ULTRASTRUCTURAL BASES FOR METABOLICALLY LINKED MECHANICAL ACTIVITY IN MITOCHONDRIA

II. Electron Transport–Linked Ultrastructural Transformations in Mitochondria

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ABSTRACT

Isolated mitochondria are capable of undergoing dramatic reversible ultrastructural transformations between a *condensed* and an *orthodox* conformation. These two conformations are the extremes in ultrastructural organization between which structually and functionally intact mitochondria transform during reversible respiratory cycles. It has been found that electron transport is required for the condensed-to-orthodox ultrastructural transformation which occurs in mitochondria under State IV conditions, i.e., under conditions in which exogenous substrate is present and ADP is deficient. Inhibition of State IV electron transport at the cyanide-, antimycin A-, or Amytal-sensitive sites in the respiratory chain results in inhibition of this transformation. Resumption of electron transport in initially inhibited mitochondrial systems, initiated by channeling electrons through pathways which bypass the inhibited sites, results in resumption of the ultrastructural transformation. The condensed-to-orthodox transformation is DNP insensitive and, therefore, does not require participation of the coupling enzymes of the energy-transfer pathway. It is concluded that this ultrastructural transformation is manifest by the conversion of the chemical energy of electron transport directly into mechanical work. The reversed ultrastructural transformation, i.e., orthodox-to-condensed, which occurs during ADP-activated State III electron transport, is inhibited by DNP and parallels suppression of acceptor control and oxidative phosphorylation. Mechanochemical ultrastructural transformation as a basis for energy transfer in mitochondria is considered with respect to the results presented.

INTRODUCTION

In the first communication of this series (1), it was reported that isolated mouse liver mitochondria undergo distinct and consistent ultrastructural transformations during respiratory transitions defined as States III and IV by Chance and Williams (2). The extremes in ultrastructural organization between which mitochondria were found to transform during reversible respiratory cycles were identified and designated the *orthodox* and *condensed* conformations. It was suggested that these observations represented the ultrastructural basis for the mechanochemical hypothesis formulated by Lehninger (3).

In an effort to resolve further the nature of

respiratory-related transformations in the ultrastructural organization of mitochondria, an investigation was undertaken to identify the specific enzymatic events of mitochondrial function upon which characteristic transformations are dependent. Of major concern is whether the observed respiratory-related ultrastructural transformations are mechanochemical or osmotic in nature, or both.

In a structural change which is mechanochemical in nature, a direct conversion of chemical into mechanical energy results in a transformation in the supramolecular organization of mitochondria. However, a structural change in mitochondria may also be osmotically induced owing to ion movements energized by electron transport (4, 5). Both of these essentially different types of electron transport–linked structural transformations appear to occur in mitochondria and will be referred to in this report as mechanochemical ultrastructural transformation and osmotic ultrastructural transformation, respectively.

In this communication, results are presented which demonstrate conclusively that the reversible ultrastructural transformation from the condensed-to-orthodox conformation, which occurs in mitochondria under conditions of State IV, occurs only during electron transport. The results show further that this ultrastructural transformation, although linked to electron transport, does not require participation of the coupling enzymes of the energy-transfer pathway of mitochondria, which are thought to be required for ATP[1] synthesis and for ion translocation. It is concluded that this ultrastructural transformation is a mechanochemical change which is manifest by the conversion of the chemical energy of electron transport directly into mechanical or conformational work.

MATERIALS AND METHODS

Mitochondria were isolated from the livers of male Sprague-Dawley rats in 0.25 M sucrose at 0°C, essentially according to the method of Schneider (6). The final concentration of stock mitochondria in 0.25 M sucrose was adjusted to 18 mg of protein/ml. Protein was determined by a biuret method (7).

Clark oxygen electrodes (8) were used to monitor oxygen consumption in both open- and closed-reaction systems. To prevent systems from becoming anaerobic during experiments in which mitochondria maintained high uncoupled rates of respiration or in which State IV respiration was carried out for extended periods, we gradually increased back diffusion into reaction media at specified times by enriching the atmosphere in contact with the media with oxygen. Reaction systems were maintained at a constant stirring speed and at a temperature of 30°C.

Samples of 100 μl (0.09 mg of mitochondrial protein) were removed from reaction systems at specified times for electron microscopy via a quick-sampling micropellet method described earlier (1). All metabolically related ultrastructural results reported in this communication were consistently reproducible, each result being reproduced five or more times with different preparations of mitochondria. All results were reproducible at oxygen tensions below 435 μ-atoms O_2/ml.

The fixative used was 2% osmium tetroxide in a Na-phosphate buffer, adjusted to equal the molarity and pH of the reaction media (buffer molarity, 10 mM; pH 7.0). Sucrose was added to adjust the osmolarity of the fixative to equal the osmolarity of the reaction media (1). Also, fixation, orientation, and sectioning were as reported earlier (1). Thin gray sections, approximately 400 A thick, were stained for 20 min at 40°C in 1.0% sodium borate solution saturated with uranyl acetate, followed by Karnovsky's mixture A lead hydroxide (9) 1/20 dilution for 5 min. Electron micrographs were taken on Kodak 3-¼- × 4-inch electron-image plates at initial magnifications of 9,000 and 40,000, with an RCA 3G electron microscope operated at 50 kv and equipped with an anticontamination cold trap and a double condenser.

Quantitative morphological analysis was carried out according to the methods of Chalkley (10, 11) and others (12, 13).

RESULTS

Controls: Ultrastructure and Acceptor Control Ratios of Rat Liver Mitochondria Isolated in 0.25 M Sucrose

Fig. 1 illustrates the condensed conformation of isolated rat liver mitochondria in 0.25 M sucrose. The inner membrane is randomly folded. The volume of the matrix is conspicuously reduced and the density increased, in comparison to the matrix of mitochondria fixed *in situ*. Consequently, the continuous outer compartment–intracristal space is increased in volume.

There is a direct relationship between the

[1] *Abbreviations used:* ATP = adenosine triphosphate; ADP = adenosine diphosphate; ADP:O = adenosine diphosphate to oxygen ratio (s); DPN = diphosphopyridine nucleotide; FP = flavoprotein; TMPD = tetramethyl-*p*-phenylenediamine; DNP = dinitrophenol.

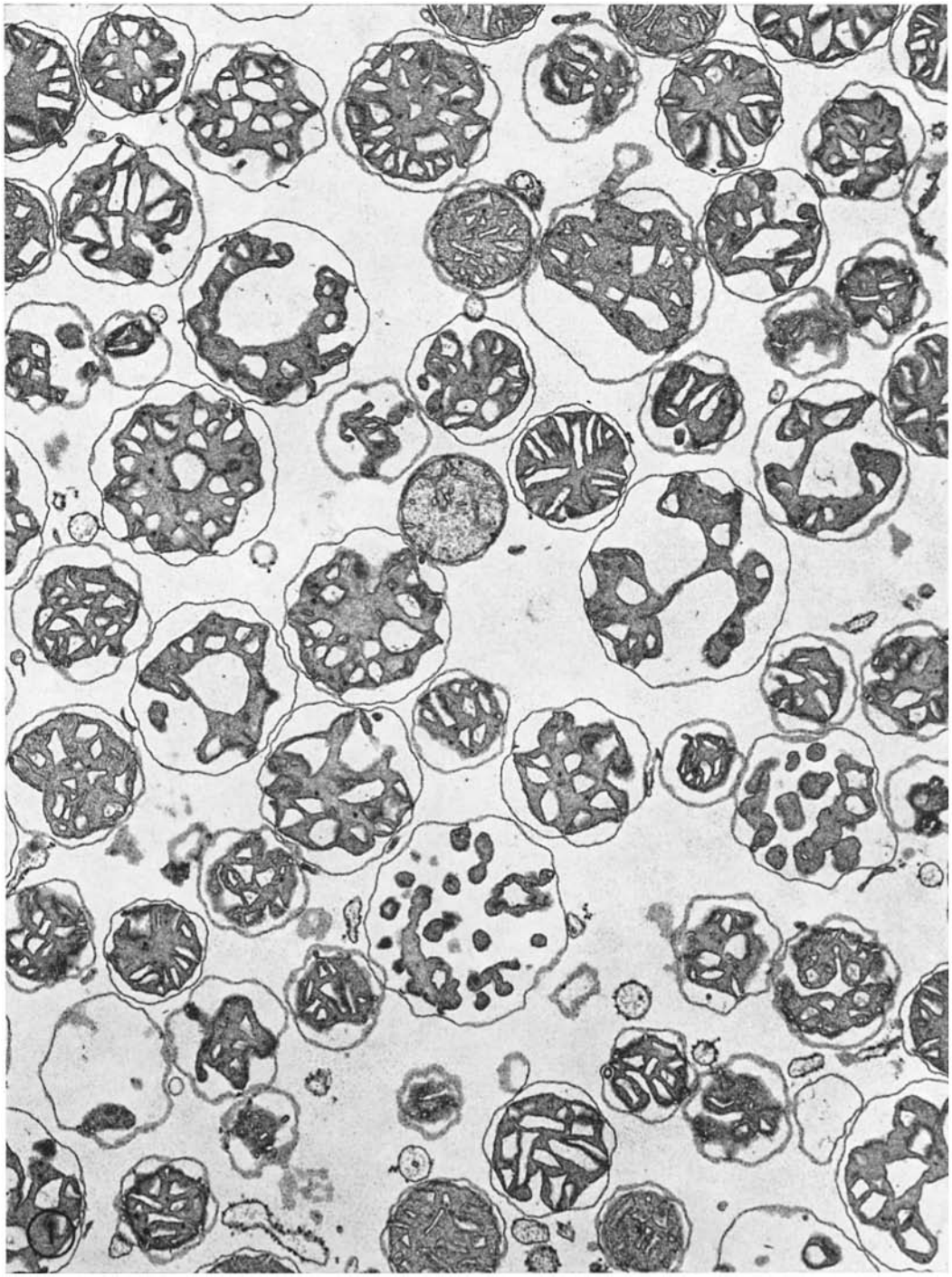

FIGURE 1 Rat liver mitochondria isolated in 0.25 M sucrose. Note the characteristics of the condensed conformation: the inner membrane is irregularly folded with only occasional suggestion of cristae; the matrix shows a decreased volume and an increased density; the outer compartment–intracristal space is volumetrically increased. The condensed conformation is to be compared with the orthodox conformation shown in Fig. 3. × 27,000.

degree of matrical condensation and the initial acceptor control ratio of mitochondria isolated in 0.25 M sucrose. Preparations which show the highest degree of matrical condensation in 0.25 M sucrose also display the highest acceptor control ratios. Fig. 1, showing mitochondria with an extreme degree of condensation, is representative of a preparation which gave an acceptor control ratio of 8.7. All mitochondrial preparations used in the present study gave acceptor control ratios of 6 to 9 and ADP:O ratios of at least 1.9 for succinate and 2.9 for β-hydroxybutyrate after 1.5 min equilibration in State IV. After 20 min of State IV respiration, most preparations gave acceptor control ratios of 4 to 5 and slightly lowered ADP:O ratios, e.g., 1.8 for succinate.

