

FIGURE 8 *a* Compared to orthodox mitochondrial structure, the condensed matrix is decreased in volume relative to the outer compartment and intracristal spaces. In addition to the very condensed form, some of the mitochondria possess a less dense matrix associated with a larger matrical volume, and a relatively smaller outer compartment and intracristal space, and are designated mitochondria of intermediate conformation (*INT*). (cf. Fig. 3 *a*). × 10,700.

FIGURE 8 *b* On the right are two profiles of mitochondria of the condensed conformation displaying large outer compartments (*OC*), while the mitochondrion on the left displays a more intermediate conformation with an outer compartment of less volume. Limiting membrane, *LM*; inner membrane, *IM*; intracristal space, *IS*. × 50,000.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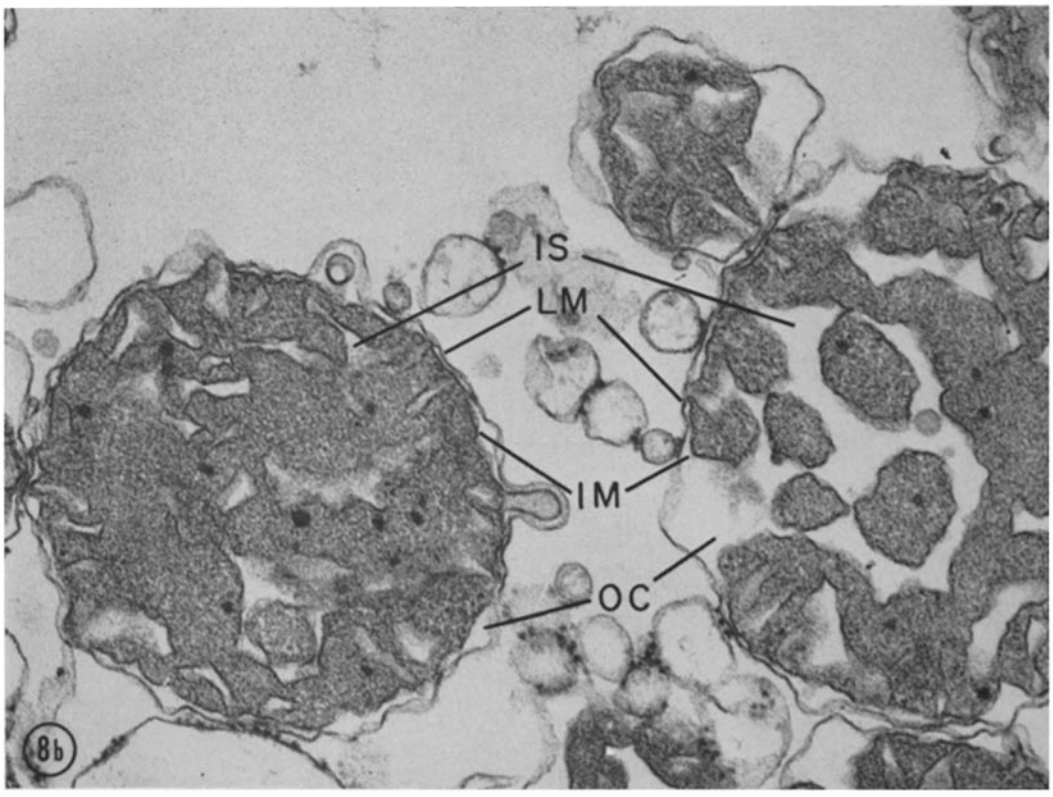
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tude optical density decrease after addition of succinate (Fig. 7); there is usually an increase in the number of damaged and swollen mitochondria, and the intact mitochondria contain a more dilute matrix (cf. Fig. 9 *a*).

Ultrastructure of Mitochondria in State III and in State IV

Fig. 11 illustrates oxygen utilization and optical density changes which occur during a 15 min State IV incubation followed by ADP-induced phosphorylation. After 1.5 min in State IV, mitochondria display condensed and intermediate conformations (Fig. 12) and are indistinguishable from those of 1.5 min State I incubations (Fig. 8). As in State I, State IV mitochondria of 1.5 min incubations routinely give ADP:O ratios of 1.9 to 2.0 for succinate with ACR of 5 to 7, and, as phosphorylation ceases, they display a characteristically condensed conformation.

As State IV mitochondria incubate, they consume oxygen at a slow steady rate (5.1×10^{-4} μatoms/sec, in Fig. 11), indicating low-level substrate consumption, display a slow and sustained optical density decrease, and concomitantly undergo a gradual reorganization of internal structure. In 15 min, approximately 95% are characterized by orthodox conformation (Fig. 13), and incubations of 25 min lead to orthodox structure in all intact mitochondria. On close inspection, the small matrical granules appear more numerous and more evenly dispersed than the matrical granules found in State I incubated mitochondria (cf. Fig. 9 *b*). State IV mitochondria incubated for 15 to 25 min are morphologically indistinguishable from spherical liver mitochondria fixed in situ.

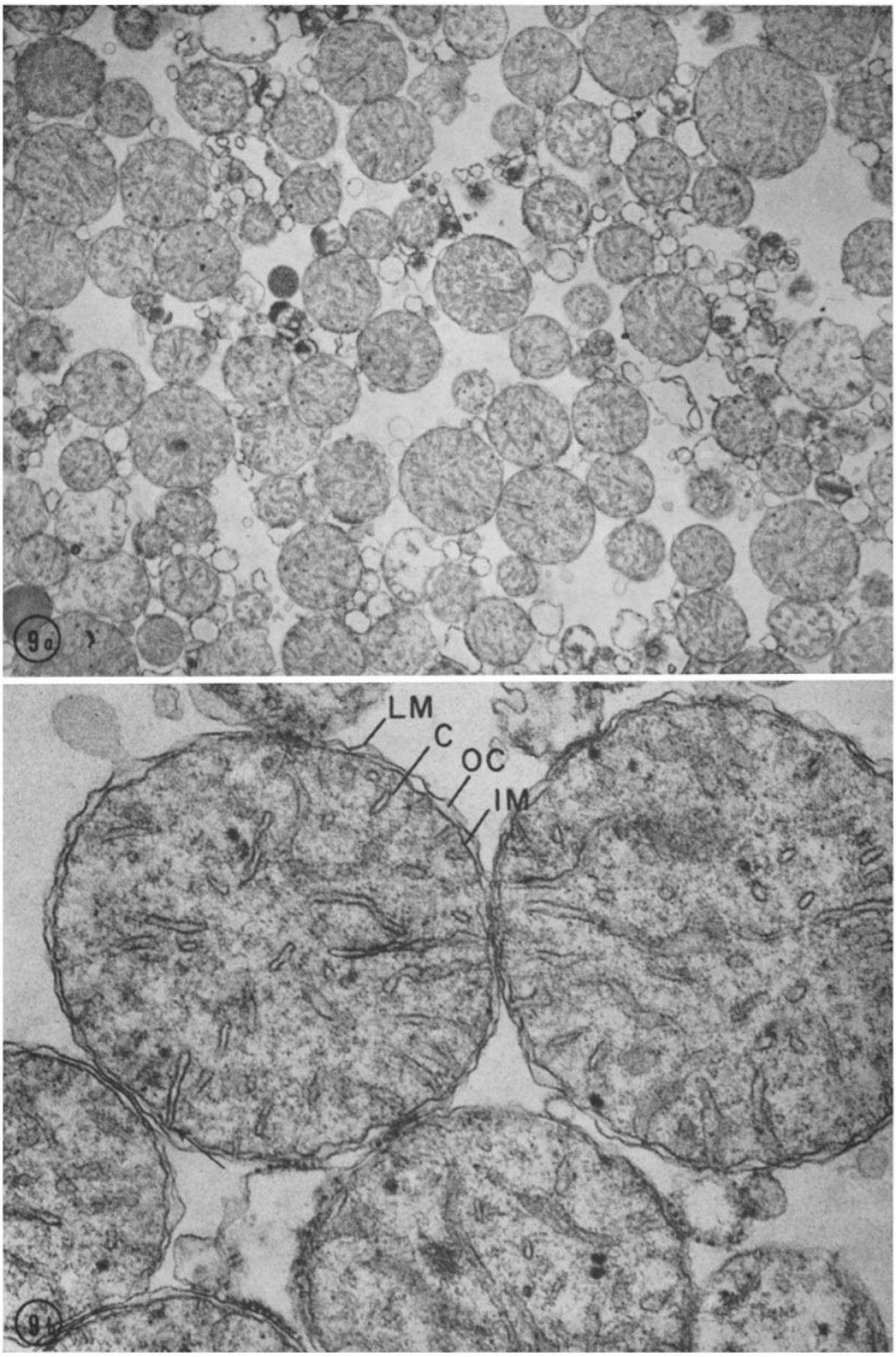
When ADP is added to State IV mitochondria, there is an abrupt but temporary low-amplitude increase in optical density which coexists with phosphorylation; this rise, as originally shown by Chance and Packer (1), is indicative of ADP-activated State III metabolism. The optical density curve in Fig. 11 shows that this ADP-dependent rise occurs, even after mitochondria are incubated for 15 min in State IV, and has a magnitude of 0.025. In this experiment, active oxygen consumption during the optical density increase was calculated to be 2.2×10^{-3} μatoms/sec, representing a 4-fold increase over oxygen consumption in State IV. Mitochondria incubated in State IV for 15 min give ADP:O ratios of approximately 1.8 for succinate.

After short-term incubations in State IV, samples of mitochondria were fixed during ADP-activated phosphorylation at the peak of the low-amplitude optical density increase. Fig. 14 shows a typical sample fixed 35 sec after the addition of ADP to a 15 min State IV incubation. It is clear that, with activation of oxidative phosphorylation, the orthodox structure of incubated State IV mitochondria undergoes a dramatic internal reorganization resulting in a highly condensed conformation. The volume of the matrix is greatly decreased with a concomitant volumetric increase in outer compartment and intracristal space. Increased electron opacity of the matrix is again clearly associated with closer approximation of the small matrical granules (Fig. 14 *b*). The inner membrane component undergoes a gross conformational change resulting in irregular folding, with only an occasional suggestion of orthodox folding into cristae. In a fair number of mitochondria, the condensed matrix appears distributed as a sphere containing an internal compartment which in some cases is, and in some cases may not be, continuous with the enlarged outer compartment. The time interval between samples represented in Figs. 13 (State IV) and 14 (State III) is 40 sec.

FIGURE 9 A mitochondrial sample fixed after 15 min' incubation in State I.

FIGURE 9 *a* A change from a condensed to an orthodox conformation has taken place (cf. Fig. 8 *a*). This structural reorganization occurs in all intact mitochondria. × 10,700.

FIGURE 9 *b* As in mitochondria fixed in situ, these reorganized mitochondria are composed of a two membrane system, the inner membrane of which may be continuous with folds of cristae (arrow). The small matrical granules appear dispersed and give the matrix a less dense appearance than the matrix of the condensed conformation (cf. Fig. 8 *b*). Limiting membrane, *LM*; inner membrane, *IM*; outer compartment, *OC*; cristae, *C*. × 50,000.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9b

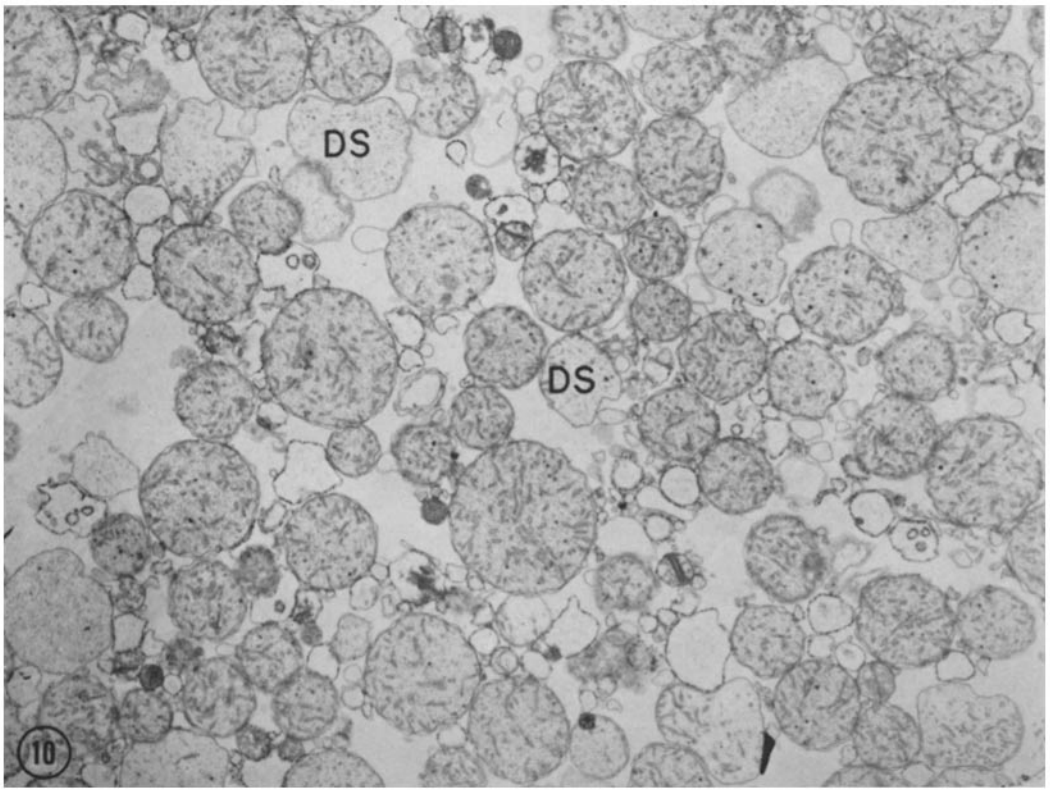


FIGURE 10 The mitochondrial sample shown here was fixed as phosphorylation ceased. Before phosphorylation was initiated, mitochondria were incubated for 15 min in State I. Note that intact mitochondria retain their orthodox conformation. However, compared to prephosphorylation mitochondria (Fig. 9 *a*), damaged and swollen mitochondria (*DS*) are increased in number and the matrices of intact mitochondria are less dense. × 10,700.

Samples fixed as phosphorylation ceases show essentially similar mitochondrial structure, but many mitochondria contain an internal compartment which definitely displays continuity with the outer compartment (Fig. 15). After phosphorylation, however, as mitochondria equilibrate once again in State IV, their internal structure gradually reverts completely to the orthodox conformation which is indistinguishable from that of prephosphorylation State IV mitochondria.

It appears that the internal components of structural organization in mitochondria have the intrinsic capacity to oscillate between a condensed and an orthodox conformation, and it appears that this reversible activity is a metabolically-linked function.

Ultrastructure of Mitochondria in State II

Curves of oxygen consumption and optical density of a 15 min incubation of mitochondria in State II, followed by phosphorylation, are shown in Fig. 16. Mitochondrial samples fixed at 1.5 min incubation possess what may be a maximally condensed matrical substance (Fig. 17). In many mitochondria the matrix appears condensed to such a degree that the outer compartment incorporates more volume than the inner compartment does. These mitochondria consistently give ADP:O ratios of 1.9 to 2.0 for succinate, and in any one mitochondrial stock preparation, they always give higher ACR than State I or State IV 1.5 min incubations. Samples fixed as phosphorylation ceases show no observable change in structure.

Incubation of State II mitochondria results in a slow rate of oxygen consumption which levels off within 10 to 12 min. Two characteristic features of the optical density curve of incubating State II mitochondria (Fig. 16) are that the initial decrease in absorbance is less abrupt, and that the final amplitude of the decrease is consistently two-

thirds that which occurs during incubations of State I and State IV mitochondria (cf. Figs. 7 and 11). In addition, the State II mitochondria appear to resist the change in structural organization from condensed to orthodox, which is indicative of State I and State IV short term incubations. After 15-min incubations, approximately 90%, and after 25-min incubations, approximately 80% or more of the intact mitochondria maintain a condensed or intermediate conformation (Fig. 18).

The curves (Fig. 16) show that succinate-initiated phosphorylation of 15 min incubated State II mitochondria results in a low-amplitude absorbance drop of 0.02 and a very rapid oxygen uptake followed by a 4-fold deceleration after 1 min, which is indicative of relatively tight respiratory coupling. Unlike State IV but especially unlike State I incubations, mitochondria incubated in State II continue to give classically high ADP:O ratios and ACR.

It is difficult to ascertain whether or not any structural changes take place during phosphorylation. The condensed and intermediate conformations persist in about 90% of the intact population observed in samples which are fixed as phosphorylation ceases (Fig. 19).

Ultrastructure of Mitochondria in State V

As mitochondria enter the anaerobic State V, a slow low-amplitude rise in optical density which has been termed autonomic reversible swelling by Beechey and Holton (2) is maintained. Samples fixed at 5-min intervals during 45-min time courses, including State IV, State IV to V transitions, and State V, show that no major reorganizational changes occur in the orthodox structure of incubated State IV mitochondria during or after entry into State V.

Fig. 20 summarizes some of the ultrastructural results.