Controls: Ultrastructural Transformations in Rat Liver Mitochondria During State IV Electron Transport and During Activation of Oxidative Phosphorylation

Fig. 2 illustrates a polarographic trace of oxygen disappearance (reduction) during respiration of rat liver mitochondria for about 20 min in State IV followed by ADP-activated oxidative phosphorylation. After a 1.5-min equilibration period in State IV, the mitochondria display condensed and some intermediate conformations; i.e., there is already a noticeable trend in transition toward the orthodox conformation (1). If ADP is added after 1.5 min, State III is initiated and the trend is immediately reversed (1). With longer periods in State IV, the curve shows oxygen to be consumed at a steady rate of 5.8×10^{-4} μatoms/sec, which is characteristic of the low-level electron transport of tightly coupled State IV mitochondria (Fig. 2). With this extended period of State IV respiration, the initially condensed mitochondria continue to undergo the gradual ultrastructural transformation observed after 1.5 min. After 15 or 20 min, the orthodox conformation is reached by all mitochondria (Fig. 3). With the subsequent addition of ADP, the trace shows a fourfold increase in oxygen disappearance as oxidative phosphorylation is initiated, and a majority of the mitochondria undergo a rapid, reversed ultrastructural transformation to a condensed conformation (Fig. 4).

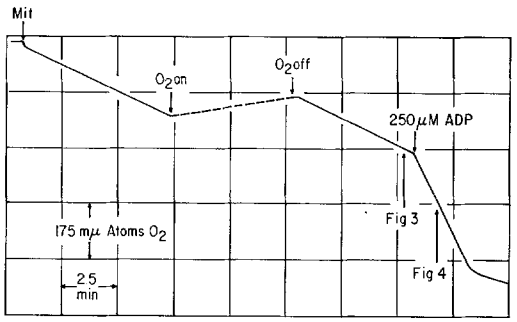

FIGURE 2 Rate of oxygen disappearance during the State IV-to-III-to-IV respiratory transition in rat liver mitochondria. Microsamples were fixed consecutively for electron microscopy after 20 min (Fig. 3) of State IV electron transport, and also after initiating State III phosphorylation (Fig. 4). The State IV reaction system contained sucrose (0.113 M), PO_4 buffer (0.01 M, pH 7.0), $MgCl_2$ (5.0 mM), succinate (10 mM), and mitochondria (1.8 mg protein). Total volume 2.0 ml. Back diffusion was increased during the dashed portion of the curve to prevent the system from becoming anaerobic.

The degree of this condensation is never so great as that found in sucrose control mitochondria (cf. Fig. 1). Also, in rat liver mitochondria, the State IV-to-III reversed ultrastructural transformation occurs in approximately 80% of the State IV orthodox mitochondria, whereas in mouse liver mitochondria, this transformation is consistently found to occur in better than 90% of the population (1). These results occur with both respiratory substrates tried, namely succinate and β-hydroxybutyrate. ATP of various concentrations consistently fails to substitute for ADP in eliciting this transformation.

It is important to reiterate that the reversible ultrastructural transformation described is noticeable after only 1.5 min of State IV electron transport. We employed 20-min periods of State IV electron transport to induce the maximally orthodox conformation in all mitochondria, in order to contrast the dramatic extremes in ultrastructural transformation.

Inhibition of State IV Electron Transport and Ultrastructural Transformation by Cyanide

Fig. 5 illustrates cyanide inhibition of oxygen consumption by mitochondria. Cyanide inhibits electron transport at the level of cytochrome a_3 (14), (Fig. 6). In the presence of 1 mM cyanide, State IV respiration was found to continue at a constant rate of 8×10^{-5} μatoms O_2/sec over a period of 20 min. Electron transport, therefore, is incompletely inhibited by approximately 85% with respect to the control State IV rate of 5.8 ×

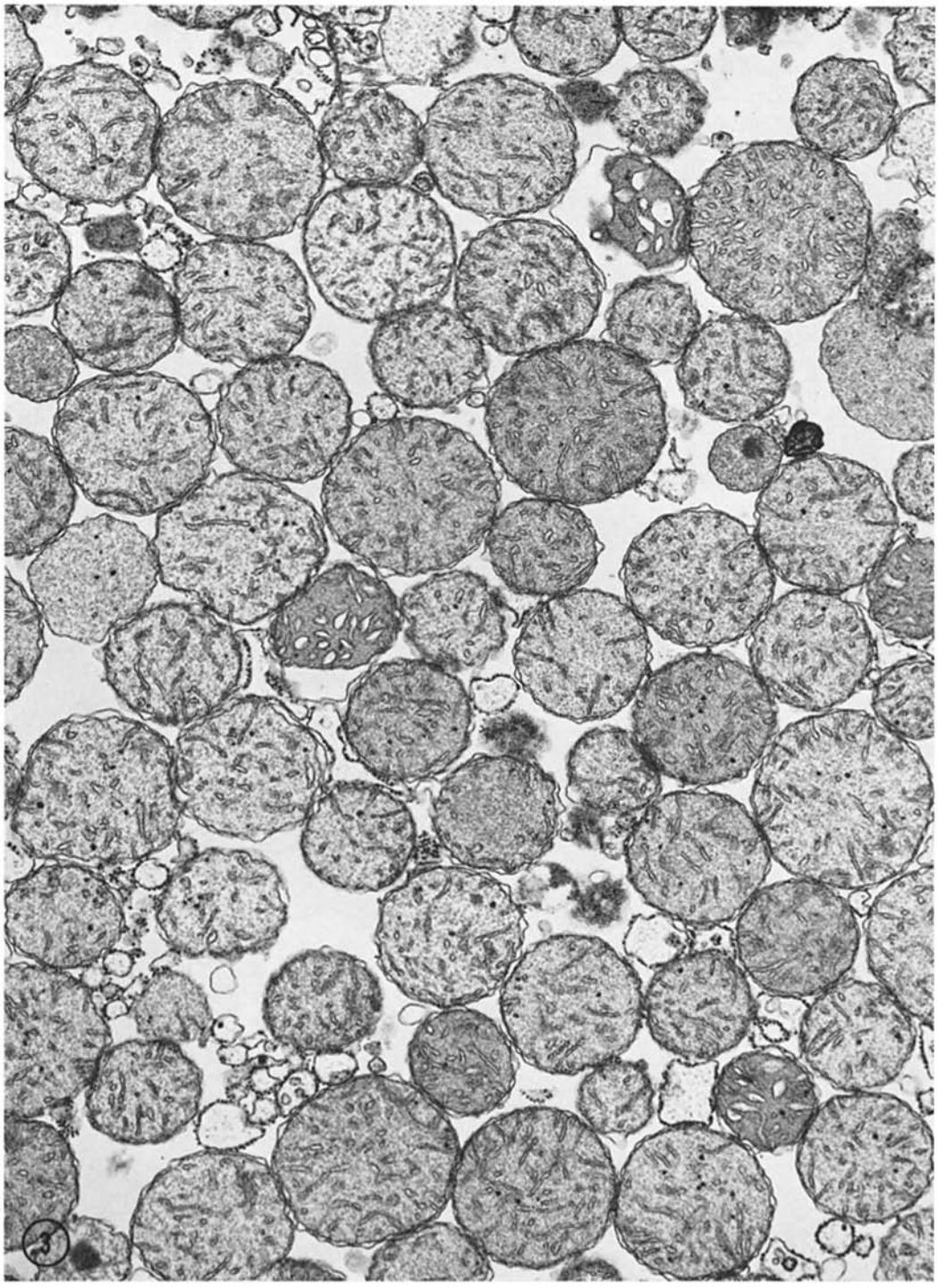

FIGURE 3 Mitochondria fixed after 20 min of State IV electron transport (Fig. 2). An ultrastructural transformation from the condensed to the orthodox conformation has occurred. Note the characteristics of the orthodox conformation: the inner membrane is regularly folded into organized cristae; the matrix shows an increased volume and a decreased density; the outer compartment–intracristal space is volumetrically decreased. The orthodox conformation is to be compared to the condensed conformation shown in Fig. 1. Note that there appears to be no significant difference in total volume between the two conformations. × 27,000.

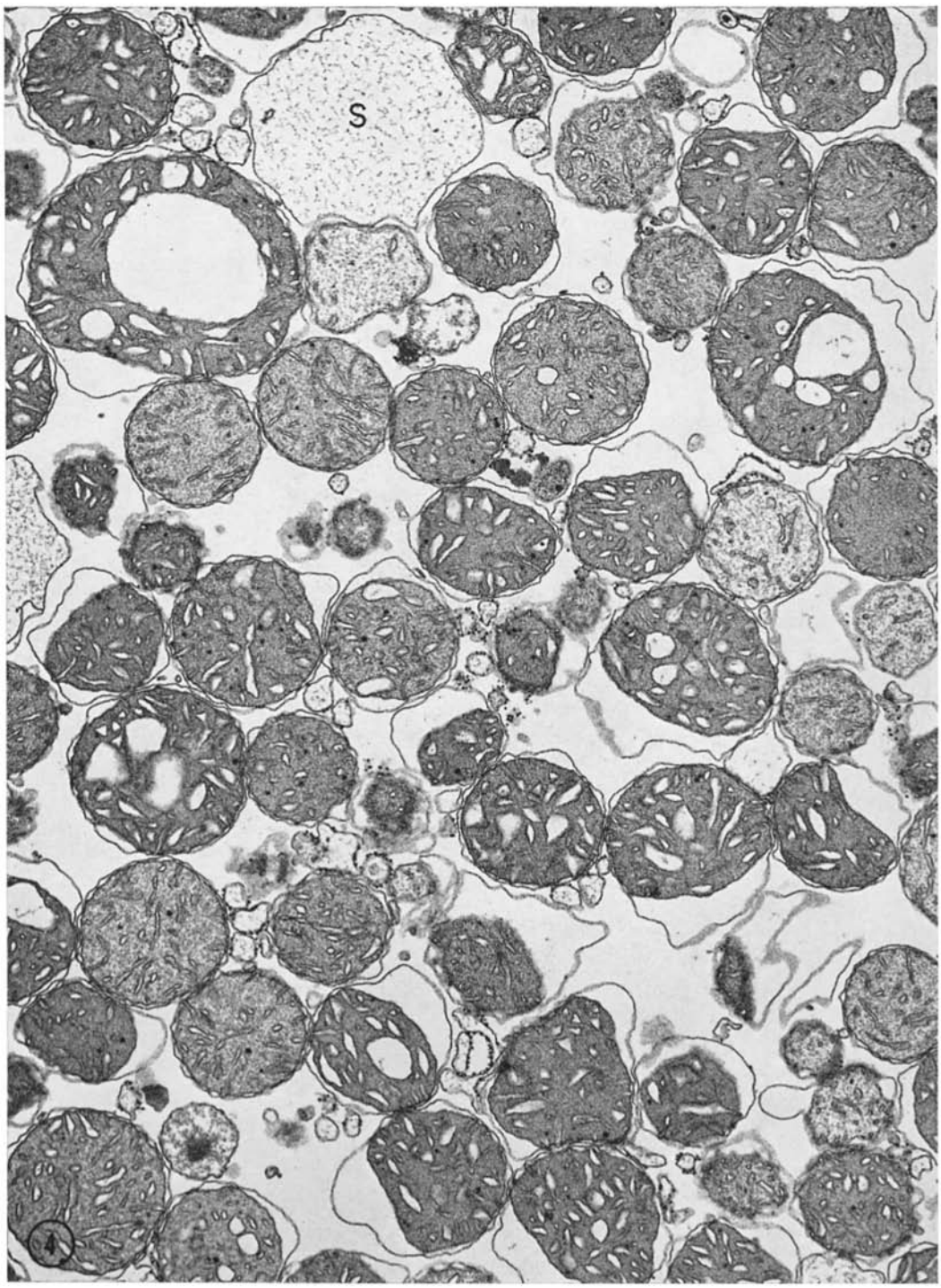

FIGURE 4 Mitochondria fixed during oxidative phosphorylation (State III) (Fig. 2). Note that the transition from State IV to State III respiration occurs concomitantly with a reversed ultrastructural transformation to a condensed conformation in the majority of the mitochondria. An occasional swollen (*S*) mitochondrion is observed. × 27,000.

10^{-4} μatoms/sec. After 1.5 min equilibration under these conditions, mitochondria appear identical in conformation to control State IV mitochondria after 1.5 min equilibration; i.e., they display a condensed conformation, with some of them showing intermediate forms.

After 20 min of partially inhibited respiration by 1 mM cyanide, approximately 75% of the mitochondrial population fail to undergo the ultrastructural transformation to the orthodox conformation which occurs in uninhibited State IV mitochondria (Fig. 7). 1 mM cyanide, therefore, was found not only to partially inhibit State IV electron transport, but also to partially inhibit State IV–related ultrastructural transformation.

It was determined by titration that, when the cyanide concentration is raised above 2 mM, oxygen disappearance becomes minimal (Fig. 5), with approximately 2.9 × 10^{-5} μatoms/sec being consumed, presumably by cyanide-insensitive oxidations, e.g., by direct oxidation of flavoproteins. This rate is 95% inhibited with respect to the control State IV respiratory rate and indicates relatively complete inhibition of respiratory chain–associated electron transfer. Microsamples taken for electron microscopy show that cyanide, above concentrations of 2 mM, also completely inhibits State IV–related ultrastructural transformation to the orthodox conformation (Fig. 8). Identical results occur when either succinate or β-hydroxybutyrate is used as an energy source and when Amytal or antimycin A is included with cyanide in the reaction medium.

It is concluded from these data that cyanide of

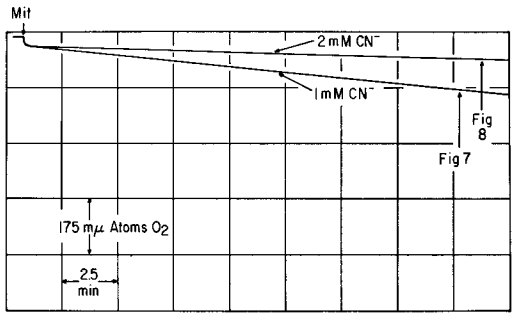

FIGURE 5 Suppression of electron transport in mitochondria by cyanide. Microsamples were fixed for electron microscopy after 20 min (Fig. 7) of partial cyanide inhibition of electron transport, and also after 20 min of complete cyanide inhibition of electron transport (Fig. 8). Reaction medium as in the legend for Fig. 2, plus the addition of cyanide.

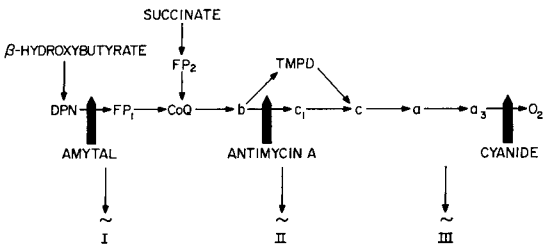

FIGURE 6 The electron-transfer pathways of mitochondria discussed in this study. Note that electron transfer from succinate bypasses Amytal inhibition and also that electron transfer from β-hydroxybutyrate and succinate bypass antimycin A inhibition in the presence of TMPD. The energy-conserving sites (~) are included.

a concentration which maximally inhibits respiratory-chain electron transport over periods of 20 min also completely inhibits the condensed-to-orthodox ultrastructural transformation which has been consistently found to occur during uninhibited State IV respiration.

Inhibition of State IV Electron Transport and Ultrastructural Transformation by Antimycin A and Bypass of Antimycin A Inhibition via TMPD

Fig. 9 illustrates the inhibitory effect of 1 μg of antimycin A/1.8 mg mitochondrial protein on electron transport and also the bypass of this inhibition upon addition of 100 μM TMPD. Antimycin A suppresses electron transport between cytochrome *b* and cytochrome *c* (14) (Fig. 6). Antimycin A–resistant respiration was found to continue throughout 20-min incubations at a constant rate of approximately 8 × 10^{-5} μatoms O_2/sec. As with cyanide, antimycin A–suppressed mitochondria consistently fail to undergo the State IV–related ultrastructural transformation from the condensed to the orthodox conformation during 20-min periods (Fig 10). Identical results occur with succinate or β-hydroxybutyrate as respiratory substrate, and also when Amytal is included in the antimycin A reaction medium. Antimycin A is made up in 95% ethyl alcohol. In control experiments, ethyl alcohol has no inhibitory effect on State IV electron transport or ultrastructural transformation.

In the presence of antimycin A, TMPD acts as an electron-transport carrier shunting respiratory substrate electrons selectively from reduced cyto-

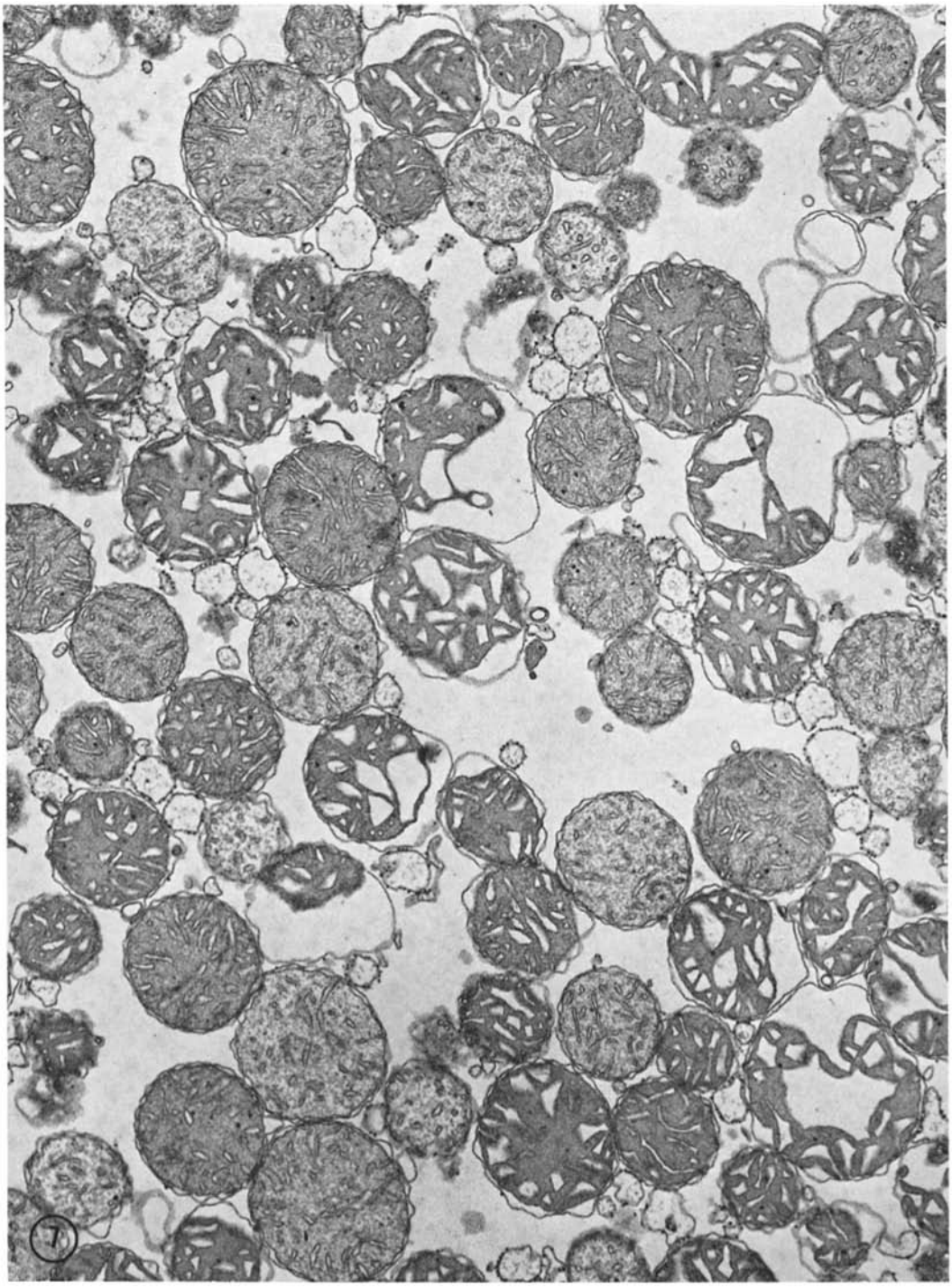

FIGURE 7 Mitochondria fixed after 20 min of partial cyanide inhibition of electron transport (Fig. 5). Note that approximately 75% of the mitochondria fail to undergo the condensed-to-orthodox ultrastructural transformation. Approximately 25% display the orthodox conformation. × 27,000.

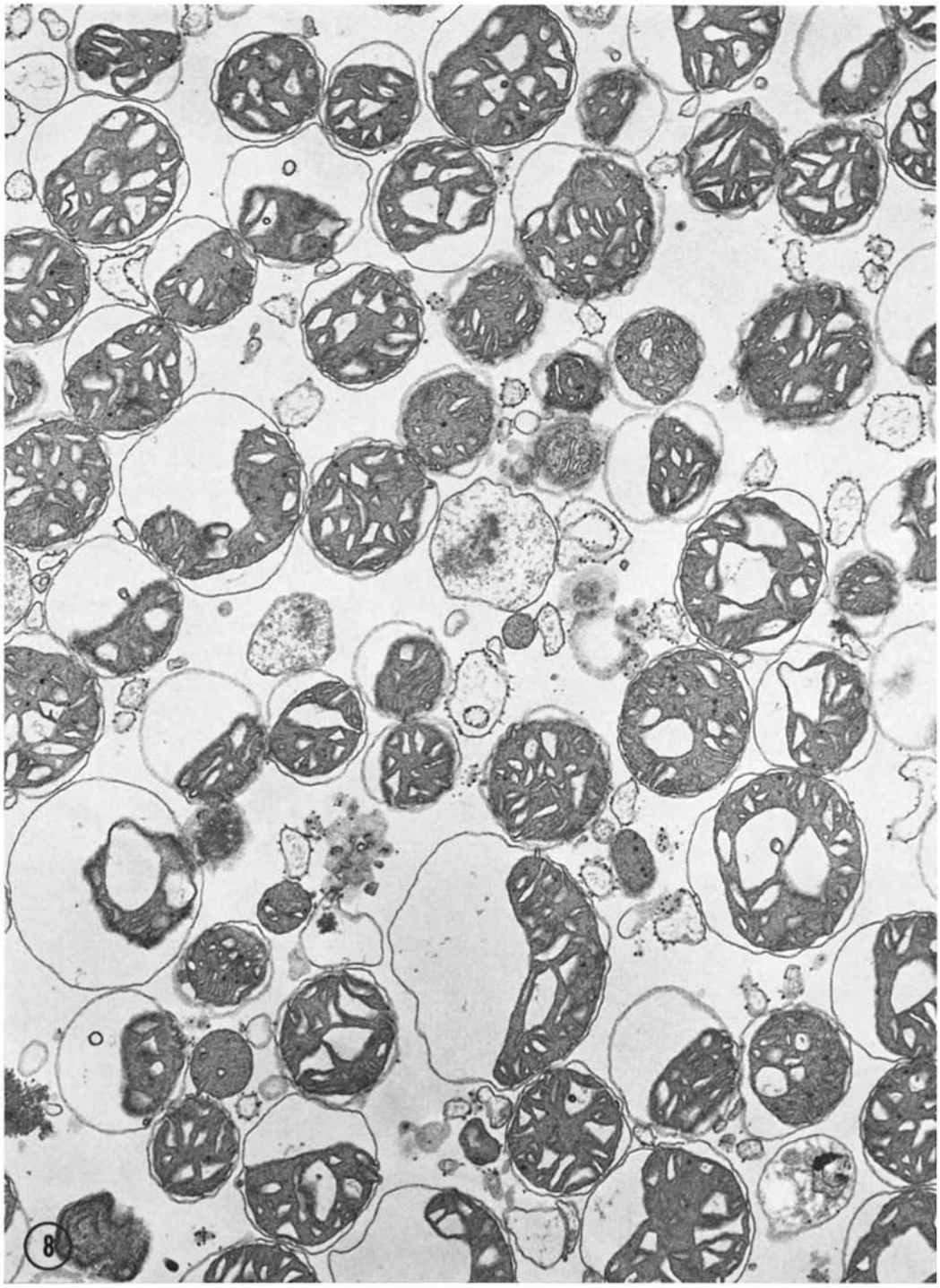

FIGURE 8 Mitochondria fixed after 20 min of complete cyanide inhibition of electron transport (Fig. 5). All mitochondria remain condensed. × 27,000.

chrome *b* to cytochrome *c* (15), thereby allowing for an electron-transport bypass of the antimycin A–sensitive site (Fig. 6). Fig. 9 demonstrates the resumption of State IV electron transport after addition of TMPD to antimycin A–suppressed mitochondria. After 20 min of TMPD-resumed State IV electron transport in mitochondria initially inhibited by antimycin A, all mitochondria undergo the typical State IV–related ultrastructural transformation from the condensed-to-orthodox conformation (Fig. 11). TMPD reverses the effect of antimycin A inhibition on electron transport and ultrastructural transformation, regardless of whether it is added to the antimycin A reaction medium after 1.5-min or 20-min inhibition. As in State IV control experiments, approximately 20 min of TMPD-resumed respiration is required before all mitochondria undergo the condensed-to-orthodox ultrastructural transformation. These results occur consistently with either succinate or β-hydroxybutyrate as an energy source.