DISCUSSION

Consistency of Ultrastructural Organization Related to Metabolic Steady States in Isolated Mitochondria

The synchronous changes of structural and metabolic parameters that this report presents were consistently reproducible. For example, with six different mitochondrial isolations, the condensed conformation characteristic of the freshly isolated mitochondria underwent change to orthodox conformation after short term incubations in State IV. This orthodox conformation was reversed in all experiments carried out by initiating State III metabolism. This consistent reproducibility was due primarily to: (*a*) the ascertainment of the functional integrity of the initial mitochondrial preparations; (*b*) identity of the functional state in

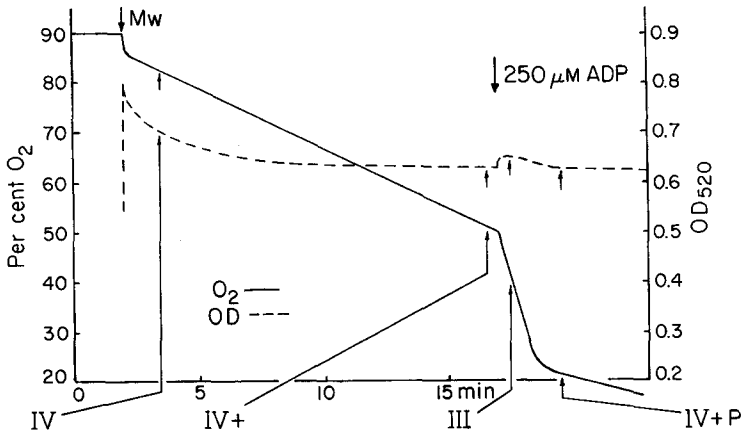


FIGURE 11 Changes in oxygen consumption and optical density during incubation of mitochondria in State IV followed by oxidative phosphorylation. Microsamples for electron microscopy were fixed consecutively at 1.5 min (arrow IV) (Fig. 12), and 15 min (arrow IV +) (Fig. 13). Samples were also fixed during State III (arrow III) (Fig. 14) and as phosphorylation ceased (arrow IV + P) (Fig. 15). Note that upon addition of ADP there is a low-amplitude upward deflection in absorbance which gradually returns to the base line coincident with the termination of State III metabolism. Reaction system as in the legend for Fig. 7.

which mitochondrial samples were fixed; (*c*) a new technical procedure of quick-sampling and preparation of micropellets for electron microscope analysis.

With the assumption that the enzymatic organization of mitochondria is the basis of both structure and function, it would seem unreasonable to investigate the structural aspects of any mitochondrial preparation without assessing its metabolic integrity. In the present study, only those mitochondrial preparations from which controls gave ADP:O ratios of 1.9 to 2.0 for succinate and which displayed tight coupling of respiration were used.

The identity of well defined functional states in incubating mitochondria was based on criteria that Chance and Williams originally formulated (13, 14). These criteria were presented in Results. The identification of metabolic steady states by polarographic, fluorometric, and optical density methods, while micropellets are being rapidly fixed for electron microscopy, has now established a characteristic ultrastructure for each state. The new method of quick-sampling of mitochondria from reaction cuvettes and rapid preparing of the microsamples for electron microscopy is most useful in relating consistent ultrastructural change to functional change throughout cyclic short term experiments.

It is appropriate to consider in detail the problem of artifact. The usual fixation of large mitochondrial pellets for electron microscopy results in varying mitochondrial structure at different levels in the pellets. This structural variation may be due to the delay in the time required for fixative to reach the depth of the pellet, which results in partially anaerobic preparations. The quick-sampling and micropellet procedures used in the present study resulted in consistent mitochondrial conformation throughout the entire depth of the pellet for any one metabolic state. It should be noted that this depth (0.2 mm) is much smaller than the size of the usual tissue block prepared for most electron microscopy.

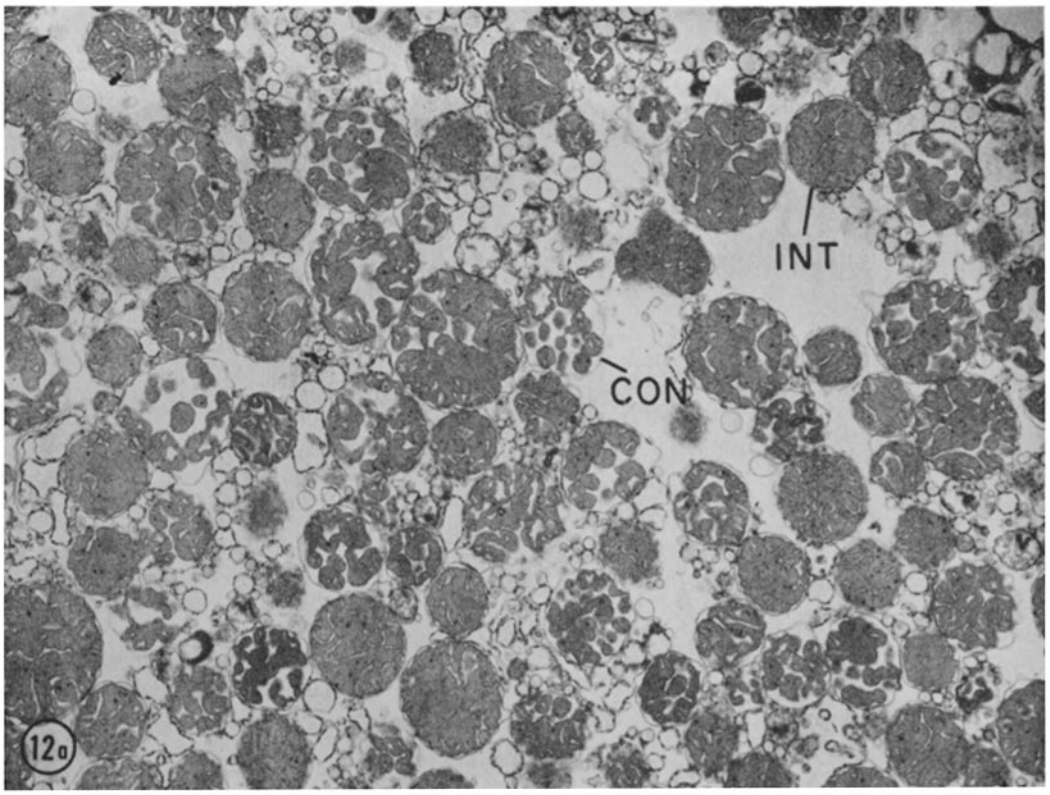
It could be argued that the 30 sec centrifugation used in the present study, even of small pellets of this size, could result in a mitochondrial anaerobic state (State V), and that the present ultrastructural results, therefore, represent conformational changes that take place in mitochondria as they are centrifuged from initially identified aerobic respiratory states to State V. These arguments, however, are inconsistent with the results. For example, mitochondria in State IV for 1.5 min display condensed to intermediate conformation (Fig. 12); in State IV for 15 min or more they display only orthodox conformation (Fig. 13); and in postphosphorylation State IV they display the condensed conformation (Fig. 15). If a conformational change, characteristic of State IV to State V transition, were occurring during the short centrifugation period, one would expect all three State IV preparations mentioned above to display similar conformational details. A preliminary study of mitochondria in State IV and in State III fixed in suspension revealed that the conformational details were consistent with those in which fixation followed the 30 sec centrifugation; however, for reasons not yet clearly understood, the mitochondrial pellets obtained were difficult to work with and could not be sectioned to give optimal electron micrographs. Work now in progress has been designed to overcome these preparative failures. Workers in laboratories where fixation of mitochondria in suspension appears to result in no consequential preparatory problems should distinguish between fixation in suspension and fixation during centrifugation.

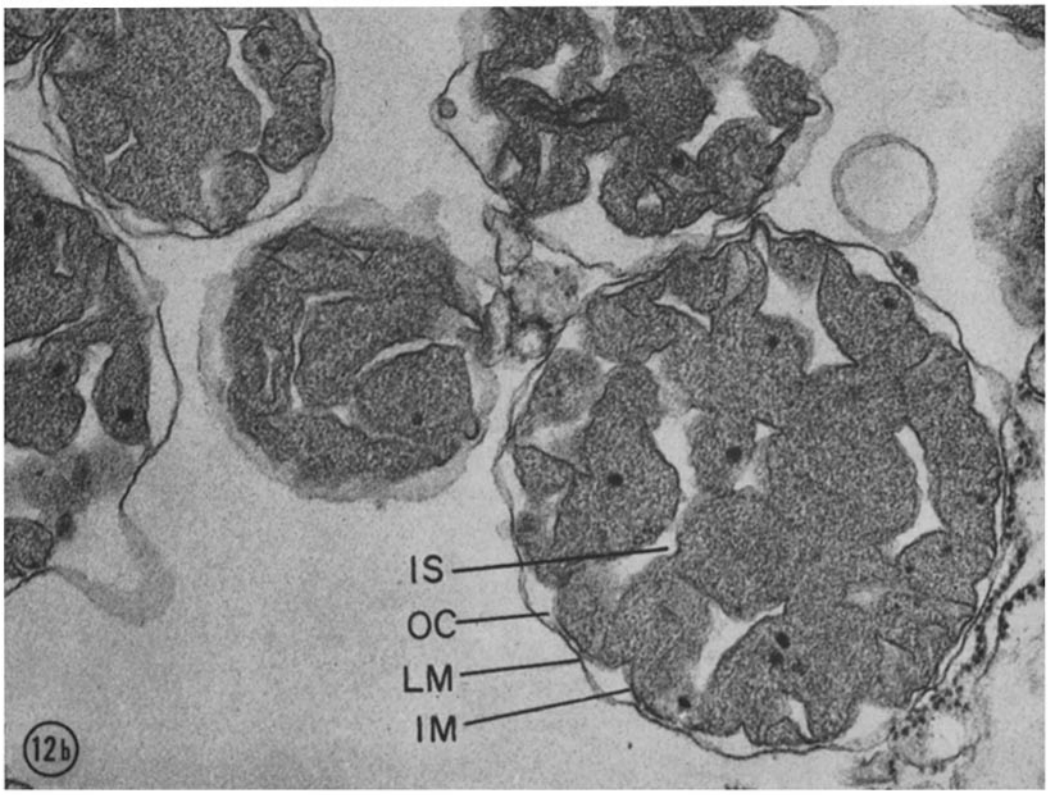
Cells and organelles in a particular functional state may possibly react differently to fixative than

FIGURE 12 Mitochondria fixed after 1.5 min' incubation in State IV.