The results clearly show that antimycin A suppresses the State IV-related ultrastructural transformation as well as electron transport, and TMPD manifests a resumption of ultrastructural transformation as well as electron transport in the antimycin A–inhibited system.

Inhibition of State IV Electron Transport and Ultrastructural Transformation by Amytal and Bypass of Amytal Inhibition via Succinate Oxidation

Fig. 12 shows a polarographic trace illustrating the suppression of electron transport by 2 mM Amytal in a system in which the exogenous electron donor is β-hydroxybutyrate and also the bypass of this inhibition by addition of 10 mM succinate as electron donor. Amytal suppresses electron transport between DPN and flavoprotein (14), (Fig. 6). In the present investigation, Amytal-insensitive respiration was found to continue at a rate of approximately 8×10^{-5} μatoms O_2/sec which may be accounted for by the oxidation of endogenous succinate.

Fig. 13 shows the ultrastructure of mitochondria after 20 min inhibition by 2 mM Amytal. The majority of mitochondria (>90%) fail to undergo the condensed-to-orthodox ultrastructural transformation characteristic of noninhibited State IV mitochondria.

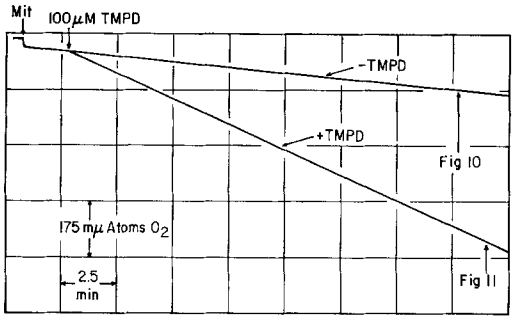

FIGURE 9 Inhibition of electron transport in mitochondria by antimycin A and bypass of antimycin A inhibition with TMPD. Microsamples were fixed for electron microscopy after 20 min of antimycin A inhibition (Fig. 10) and after 20 min of TMPD resumed electron transport in initially inhibited mitochondria (Fig. 11). Reaction medium as in the legend for Fig. 2, plus the addition of 1 μg antimycin A.

When succinate is added to Amytal-inhibited mitochondria, electron flow into the respiratory chain occurs at the level of cytochrome *b*, thereby selectively bypassing the Amytal-sensitive site (Fig. 6). Fig. 12 shows the resumption of State IV respiration by addition of 10 mM succinate. During 20 min of resumed electron transport, all mitochondria undergo the condensed-to-orthodox ultrastructural transformation indicative of State IV mitochondria (Fig. 14).

Amytal then suppresses State IV electron transport through its effect on DPN-linked oxidations and also suppresses the State IV–related ultrastructural transformation from condensed-to-orthodox conformation in mitochondria. Furthermore, the effects of inhibition by Amytal on these metabolic and ultrastructural parameters are reversed in a bypass pathway by addition of an energy source in the form of succinate.

Electron Transport and Ultrastructural Transformation in DNP-Uncoupled Mitochondria

Fig. 15 illustrates the uncoupling effect of DNP on mitochondria. As shown, the reaction system can be kept aerobic by gradually increasing back diffusion of oxygen into the system at specified times. Various concentrations of DNP were used. By titration, it was found that concentrations of DNP greater than 20 μM result in gradually progressive respiratory inhibition after 1 or 2 min of uncoupling. 20 μM DNP added to succinate-

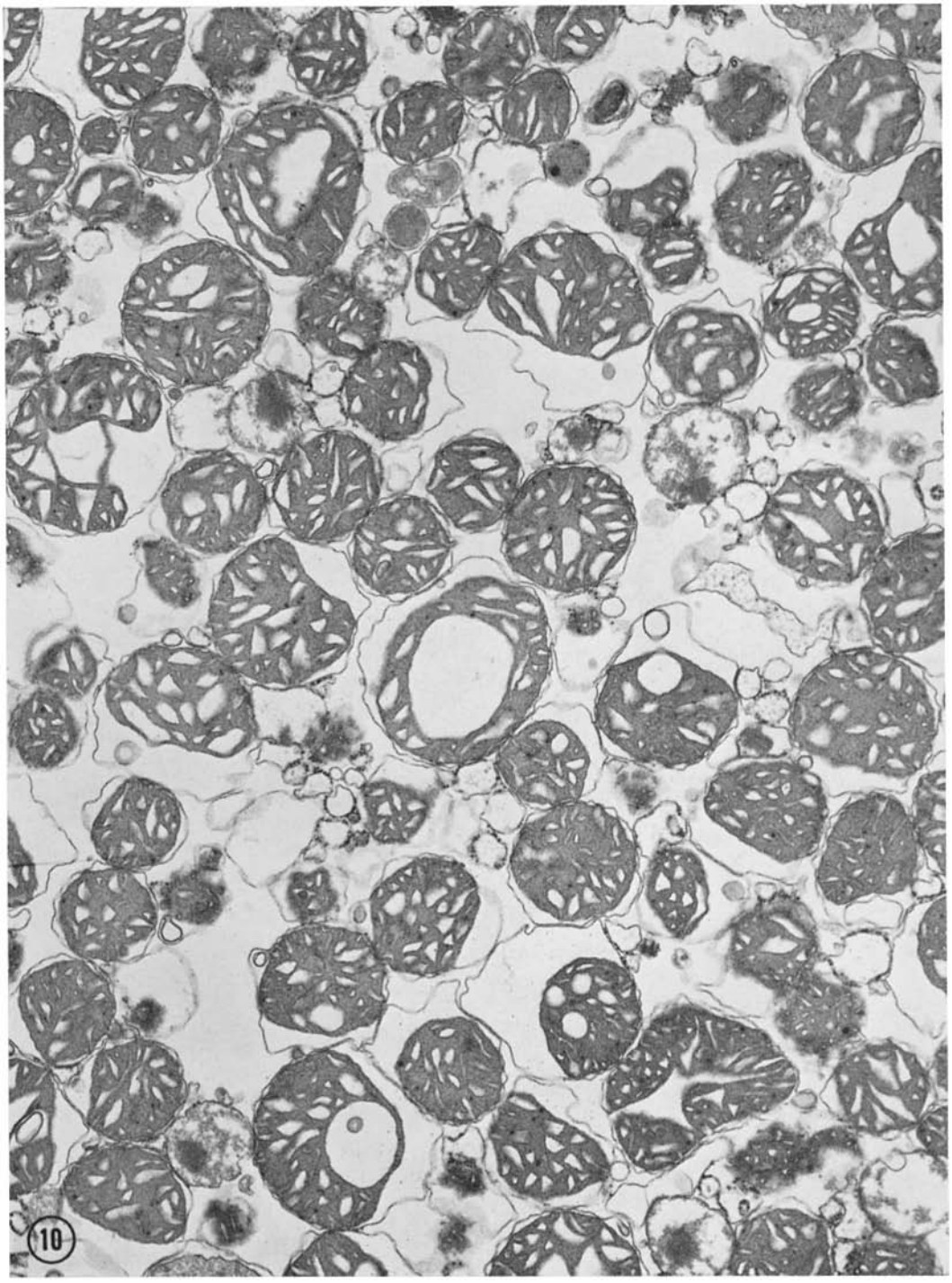

FIGURE 10 Mitochondria fixed after 20 min of antimycin A inhibition of electron transport (Fig. 9). All mitochondria remain condensed. × 27,000.

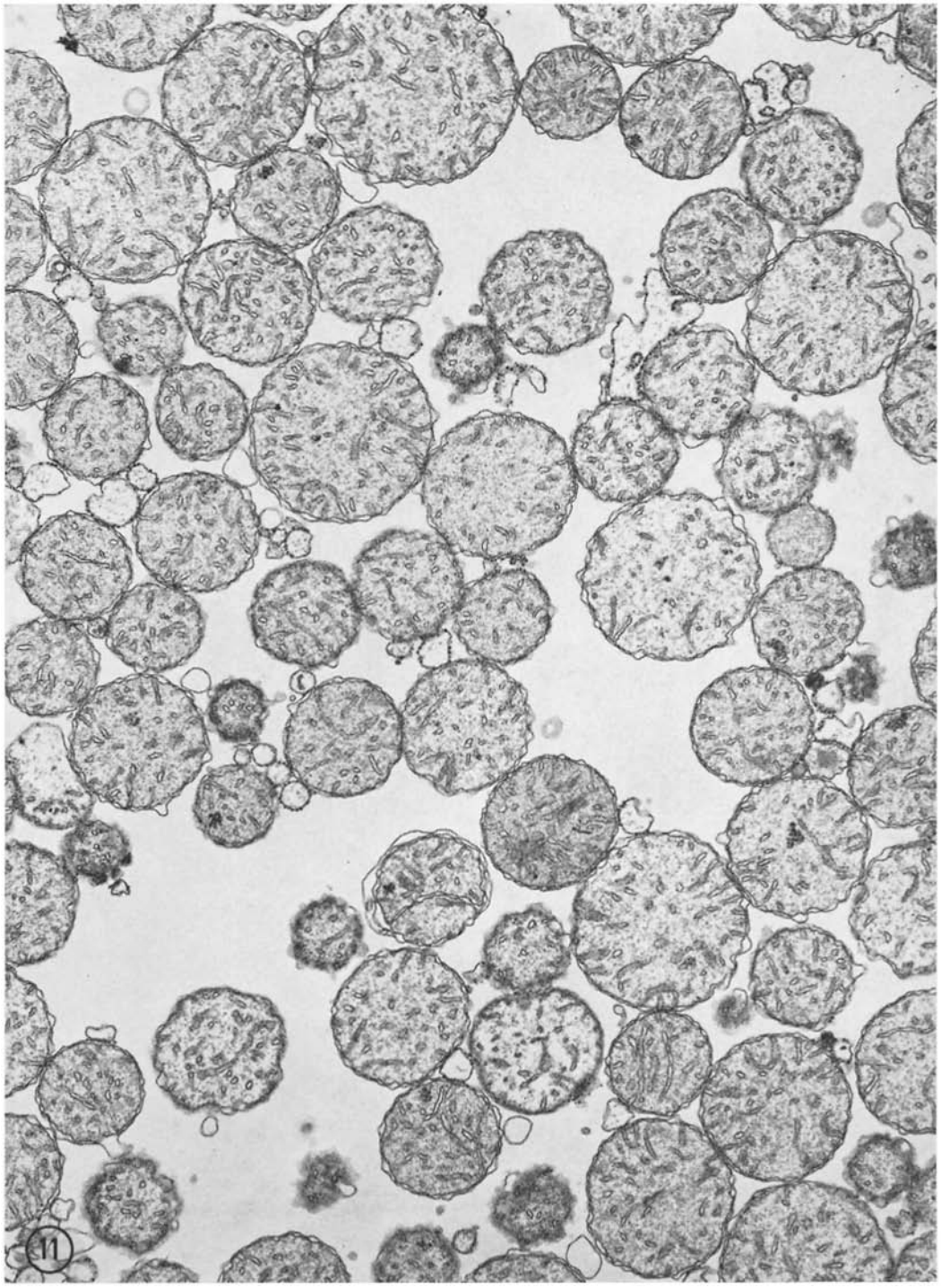

FIGURE 11 Mitochondria fixed after 20 min of TMPD resumed electron transport following initial inhibition by antimycin A (Fig. 9). Note that all mitochondria undergo the condensed-to-orthodox ultrastructural transformation. × 27,000.

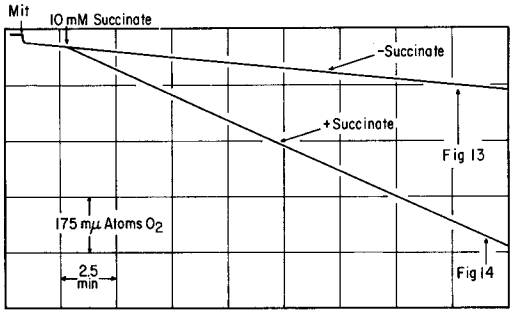

FIGURE 12 Inhibition of electron transport by Amytal in β-hydroxybutyrate-supported mitochondria, and bypass of Amytal inhibition with succinate. Microsamples were fixed for electron microscopy after 20 min of Amytal inhibition (Fig. 13) and after 20 min of succinate resumed electron transport in initially inhibited mitochondria (Fig. 14). Reaction medium as in Fig. 2, but with β-hydroxybutyrate substituted for succinate, plus 2 mM Amytal.

supported medium, although not a maximal uncoupling concentration for maximum ATPase activity (16), initiates and maintains a *constant* uncoupled respiratory rate of approximately 5×10^{-3} μatoms O_2/sec over a period of 20 min. Fig. 15 shows a 12-min trace. 20 μM DNP is close to the maximal uncoupling concentration for maximum respiration (16, 17).

Mitochondria under succinate-supported conditions uncoupled with 20 μM of DNP for 10 min show a gradual ultrastructural transformation from the condensed-to-orthodox conformation characteristic of uninhibited State IV mitochondria (Fig. 16). This finding differed from the State IV–related ultrastructural transformation only in that the transformation occurred at a faster rate, i.e., only 10 min was required for all mitochondria to undergo the transformation. 100 μM DNP, which gives maximal ATPase activity (16), results in a gradually progressive inhibition of electron transport after an initial 1-min burst of uncoupled respiration, and concomitantly results in a more gradual ultrastructural transition from the condensed-to-orthodox conformation which agrees quantitatively as well as qualitatively with the State IV–related transformation.

Mitochondria under succinate-supported conditions were uncoupled with 20 and 100 μM DNP as in the above experiments. After 2 min of uncoupled respiration, 2 mM Amytal and 1 μg of antimycin A/1.8 mg protein were added, inhibiting both reversed and forward electron transport (Fig. 17). Fig. 18 shows that 20 min of inhibition of electron transport in initially uncoupled mitochondria essentially prevents the ultrastructural transition from the condensed to the orthodox conformation which characteristically occurs in both DNP-uncoupled mitochondria and coupled State IV mitochondria. Addition of only antimycin A to the uncoupled system gives identical results.

It should be noted that, unlike the response in coupled State IV mitochondria, the subsequent addition of ADP to uncoupled mitochondria fails to elicit the State III respiratory transition (Fig. 15) as well as the reversed ultrastructural transformation characteristic of oxidative phosphorylation. No immediate ultrastructural transformation due to DNP uncoupling was ever observed during this investigation.

These findings show conclusively that the condensed-to-orthodox ultrastructural transformation in DNP-uncoupled mitochondria is not only dependent on electron transport, but is also directly related to the rate of electron transport, and further, that the transformation can be qualitatively and quantitatively identical to the transformation which takes place in tightly coupled State IV mitochondria.

The Effect of ATP on Ultrastructural Transformation

ATP of various concentrations was found unable to substitute for electron transport in eliciting the condensed-to-orthodox ultrastructural transformation in both coupled and DNP-uncoupled mitochondria.

DISCUSSION

The Condensed Conformation

The condensed conformation (Fig. 19) has two major characteristics which distinguish it from the orthodox conformation (Fig. 20): (*a*) a randomly folded inner membrane with very little suggestion of organized mitochondrial cristae; (*b*) a decreased matrical volume. As a consequence of the volume decrease in the matrical compartment, there is a volume increase in the outer compartment–intracristal space which is presumably a sucrose-accessible space. By quantitative morphological methods (10–13), this space

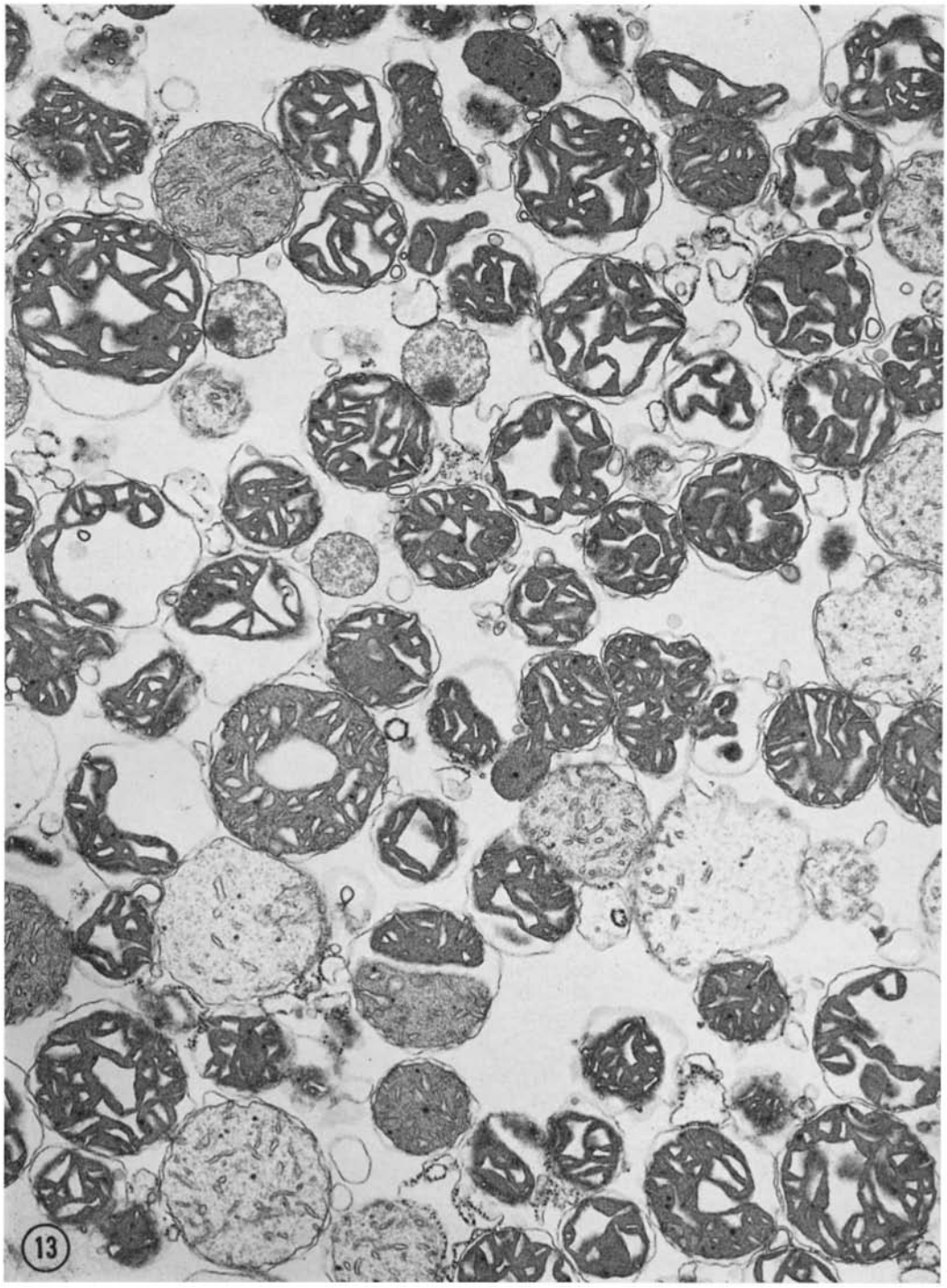

FIGURE 13 Mitochondria fixed after 20 min of Amytal inhibition of β-hydroxybutyrate–supported electron transport (Fig. 12). Note that approximately 90% of the mitochondria fail to undergo the condensed-to-orthodox ultrastructural transformation. × 27,000.