FIGURE 12 *a* These mitochondria are characterized by condensed (*CON*) and intermediate (*INT*) conformations which are indistinguishable from mitochondria incubated in State I for 1.5 min (cf. Fig. 8 *a*). × 10,700.

FIGURE 12 *b* The matrical granules are more closely packed in mitochondria of highly condensed conformation (upper right) than in mitochondria possessing a less condensed, more intermediate structure (lower right). Limiting membrane, *LM*; inner membrane, *IM*; outer compartment, *OC*; intracristal space, *IS*. × 50,000.


INT
CON
12a


IS
OC
LM
IM
12b

the same kinds of cells and organelles in a different functional state do. For example, in the present investigation, the per cent reduction and therefore presumably the conformation of the respiratory enzymes differ in the various metabolic states. Because it is generally thought that these enzymes are contained within the inner mitochondrial membrane, the permeability and reactivity of this membrane to fixative may be quantitatively different in different metabolic states. Also, dissimilar internal concentrations of ATP, ADP, and substrate, chemiosmotic and pH differences, etc. in the various metabolic states may be expected to influence the rate and manner in which the structural components of mitochondria will react with fixative and therefore to result in an artifactual inhibition or accentuation of any truly mechanical or conformational change. Unfortunately, information is not yet available nor is technology at a stage to permit a decisive evaluation of the possible combined influence of the different chemical and physical conditions of each metabolic state on the interactivity of structural components and fixative. Any investigation concerned with structural kinetics and structure-function relationships must take into account the possible influence of this type of artifact. The following discussions will present the ultrastructural results of this investigation as a rational basis for metabolically linked mechanical activity in coupled mitochondria. The theoretical foundation and the biochemical and physical bases for mechanochemical activity in mitochondria have already been well formulated by Lehninger (9–12).

Ultrastructure in Relation to Low-Amplitude Optical Density Changes in Isolated Mitochondria

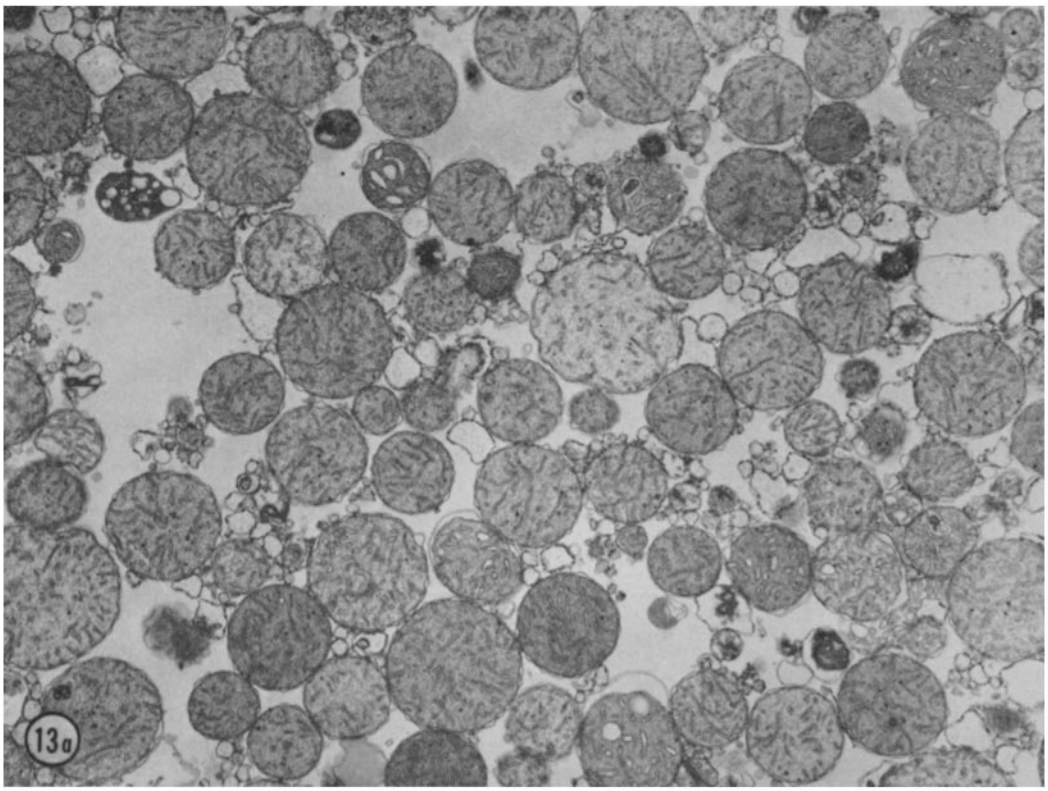
Chance and Packer (1) were the first to identify a nonspecific low-amplitude optical density increase in mitochondria (rat heart) during ADP phosphorylation. They recorded single- and double-beam tracings simultaneously and therefore were able to distinguish between nonspecific low-amplitude optical density changes and specific cytochrome absorbance effects. Packer (3–7) has since photometrically identified "structural states" for each metabolic state; mitochondria in States I and IV are in a swollen structural state, whereas mitochondria in States II, III, and V are in a shrunken state. Since the addition of ADP to mitochondria in States I and IV results in a change to the shrunken state, the conclusion is that any condition leading to increased intramitochondrial ADP results in mitochondrial shrinkage.

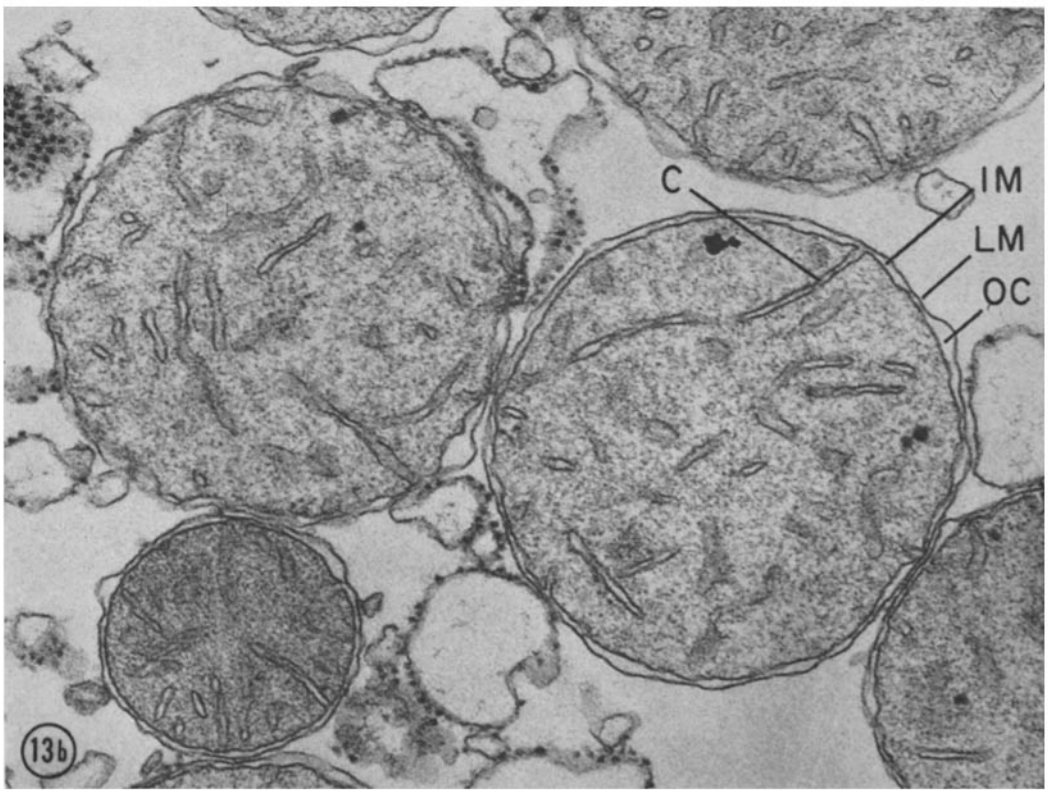
In the present investigation, analysis of mitochondrial diameters measured from a series of electron micrographs indicates that mitochondria incubated up to 27 min in State I and State IV tend to increase gradually in total volume to approximately 20%. This volume increase occurs during and after a change from a condensed to an orthodox conformation (Figs. 9 and 13). ADP-induced phosphorylation of State IV mitochondria results in a low-amplitude absorbance increase (Fig. 11) and a dramatic ultrastructural reorganization of inner membrane folding and increased matrical density (Fig. 14), but in no obvious decrease in total mitochondrial volume. In experiments where succinate and ADP are added (in that order) to activate phosphorylation in short-term incubated State I mitochondria, succinate addition induces a low-amplitude optical density decrease, but there is no subsequent ADP-linked change in optical density although some phosphorylation is recorded (Fig. 7). In this case electron microscopy indicates that no conformational change in inner membrane takes place, but a dilution of matrical substance does occur (Fig. 10), along with a volume increase as great as 50% in

FIGURE 13 Mitochondria from a sample fixed after incubation for 15 min in State IV.