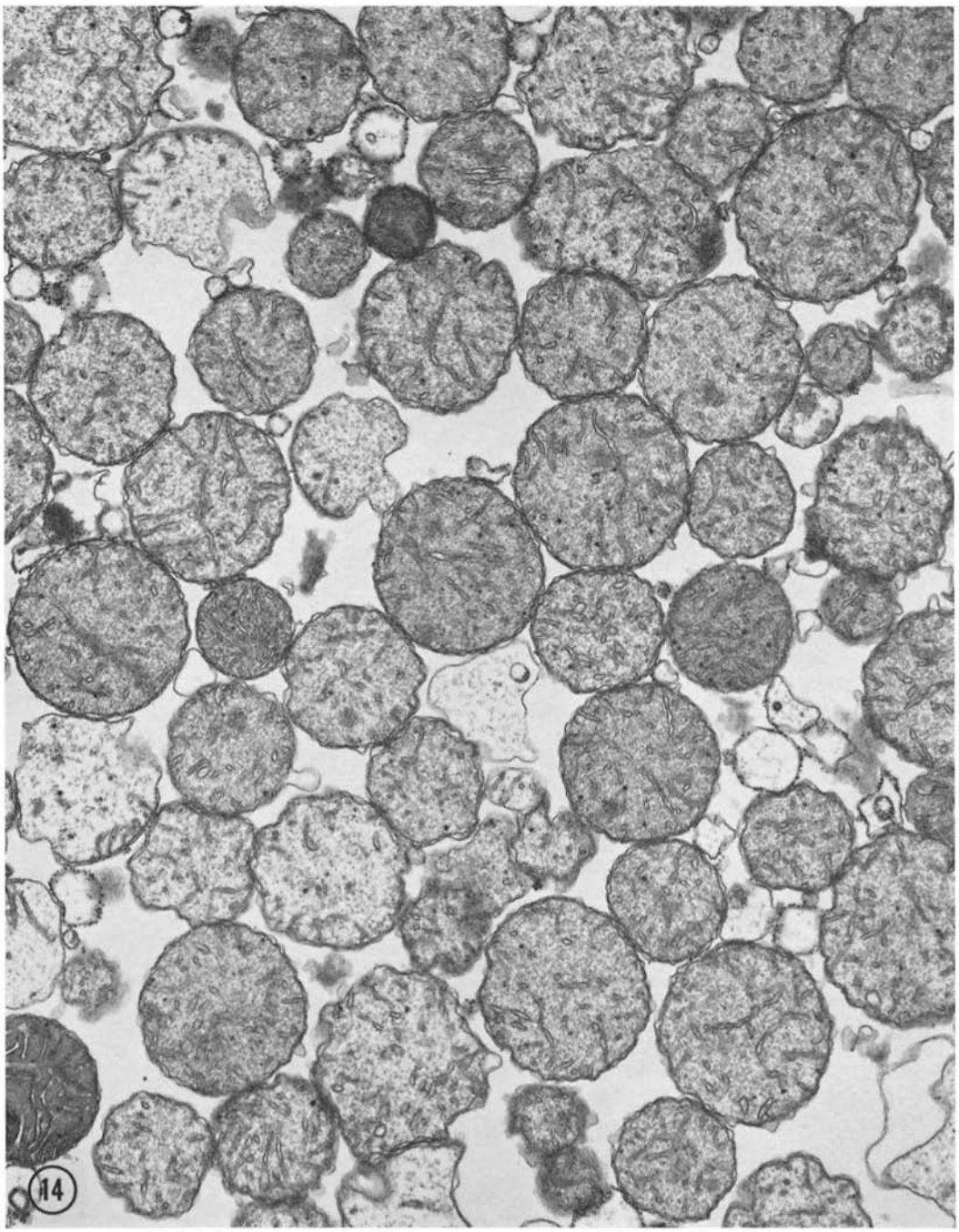

FIGURE 14 Mitochondria fixed after 20 min of succinate resumed electron transport following initial inhibition by Amytal (Fig. 12). Note that all mitochondria undergo the condensed-to-orthodox ultrastructural transformation. × 27,000.

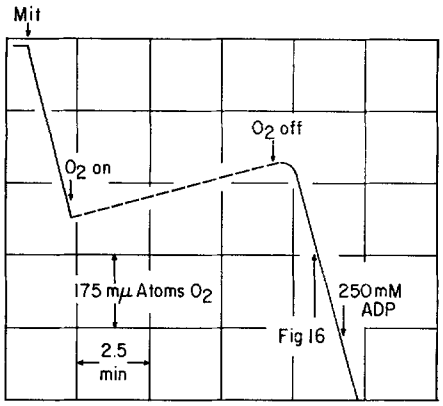

FIGURE 15 Uncoupling in mitochondria by DNP. A microsample was fixed after 10 min of electron transport (Fig. 16). Reaction medium as in the legend for Fig. 2, plus the addition of 20 μM DNP. Back diffusion was increased during the dashed portion of the curve to prevent the system from becoming anaerobic. Addition of ADP shows no change in rate of respiration. Note that 20 μM DNP results in a high and continuous uncoupled rate of respiration.

was found to agree with the suggested "osmotic dead space" of 40–50% (18). Further, the volume increase of the matrical mass required during the condensed-to-orthodox transformation was found to be 100% (Figs. 19 and 20). We shall utilize this finding later in the discussion. The *total* mitochondrial volume does not change significantly during State III-to-IV ultrastructural transformation; therefore, mitochondrial swelling, as such, does not occur under the conditions of these experiments.

Electron Transport–Linked Ultrastructural Transformation in State IV Mitochondria

The results presented in this report show conclusively that ultrastructural transformation from a condensed to an orthodox conformation, which gradually takes place in State IV mitochondria, is an electron transport–linked function. Three major lines of evidence represent the basis for this conclusion: (*a*) The condensed-to-orthodox transformation occurs only during electron transport and can be selectively inhibited at any one of three specific sites in the respiratory chain which are sensitive to the action of specific electron-transport inhibitors. Thus, Amytal, antimycin A, and cyanide, which inhibit electron transfer, concurrently inhibit the condensed-to-orthodox transformation which occurs in uninhibited State IV mitochondria. (*b*) Resumption of electron transport, paralleled with resumption of the condensed-to-orthodox ultrastructural transformation, can be initiated in inhibited mitochondrial systems by selectively bypassing the inhibitor-sensitive sites. Thus, exogenous succinate, which activates resumption of electron transport in Amytal-inhibited mitochondria, and TMPD which activates resumption of electron transport in antimycin A–inhibited mitochondria, concomitantly elicit resumption of the gradual State IV–related ultrastructural transformation. (*c*) The complete inhibition of State IV–related ultrastructural transformation requires complete inhibition of respiratory-chain electron transport. In inhibited mitochondrial systems in which respiratory inhibition is known to be incomplete, inhibition of ultrastructural transformation is also incomplete. Thus, in the presence of Amytal incomplete respiratory inhibition supports limited ultrastructural transformation (Fig. 13). Also, the low-level electron transport which occurs in the presence of 1 mM cyanide supports limited ultrastructural transformation (Fig. 7).

Although the influence of electron transport through specific segments of the respiratory chain on State IV–linked ultrastructural transformation was not systematically examined, it is worth mentioning that the energy-conserving Site I (Fig. 6) is bypassed in the Amytal-succinate reaction system without interfering with the State IV–linked ultrastructural transformation (Fig. 14). Both energy-conserving Sites I and II are bypassed in the antimycin A-TMPD-succinate reaction system without affecting the State IV–linked ultrastructural transformation (Fig. 11). More information is needed to determine whether only the terminal energy-conserving site at cytochrome oxidase is essential, or whether each energy-conserving site contributes to the State IV–linked condensed-to-orthodox ultrastructural transformation.

It is apparent from the results that the electron-transport inhibitors do not spontaneously effect changes in mitochondrial ultrastructure via some nonspecific physical or mechanical alteration of mitochondrial membrane or matrix. The release of inhibition of ultrastructural transformation is readily accomplished by resumption of electron transport via pathways which bypass the inhibited sites. Under no circumstances did any concentra-

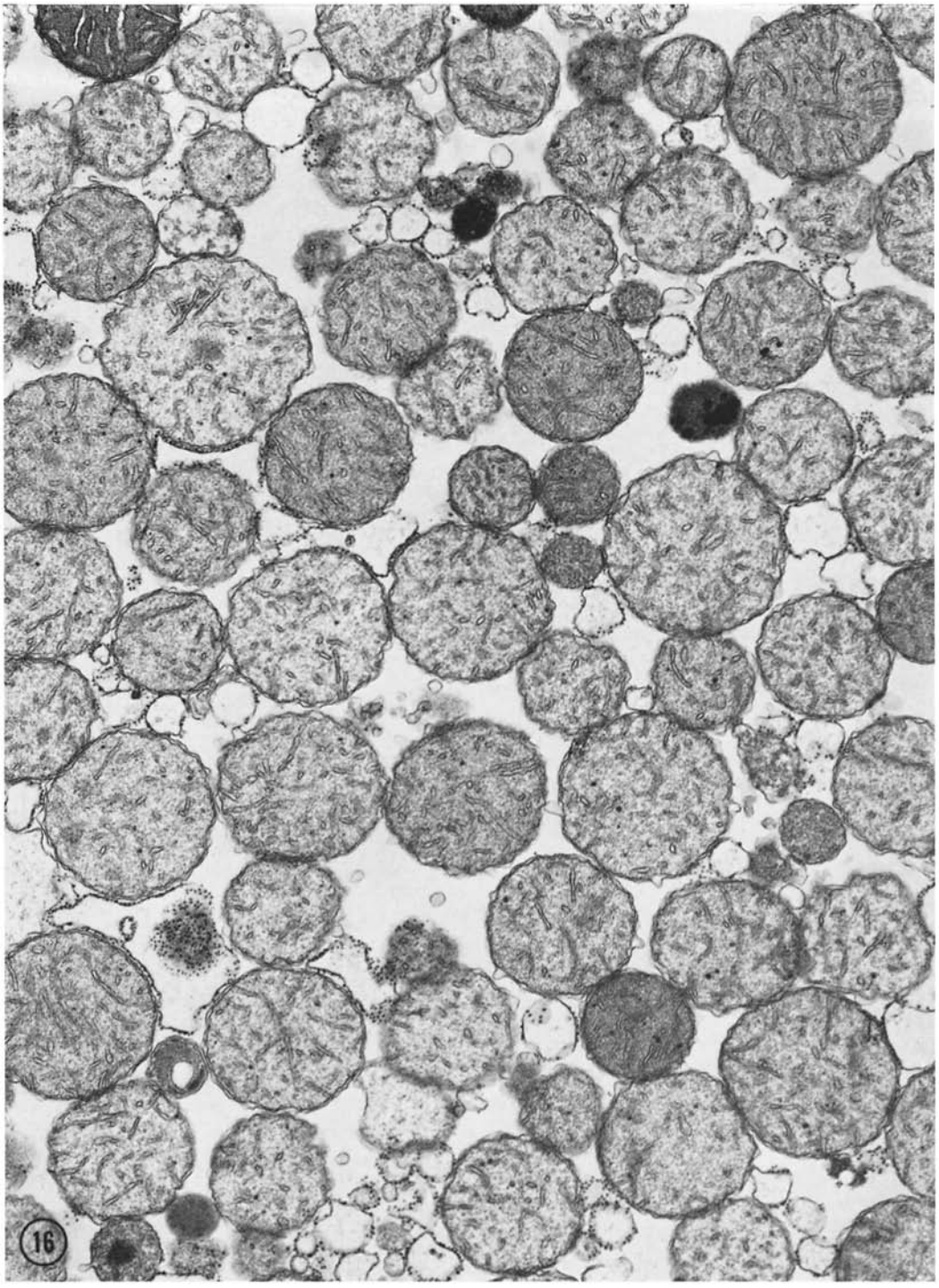

FIGURE 16 Mitochondria fixed after 10 min of DNP uncoupling (Fig. 15). Note that all mitochondria undergo the condensed-to-orthodox ultrastructural transformation. × 27,000.

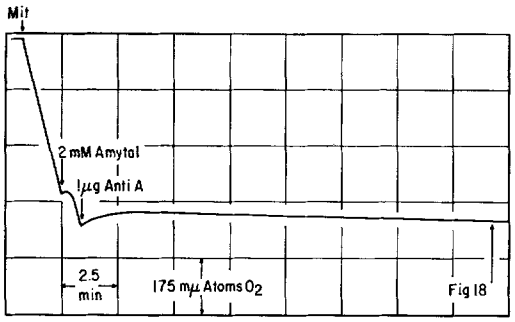

FIGURE 17 Inhibition of electron transport by Amytal and antimycin A in mitochondria initially uncoupled with DNP. A microsample was fixed after 20 min of respiratory inhibition in uncoupled mitochondria (Fig. 18). Reaction medium as in Fig. 2, plus the addition of 20 µM DNP.

tion of any electron-transport inhibitor used in this study result in a spontaneous change in membrane folding or in matrical condensation in mitochondria as does, e.g., ADP (Fig. 4). Rather, inhibitors maintained or stabilized membrane and matrical conformation, preventing a transformation from materializing. It appears that the suppressing effect of respiratory inhibitors on ultrastructural transformation in mitochondria is a consequence of their direct chemical inhibition of electron transport.