FIGURE 13 *a* It is clear that as mitochondria incubate in State IV they undergo a structural reorganization which leads to an orthodox conformation essentially indistinguishable from that of mitochondria fixed in situ (cf. Fig. 12 *a*). × 10,700.

FIGURE 13 *b* The orthodox form of folding of cristae (*C*) is apparent. Note that the small matrical granules are consistently more evenly distributed in these mitochondria than in the matrices of mitochondria incubated in State I for 15 min (cf. Fig. 9 *b*). Limiting membrane, *LM*; inner membrane, *IM*; outer compartment *OC*. × 50,000.


13a


C
IM
LM
OC
13b

intact mitochondria. The slow rise in optical density during the State IV to State V transition, called autonomic reversible swelling (2), occurs without any observable ultrastructural change or total volume change. Yet an absorbance rise of the same low magnitude (0.02) during the State IV to State III transition occurs during a change from orthodox to condensed ultrastructure.

The interpretation of nonspecific low-amplitude changes in absorbance simply as changes in total mitochondrial volume appears questionable when the more direct results obtained by electron microscopy as observed in the present study are considered. It would seem that low-amplitude, nonspecific optical density and light-scattering changes could result from a number of phenomena in combination with total volume change, e.g. reorganizational changes in internal structure as revealed by this investigation, or from other less demonstrable changes such as phase or redistributional changes of metabolites within mitochondrial compartments, geometrical changes in various molecular components, etc. Any such phenomena could be expected to influence the total light-scattering and absorbance properties of mitochondrial suspensions and therefore to influence the direction and amplitude of nonspecific optical density change.

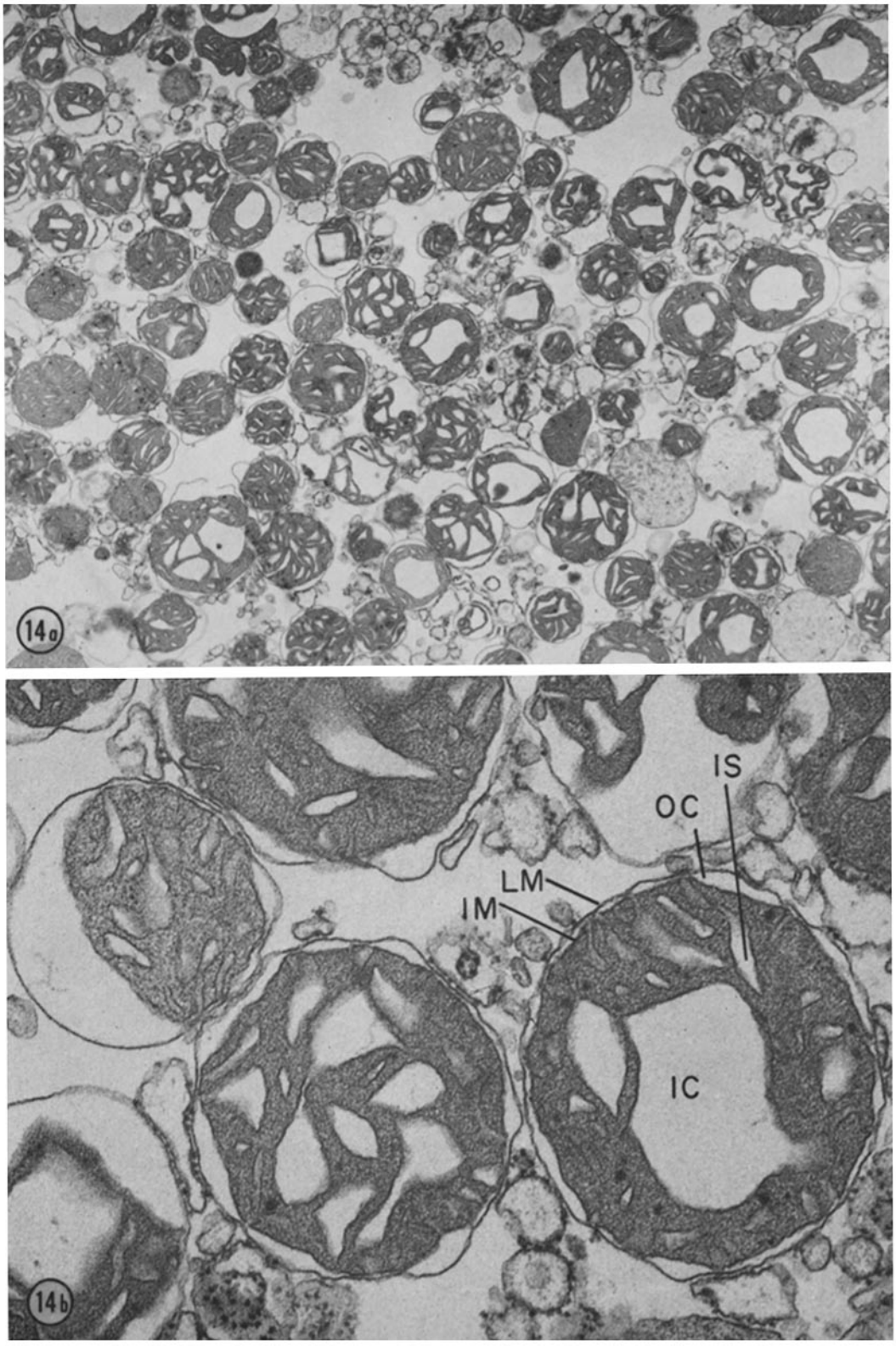
An Ultrastructural Basis for Metabolically-Linked Mechanical Activity in Isolated Mitochondria

Four distinct conformations of mitochondria are described in the present study: orthodox, condensed, intermediate, and swollen. Isolated mitochondria displaying orthodox structure (Fig. 13) closely resemble the traditionally "normal" mitochondria observed within fixed tissue. The mitochondria of condensed conformation described in this report display a highly electron-opaque matrix surrounded by an irregularly folded inner membrane. Although both the matrix and inner membrane appear to be generally "contracted," they display subtle conformational differences in various functional states, e.g., in sucrose (Fig. 3), in State I (Fig. 8), in State IV (Fig. 12), in State III (Fig. 14), and in State II (Fig. 17). Mitochondria of intermediate conformation contain an inner membrane of intermediate complexity, with respect to folding, and a matrix of intermediate electron opacity, giving the appearance of only slight internal "contraction." They are always observed in situations where the condensed form is progressing toward the orthodox form, e.g., during short term incubations in State I and State IV. Considering that the change from condensed, through intermediate, to orthodox ultrastructure in State IV is consistently and rapidly reversible under conditions of phosphorylation, it is suggested that these changes are of physiological significance. Swelling as described in this report (Fig. 6) results in the rupture of membranes and is considered an irreversible, nonphysiological condition. The heterogeneous mitochondrial "types" which Burgos et al. (26, 27) have described are more than likely the result of a low-level respiration-dependent swelling tendency which occurs at the expense of endogenous substrate during short term

FIGURE 14 Phosphorylating mitochondria. This sample of mitochondria was fixed 35 sec after the addition of ADP to a 15 min State IV incubation.

FIGURE 14 *a* It is clear that, during the State IV to State III ADP-activated transition, the internal mitochondrial components undergo a dramatic conformational reorganization into a highly condensed form (cf. Fig. 13 *a*). In all intact mitochondria, the inner membrane undergoes a conformational change in folding; there is a parallel decrease in matrical volume with increase in electron opacity and a volumetric increase in outer compartment. In many mitochondria the matrix condenses into a peripheral mass which surrounds an internal compartment. × 10,700.

FIGURE 14 *b* Reconformational folding of the inner membrane (*IM*) leaves little suggestion of orthodox cristae, and matrical density increases again with increased packing of the small matrical granules (cf. Fig. 13 *b*). A large membrane-bounded internal compartment (*IC*) is completely surrounded by the matrix and may be an extension of the volumetrically enlarged outer compartment (*OC*) or may be an enlarged intracristal space (*IS*). Limiting membrane, *LM*. × 50,000.


14a
IS
OC
LM
IM
IC
14b

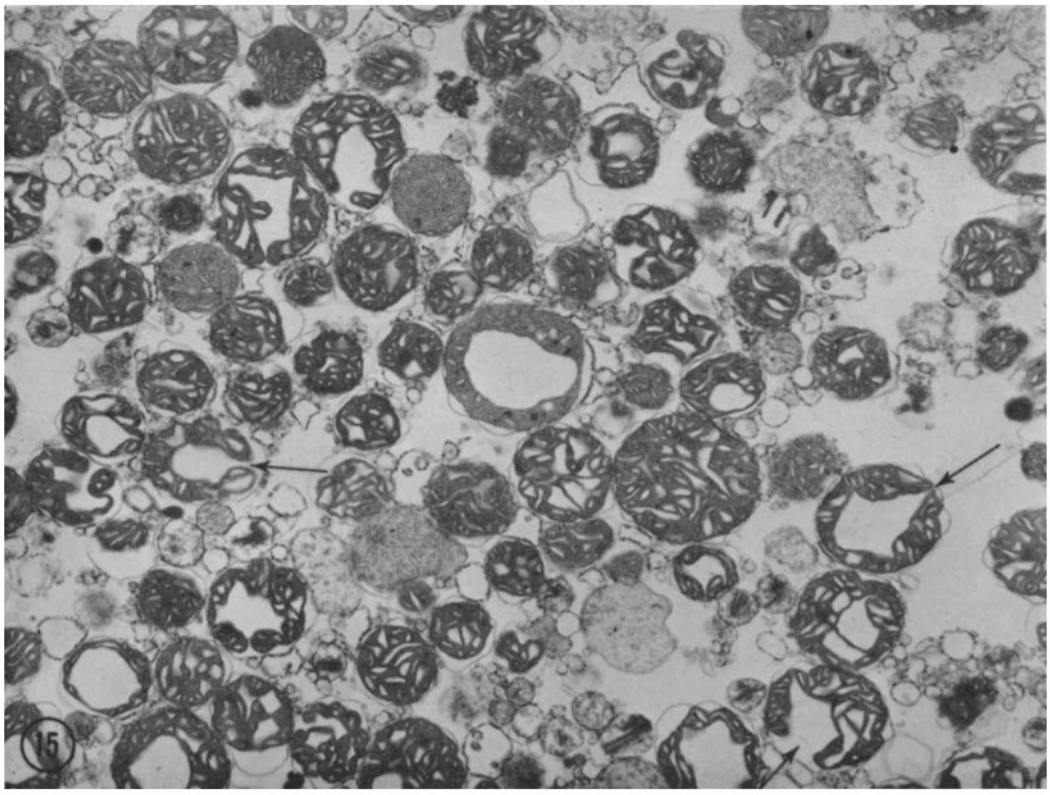


FIGURE 15 This mitochondrial sample is from a 15 min State IV incubation followed by oxidative phosphorylation. The sample was fixed as phosphorylation ceased. Structure is essentially indistinguishable from that of State III mitochondria (cf. Fig. 14 *a*). In many mitochondria the matrix surrounds a large internal compartment, which is often in continuity with the outer compartment (arrows). Short term equilibration in this postphosphorylation State IV consistently reverts the condensed conformation to an orthodox conformation indistinguishable from that of prephosphorylation State IV mitochondria shown in Fig. 13 *a*. × 10,700.

incubations in sucrose (Fig. 4). Mitochondrial swelling, measured by optical density change, is thought to depend on oxidation (28) and, more specifically, on electron flux (29).

The lack of consistency in the ultrastructure of isolated mitochondria is well established in the literature (30–36), and isolated mitochondria displaying structure other than orthodox are traditionally characterized as swollen, damaged, or poorly preserved. Various isolation media have been devised to maintain "structural integrity" in mitochondria, based on the rationale that this is a prerequisite for functional integrity. But a careful review of the literature reveals that factors more complex than isolation media alone are responsible for the great diversity in the morphology of isolated mitochondria. Structural integrity is equated with orthodox mitochondrial morphology as observed after fixation within intact tissue (37). But it is of major significance that disintegration of mitochondrial organization by digitonin treatment, sonic vibration, and other methods yields membrane fragments (vesicles) capable of effectively carrying out oxidative phosphorylation (19, 38–43). It is also known that intact swollen mitochondria efficiently carry out oxidative phosphorylation (30, 44, 45). After swelling to high-amplitude levels, mitochondria lose phosphorylating integrity, but this function can be restored as long as the swelling is reversible under conditions of phosphorylation (46). However, when organizational change reaches a level where mitochondria can no longer undergo reversible structural change (revealed by optical density

studies), phosphorylation integrity is irreversibly lost (6). Significantly, digitonin-prepared mitochondrial membrane fragments undergo organizational changes which are tightly linked to the activities of electron transport and oxidative phosphorylation (47, 48). The present results show that after short term incubations in State I, mitochondrial inner membranes lose the capacity to undergo ultrastructural reorganization (refolding) during induced change in metabolic state, and phosphorylating integrity markedly decreases; however, these mitochondria display excellent orthodox conformation (Fig. 9). It would appear that functional integrity, at least of oxidative phosphorylation, does not require stabilized or orthodox mitochondrial conformation. On the contrary, it requires that mitochondrial *membranes* maintain their property of conformational *flexibility* which appears to coexist with their molecular integrity. The results clearly demonstrate that mitochondria isolated in 0.25 M sucrose possess, in addition to a high phosphorylating capacity and acceptor control, an intrinsic capacity to undergo reversible changes in inner membrane folding and matrical density. This capacity is such that the internal structural organization of isolated mitochondria can oscillate between an orthodox and an unorthodox condensed conformation, provided conditions are suitable for controlling their metabolic state.

The ultrastructural changes which accompany change in metabolic state can be attributed to properties residing in the limiting membrane, the inner membrane, the matrix, or a combination of these structural components.

The limiting membrane, although likely to possess elastic properties, does not appear to fold or contract during ADP-activated phosphorylation (Fig. 14; see also Fig. 17). Perhaps the limiting membrane plays a role in restraining respiratory-dependent expansion of the matrix and unfolding of inner membrane. With rupture of the limiting membrane, as in excessive swelling, the inner membrane undergoes maximal unfolding as the matrix greatly expands, suggesting the uptake of water (Fig. 6). During ADP-activated phosphorylation in short term incubated State IV mitochondria, the inner membrane undergoes a reversible change in folding and the matrix undergoes a reversible condensation. In short, the entire membrane-bounded matrical mass volumetrically decreases, as in a syneresis. The extrusion of water, equated with volume decrease, during ATP-linked high-amplitude contraction of mitochondria has been measured directly (10) and appears to be independent of ionic environment, electron flux, and osmotic pressure (49). Water extrusion also takes place during oxidative phosphorylation (50). The fact that the inner membrane undergoes ultrastructural refolding concomitantly with oxidative

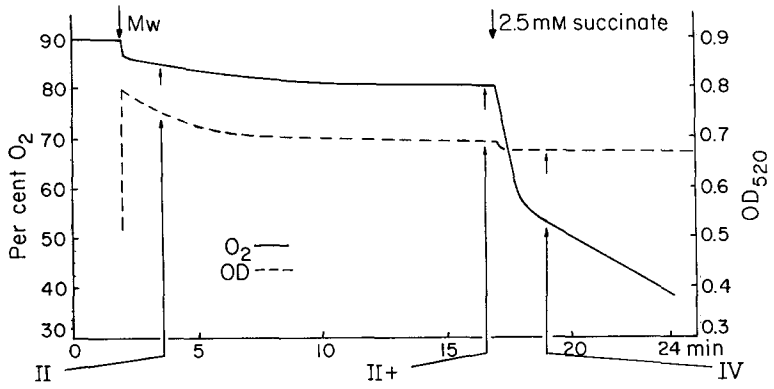


FIGURE 16 Curves show changes in oxygen consumption and optical density of State II mitochondria during a 15 min incubation followed by oxidative phosphorylation. The initial drop in absorbance is comparatively slow and of lower amplitude than in either State I or State IV incubations. Note also the low-amplitude decrease in absorbance upon substrate-activated phosphorylation which should be contrasted with the short term increase in absorbance during ADP-activated phosphorylation in mitochondria incubated in State IV (cf. Fig. 11). Microsamples for electron microscopy were fixed consecutively during State II incubations at 1.5 min (arrow II) (Fig. 17), at 15 min (arrow II +) (Fig. 18), and at the termination of phosphorylation (arrow IV) (Fig. 19). Reaction system as in the legend for Fig. 7.

phosphorylation indicates that this membrane influences water efflux and is the one most likely linked to ATP synthesis. Various investigations (44, 51–53) and the recent location and identity of the inner membrane subunit as an ATPase complex (54) support this view.