Electron Transport–Linked Ultrastructural Transformation in DNP-Uncoupled Mitochondria

Uncoupling of oxidative phosphorylation in mitochondria is experimentally observed as an increase in electron transport without phosphate esterification and is classically thought to occur via a catalyzing of the hydrolysis of a hypothetical high-energy, nonphosphorylated intermediate of the energy-transfer pathway (19–21). On a purely physical basis, uncoupling is a discontinuity in the energy-transfer pathway, and, therefore, has been thought of in broader terms as an abolishment of all known energy-requiring functions of mitochondria. Thus, DNP uncouples oxidation, from phosphorylation (17, 22), from energy-requiring volume changes (23), from ion translocation (24), and from pyridine nucleotide transhydrogenation (25). This major consequence of uncoupling is also implicit in other hypothetical schemes in which DNP uncoupling is thought to be independent of a high-energy intermediate as such (26, 27).

The results presented in this report illustrate that an identical condensed-to-orthodox ultrastructural transformation can occur in both State IV–coupled and DNP-uncoupled mitochondria, and, in both cases, requires electron transport. That electron transport promotes this ultrastructural transformation independent of the DNP-sensitive site in the major energy-transfer pathway in mitochondria, is supported by three definitive findings: (*a*) Provided a high rate of DNP-uncoupled electron transport is maintained, an ultrastructural transformation occurs which is qualitatively identical to, and quantitatively twice as fast as, the condensed-to-orthodox ultrastructural transformation which occurs in coupled State IV mitochondria (Fig. 16). (*b*) Increase in concentrations of DNP cause a gradually increasing inhibition of electron transport. This inhibition is paralleled by a more gradual condensed-to-orthodox ultrastructural transformation which can be quantitatively, as well as qualitatively, identical to the ultrastructural transformation of State IV–coupled mitochondria. It is important to reiterate that when the DNP concentration is increased to a point at which the rate of electron transport is reduced, there is a parallel decrease in the rate of the condensed-to-orthodox transformation. This important finding obviously indicates that it is electron transport which supports the condensed-to-orthodox ultrastructural transformation, rather than a passive permeability change due to some nonspecific effect of DNP on mitochondrial structure. (*c*) Complete inhibition of electron transport by respiratory-chain inhibitors, subsequent to DNP uncoupling, results in essentially complete inhibition of the condensed-to-orthodox ultrastructural transformation (Fig. 18).

Recent findings by Weinbach et al. (28) that uncoupling with 500 µM DNP does not lead to ultrastructural transformation may be due to the high concentration of DNP used, which apparently has a significant suppressing effect on electron transport as described in this report, and as described in earlier studies (29, 30). Complete uncoupling of phosphorylation occurs with approximately 100 µM DNP (16).

It is clear from the results of the systematic uncoupling experiments presented, that the respiratory-linked condensed-to-orthodox ultra-

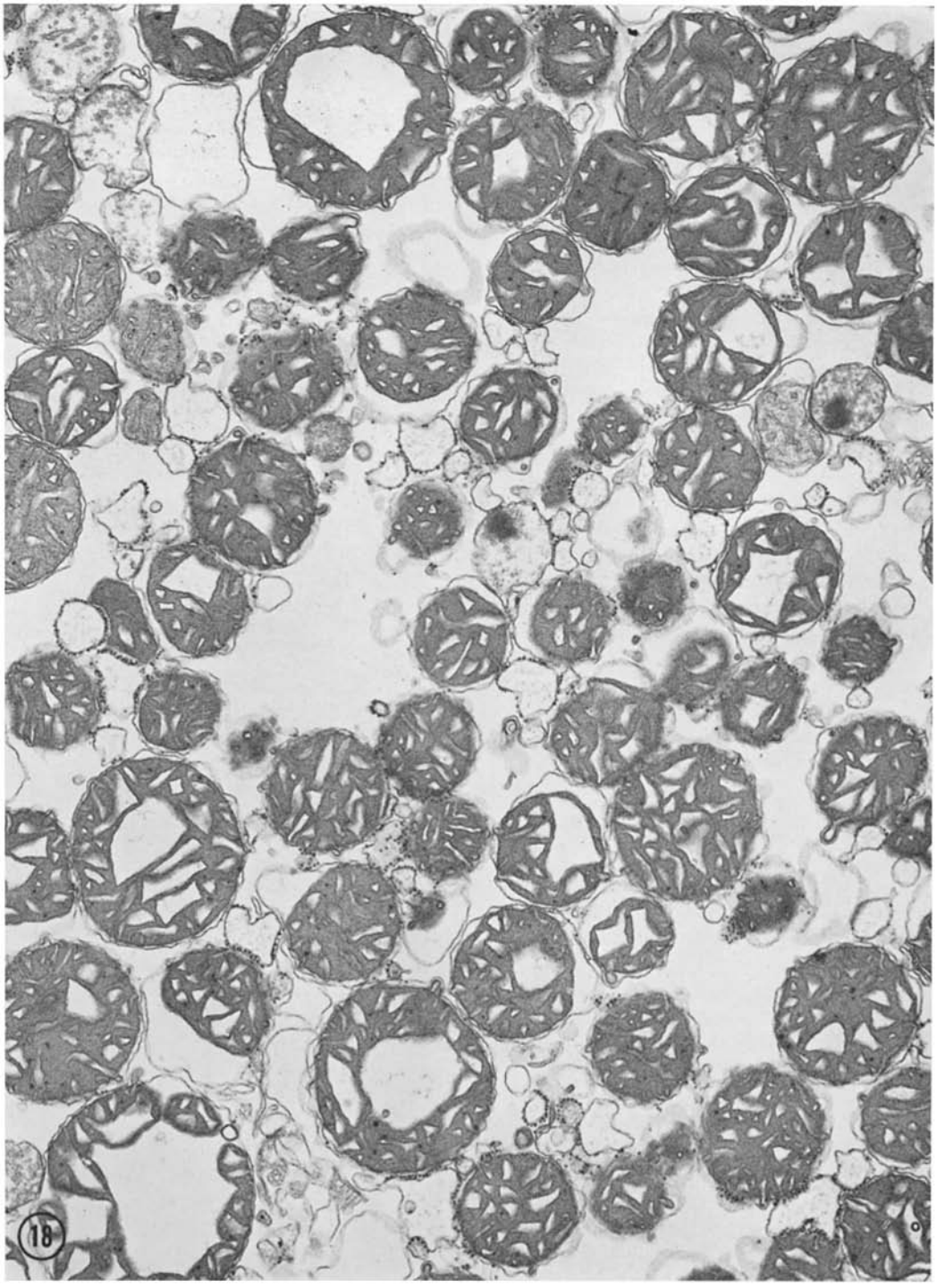

FIGURE 18 Mitochondria fixed after 20 min of Amytal and antimycin A inhibition of electron transport following DNP uncoupling (Fig. 17). Mitochondria remain condensed. × 27,000.

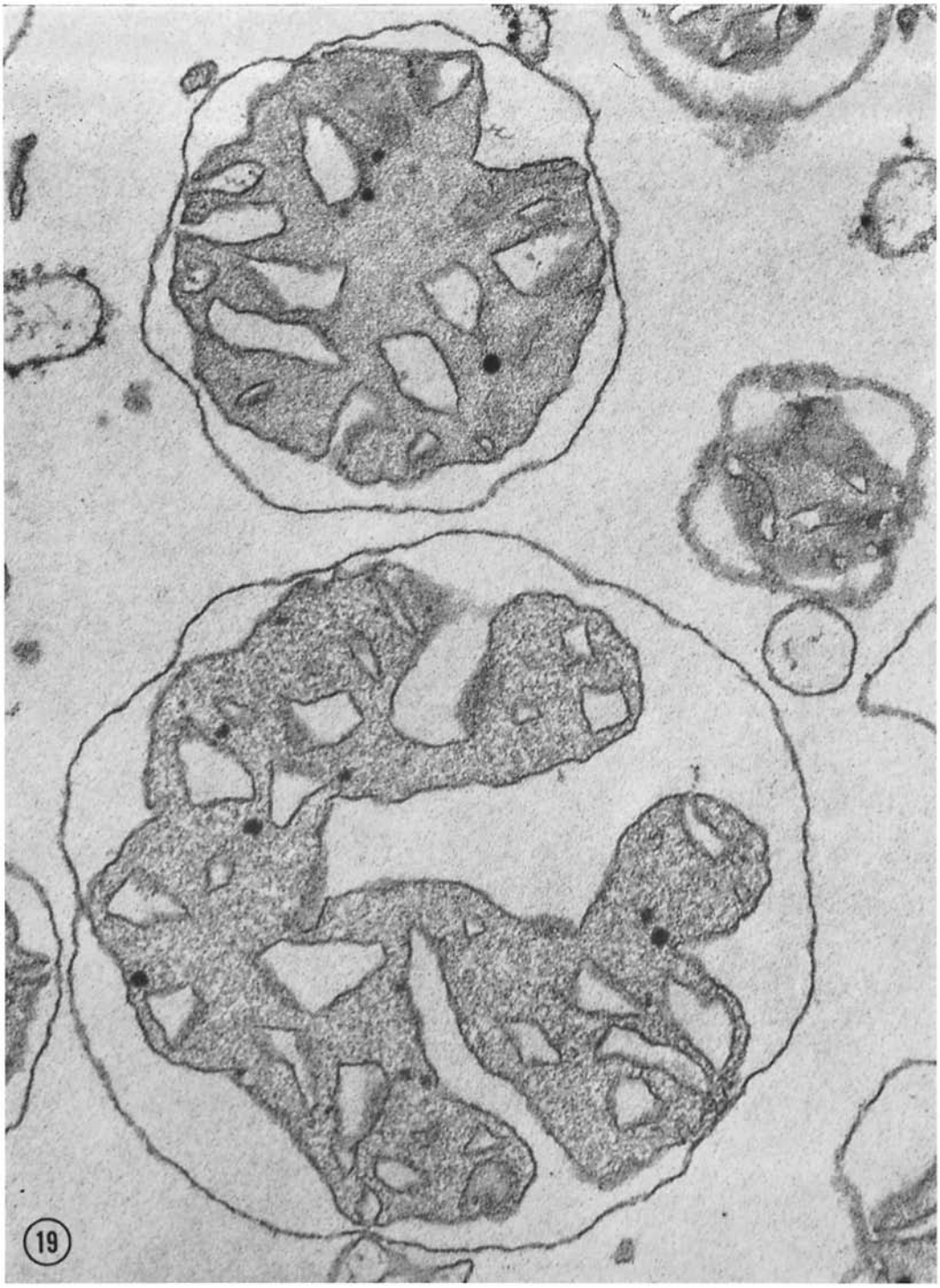

FIGURE 19 The condensed conformation. The spatial folding of the electron-transport membrane is random. The volume of the matrix is approximately 50% of the total mitochondrial volume. × 110,000.