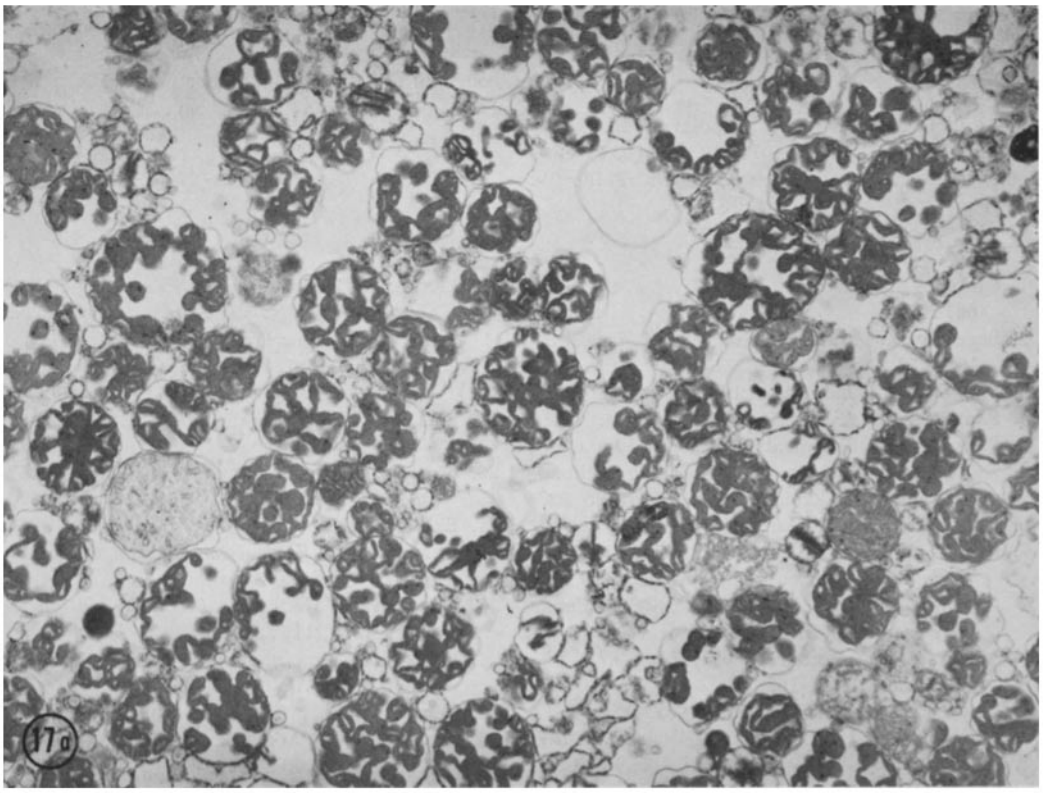
The present results show that the two structural components which clearly undergo organizational or positional changes during the transition from orthodox to condensed mitochondrial ultrastructure are the inner membrane which refolds and the small matrical granules which repack. The change in geometry of these two components during change in functional state represents an ultrastructural basis for metabolically linked mechanical activity in coupled mitochondria. Although the inner membrane is believed to contain "contractile" elements or "mechano-enzymes" which may explain the capacity for membrane refolding (9–12), the properties or function of the small matrical granules are unknown. These granules appear as spheres and filamentous rods which are 60 to 100 A in diameter. When the matrix undergoes a volumetric decrease, as in the State IV to State III transition, these granules repack more closely together and the matrix becomes more electron opaque. As indicated earlier, this increased matrical condensation more than likely contributes to an optical density rise. Matrical condensation possibly comes about during aggregation or polymerization of the small matrical granules. Polymerization of a matrical restricted solute resulting in a decrease in colloidal osmotic pressure has been considered in relation to mitochondrial contraction and extrusion of water (9). In excessively swollen mitochondria the matrical mass appears as a dilute dispersion of small granules and rods (Fig. 6). The residual cristae of these mitochondria possess attached granules of approximately 80 A (Fig. 6 *b*). Although the configurations of the cristae with attached granules are reminiscent of negatively stained preparations, the relationship of these granules, or the small matrical granules in general, to the elementary particle (24, 25) of negatively stained, ruptured mitochondria is presently unknown. Unlike the 40 to 50 A matrical granules described by Burgos et al. (27), the matrical granules described in the present study stain with both lead hydroxide and uranyl acetate.

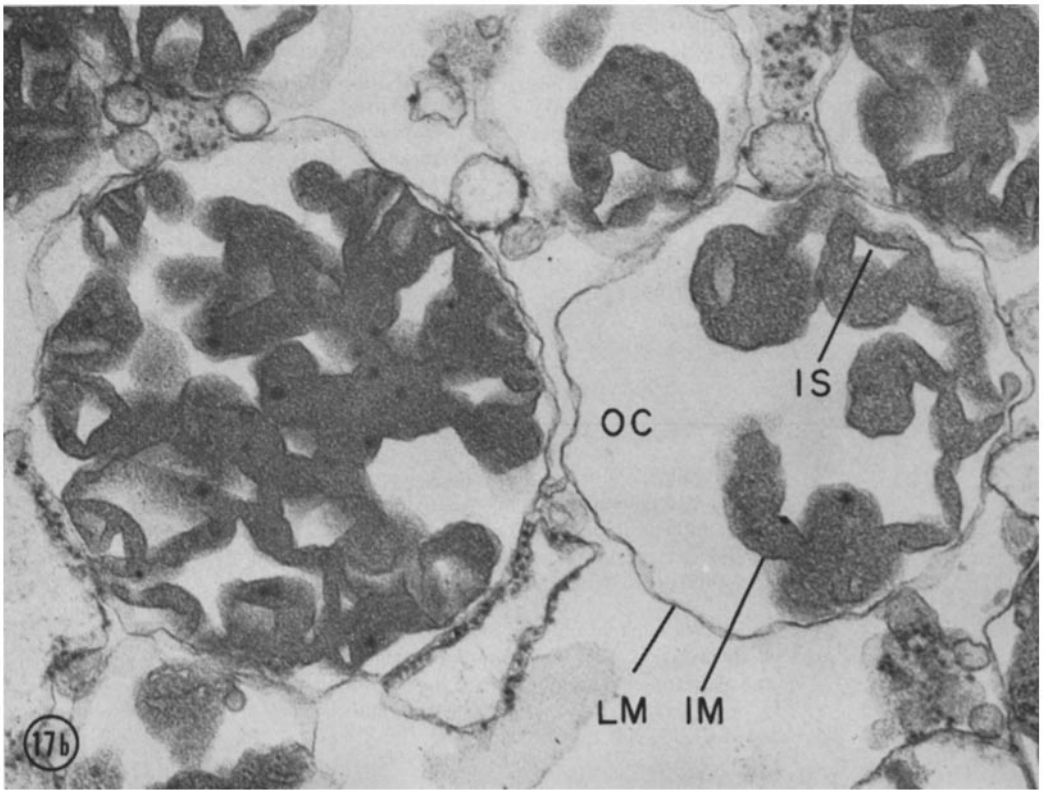
Energy transduction into ATP synthesis and also into mechanochemical activity has been hypothesized by Lehninger to occur via activity of common "mechano-enzyme" coupling intermediates in the respiratory assembly (10, 11, 45, 55). These proposed couplers, traditionally bound in or onto mitochondrial membranes, are thought to be converted into high-energy phosphate intermediates during oxidative phosphorylation as well as during contraction (56, 57). It is tempting to suggest that ADP-phosphorylation and mechanical activity (revealed by inner membrane refolding and matrical condensation) are linked together by common energy-transducing enzymes. Change in the energized state of enzymes in the respiratory assembly may be responsible for the ultrastructural transformations described in this study. In coupled mitochondria, the results clearly show that the oxidative synthesis of ATP, and the mechanical activity of the inner membrane component and matrix are not mutually exclusive: they can occur concomitantly, or at least additively, as observed by ultrastructural changes during the ADP-activated State IV to State III transition. Whether or

FIGURE 17 Mitochondria fixed after 1.5 min' incubation in State II.

FIGURE 17 *a* These mitochondria are characterized by an apparently maximal condensation of membrane-bounded branched matrical substance. × 10,700.

FIGURE 17 *b* A decrease in matrical volume appears to result in an exceedingly large outer compartment (*OC*). Note that, although the limiting membrane (*LM*) does not appear under tension, it maintains a generally spherical geometry and is never observed to be collapsed against the matrix. The condensed conformation displayed by these mitochondria should be contrasted with the condensed conformations of mitochondria incubated for 1.5 min in 0.25 M sucrose (Fig. 3 *b*), in State I (Fig. 8 *b*), and in State IV (Fig. 12 *b*), and also with phosphorylating mitochondria of State III (Fig. 14 *b*). Inner membrane (*IM*), intracristal space (*IS*). × 50,000.




IS
OC
LM
IM

not these ultrastructural changes are *energy-linked* functions of metabolism is the subject of an investigation currently in progress.