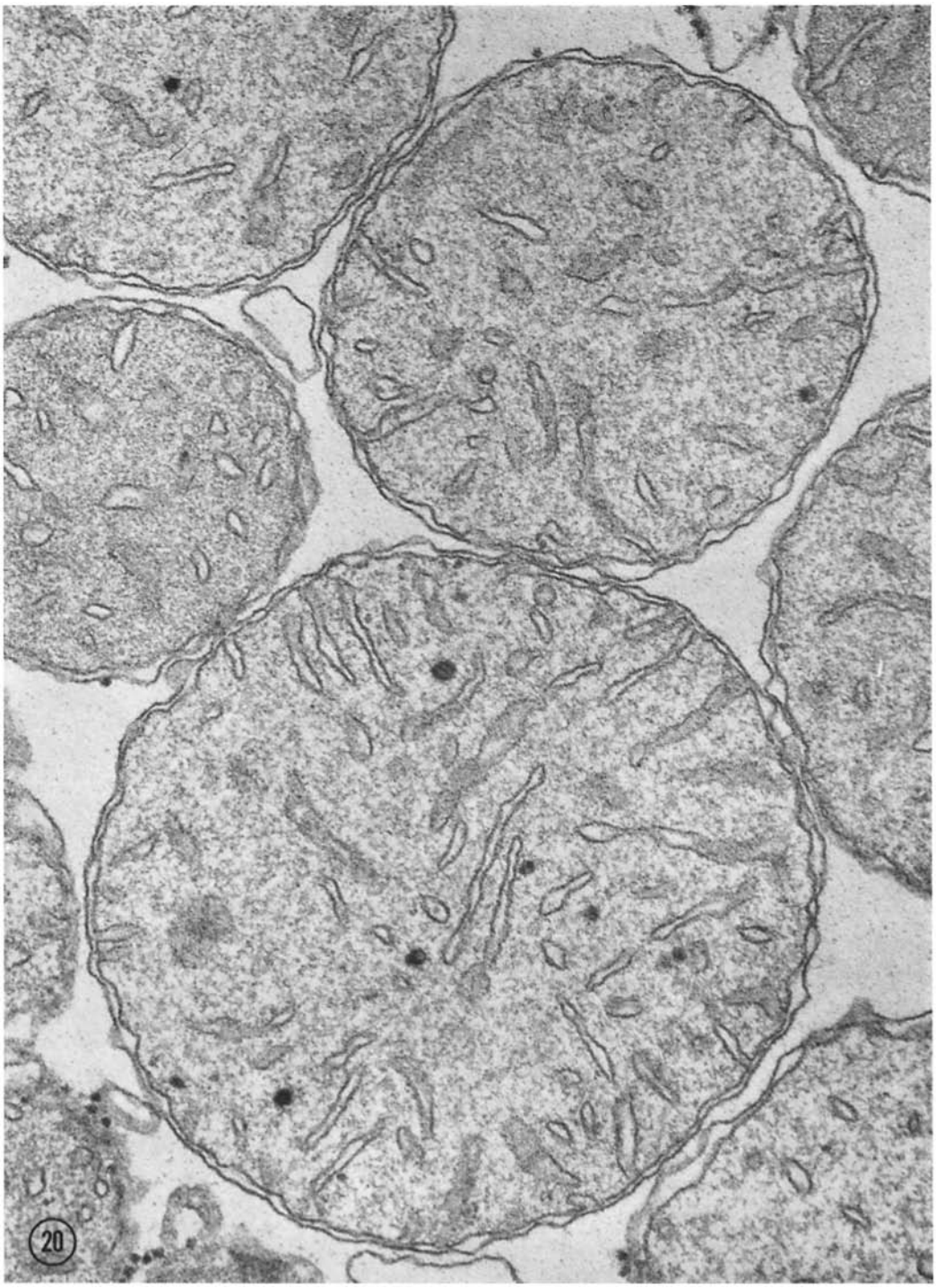

FIGURE 20 The orthodox conformation. The spatial folding of the electron-transport membrane is organized into cristae. The volume of the matrix increases by 100% during the condensed-to-orthodox mechanochemical ultrastructural transformation. (cf. Fig. 19). × 110,000.

structural transformation is unaffected by DNP uncoupling.

The Nature of the Mechanism of Electron Transport–Linked Ultrastructural Transformation in Mitochondria

Respiratory energy-linked functions of mitochondria requiring the generation of a high-energy intermediate can usually be supported by hydrolysis of ATP in the absence of respiratory energy. In the present investigation, ATP was found unable to substitute for respiratory energy in effecting the condensed-to-orthodox ultrastructural transformation. ATPase activity is slightly activated in fresh mitochondria in reaction media containing antimycin A or Amytal (31), but is considerably inhibited when the respiratory chain is reduced with substrate plus cyanide (32). Amytal has a considerable inhibitory effect on DNP-activated ATPase activity, whereas antimycin A displays no effect (31). Regardless of this variable potential of ATPase activity in the presence of different respiratory inhibitors, the condensed-to-orthodox ultrastructural transformation materializes only during electron transport. That a high-energy intermediate of oxidative phosphorylation does not influence the condensed-to-orthodox ultrastructural transformation is suggested by the failure of ATP to substitute, under any conditions, for electron transport in supporting this ultrastructural transformation. Other investigators have suggested that high-energy intermediates of oxidative phosphorylation do not play a role in mitochondrial volume changes (16, 23, 33).

Of significant concentration in our reaction medium are the ions of magnesium, sodium, phosphate, and substrate. If one considers the results of the DNP-uncoupling experiments, it is difficult to implicate osmotic activity of active ion accumulation as the cause of the matrical volume increase and inner membrane refolding which takes place during the condensed-to-orthodox ultrastructural transformation, since it is generally accepted that energized ion accumulations are suppressed in DNP-uncoupled systems (24). Under coupled State IV conditions, Mg^{2+} is not accumulated to any appreciable amount by rat liver mitochondria (34, 35). Mg^{2+} accumulation requires inducement by Ca^{2+} plus phosphate (34), parathyroid hormone (36), or Zn^{2+} ions (37). Na^{+} is like Mg^{2+} in that it, too, requires an "inducer," in this case gramicidin (38), for any significant accumulation. Under State IV conditions, phosphate, the major permeant anion of the system, is taken up in liver mitochondria only as a consequence of active accumulation of certain divalent cations, e.g., Ca^{2+} (24). Pressman and Parks (35) and more recently Carafoli et al. (34) found no appreciable phosphate accumulation by rat liver mitochondria in 20 min under State IV conditions.

It is a well established fact that the endogenous freely mobile phosphate, of mitochondria isolated in 0.25 M sucrose, is equivalent to the endogenous freely mobile K^{+} (39), i.e., about 150 μmoles/g of mitochondrial protein (39–43). In the absence of respiration, these mobile ions are spontaneously released from mitochondria (39) and, therefore, are actively maintained within the matrical compartment essentially as osmotically active ions. As mentioned earlier in the discussion, the calculated volume increase in the matrical mass of condensed mitochondria which is required during ultrastructural transformation to the orthodox conformation is 100% (Figs. 19 and 20). If one considers that the condensed matrical compartment of 1 g of mitochondrial protein already contains the osmotic equivalence of at least 150 μmoles of mobile phosphate and 150 μmoles of mobile K^{+}, it would be difficult to implicate seriously the uptake of phosphate, Mg^{2+}, or Na^{+} in an osmotic induction of a 100% volume increase in the matrical compartment during the State IV–linked condensed-to-orthodox ultrastructural transformation.

Ion exchange–diffusion mechanisms, which may influence the entry of permeant anions in mitochondria (44), require an energy-dependent H^{+}-pump in the inner membrane of mitochondria (26) and, therefore, could not account for an osmotically induced condensed-to-orthodox ultrastructural transformation in the DNP-uncoupling experiments of this study. Under our State IV conditions, Na^{+} would be the most likely ion to exchange for H^{+}. However, it is known that Na^{+} is not accumulated by rat liver mitochondria in 20 min under Mg^{2+} and phosphate-supported State IV conditions (34). It is important to note that in the presence of the antibiotics gramicidin and valinomycin, exchange-diffusion mechanisms for alkali metal/H^{+} may cause what appears to be phosphate-associated osmotic swelling, i.e., decrease in light scattering (44). But even under

these specialized conditions, what appears to be swelling is quantitatively greater than that which would be predicted on the basis of ion uptake alone (40, 45, 46). Active accumulation of potassium salts of divalent anionic respiratory substrates is thought to occur in response to K^+/H^+ exchange; however, the sodium salt of the substrate, as used in the present study, apparently is not actively accumulated (47).

The evidence shows that the conditions used in the present investigation are not favorable for significant ion uptake and that the electron transport–linked ultrastructural transformation which occurs in both coupled State IV and DNP-uncoupled mitochondrial systems is not due to the osmotic activity of energized ion accumulation. However, the volume increase in the matrical mass during this ultrastructural transformation does require water uptake. Water uptake has been shown to occur during electron transport (48). As discussed above, the accumulation of ion required for the osmotic induction of the volume of water which must be moved under conditions of State IV does not occur. Even under conditions favorable for ion accumulation, "swelling" (water uptake) may be several times greater than that predicted from the actual amount of ion accumulated, and it has been accounted for by the contribution of mechanochemical activity (40, 45, 46). The water uptake which occurs during the condensed-to-orthodox ultrastructural transformation described in this report occurs most likely via a passive movement into space in the inner mitochondrial compartment made accessible by an active mechanochemical-induced refolding of the inner membrane system and expansion of the matrical mass.

It is suggested that changes in conformation or orientation of respiratory-chain carriers, which occur during electron transfer, represent the basis for a State IV mechanochemical ultrastructural transformation, as illustrated in this report. Although State IV respiratory-chain carriers remain at steady-state levels with respect to oxidation-reduction, they continue to transfer electrons at a rate of 30–100 times/sec (21, 49, 50). Differences in the oxidation-reduction levels of the carriers, or differences in concentration levels of high-energy intermediates of oxidative phosphorylation, appear to have no effect on ultrastructural transformation. Thus, in the presence of substrate and cyanide, the respiratory chain is reduced and high-energy intermediates are accumulated, whereas in the presence of Amytal and antimycin A, the chain is predominantly oxidized and high-energy intermediates are run down. Yet, in both instances, ultrastructure is maintained in a maximally condensed conformation, apparently because of the lack of electron transport. Mechanochemical ultrastructural transformation is not an instantaneous all-or-none transformation in State IV, but is gradually energized as long as electron transport continues. It is important to realize that this gradual ultrastructural transformation is noticeable after only 20 sec of State IV electron transport and presumably commences with respiration. Electron transport is the rate-determining factor in the kinetics of mechanochemical events which progress gradually in mitochondria during the slow respiration of State IV. This finding is consonant with results in other laboratories which show that State IV "resting" respiration is capable of supporting Ca^{2+} and Sr^{2+} accumulation and also ADP phosphorylation (51).

The conclusion, based on the present results, is that an electron transport–linked mechanochemical ultrastructural transformation occurs in coupled State IV mitochondria. This ultrastructural transformation is DNP-insensitive and, therefore, does not require participation of the coupling enzymes of the energy-transfer pathway. The results indicate that the chemical energy of electron transport is converted directly into mechanical or conformational work. It is clear that an ion-induced osmotic ultrastructural transformation does not occur. This type of ultrastructural transformation has also been systematically studied and will be reported in a forth coming paper.[2]

Mechanochemical Ultrastructural Transformation as a Basis for Energy Transfer in Mitochondria

Loss of acceptor control and phosphorylation efficiency invariably occurs with a concomitant loss in the capacity for reversible ultrastructural transformation (1, 52). There is reason to propose that ultrastructural transformation and energy transfer are integrated in a mechanochemical event in the inner membrane of mitochondia. It is likely that conformational changes in the car-

[2] A. I. Caplan and C. R. Hackenbrock. In preparation.

riers of the inner, electron-transport membrane represent the transduction of chemical into mechanical energy, which is manifest as an energized ultrastructural transformation.

The effect of DNP in the parallel deactivation of State III energy transfer and the related reversed ultrastructural transformation raises the question of the possible influence of conformational transition in ATP synthesis. A point for consideration is whether electron transport–linked mechanochemical ultrastructural transformation as observed in State IV represents a potential source of conformational energy, and, if so, whether this conformational energy is subsequently transduced in support of the phosphorylation reaction during the ADP-induced reversed ultrastructural transformation. This idea is consistent with the recent suggestion by Chance et al. (53) that high-energy intermediates may be identified with conformational states in electron carriers.

The very nature of a system of energy transfer through conformational transition requires an integrated process of reciprocal dependency between ultrastructural transformation and energy metabolism in mitochondria which is consistent with the results reported. In this system, energized conformational transitions in the ultrastructural organization of the electron-transport membrane of mitochondria may participate, in an intermediate capacity, in the coupling of the free energy of electron transport to the phosphorylation reaction.

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