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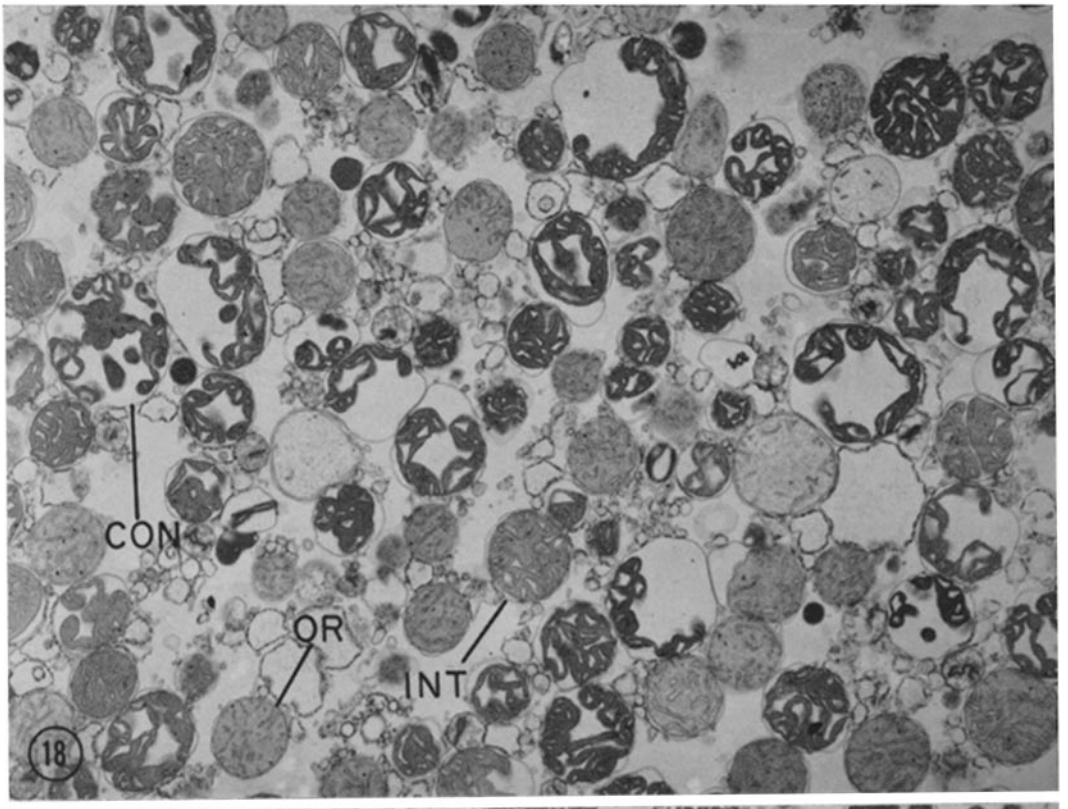
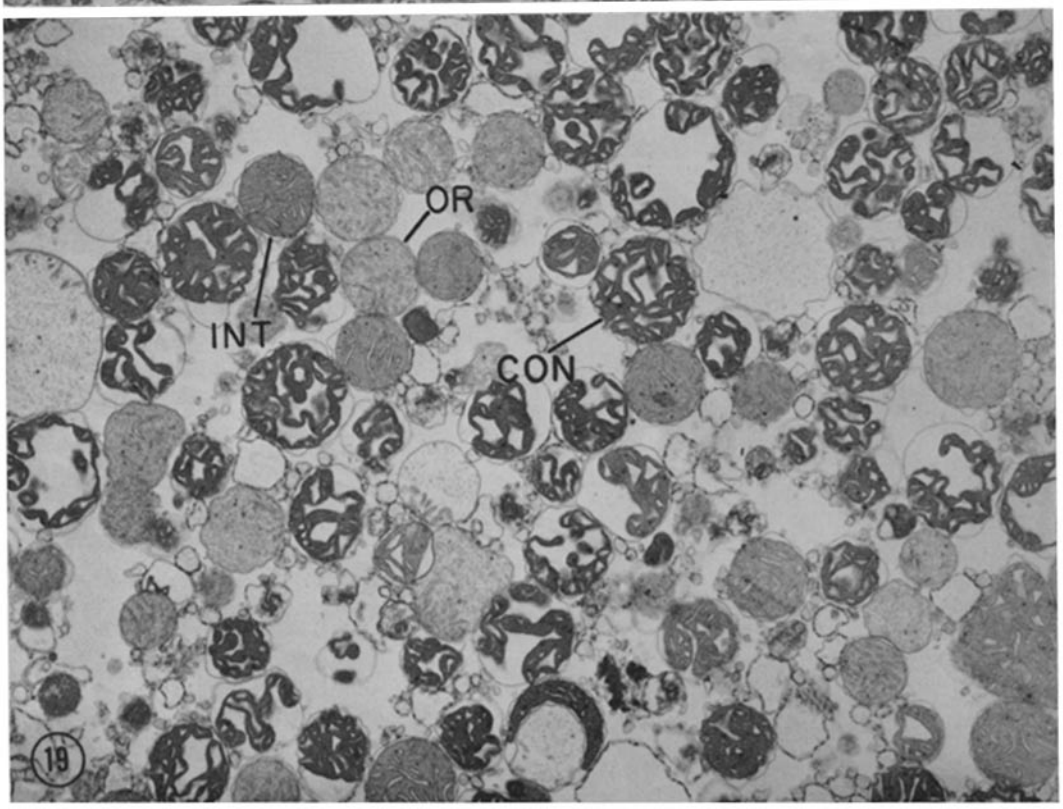
FIGURE 18 Mitochondria incubated for 15 min in State II. The majority of these mitochondria tend to maintain a condensed (*CON*) to an intermediate (*INT*) conformation. Unlike 15-min incubations in State I (Fig. 9 *a*) and in State IV (Fig. 13 *a*), only approximately 10% of the total intact mitochondrial population can be considered to be represented by orthodox (*OR*) conformation. × 10,700.

FIGURE 19 A sample of State II mitochondria incubated for 15 min followed by oxidative phosphorylation. This sample was fixed as phosphorylation ceased. These mitochondria do not display any obvious difference in the proportion of condensed (*CON*), intermediate (*INT*), or orthodox (*OR*) forms present from mitochondria fixed before phosphorylation is initiated (cf. Fig. 18). × 10,700.


CON
OR
INT
18


OR
INT
CON
19

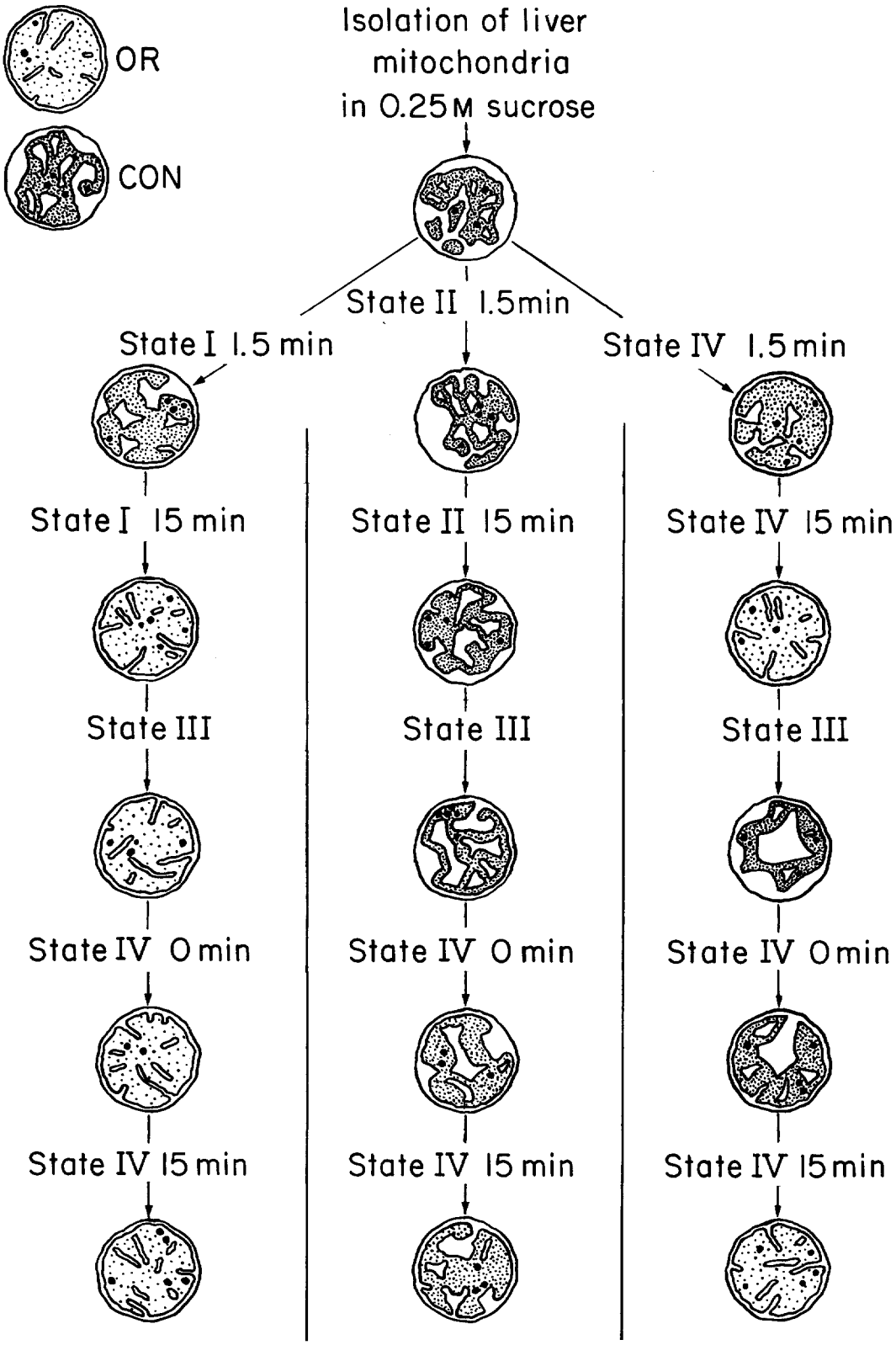


FIGURE 20 Composite of consecutive changes in mitochondrial conformation which take place during consecutive changes in metabolic steady states. Any mitochondrion shown above a particular label undergoes change in conformation to that shown below the label as it enters into or passes time in the metabolic state identified by that particular label. For simplicity, the intermediate conformation has been omitted. Condensed conformation, *CON*; orthodox conformation, *OR*.

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