MORPHOLOGY OF ISOLATED RABBIT HEART MUSCLE MITOCHONDRIA AND THE OXIDATION OF EXTRAMITOCHONDRIAL REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE

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ABSTRACT

The morphology of rabbit heart muscle mitochondria isolated in several media has been compared by electron microscopy. The internal structure of isolated mitochondria differs from that of *in situ* mitochondria, with the type and degree of alteration depending on the isolation medium. Examination of the isolated mitochondria after incubation revealed that additional morphological changes occurred during incubation, but these changes were less pronounced when the incubation was conducted in a complete medium containing substrate. The isolated mitochondria have been shown to be capable of catalyzing a slow aerobic oxidation of extramitochondrial reduced diphosphopyridine nucleotide. The rate of DPNH oxidation observed is sufficient to account for the ability of the mitochondria to oxidize lactate in the presence of catalytic amounts of DPNH. The suspensions used were essentially free of mitochondrial fragments, which are known to oxidize DPNH. Possible relationships of these findings to metabolism *in situ* are discussed. The results indicate the desirability of correlating biochemical activities with the morphology of isolated mitochondria.

Previous reports from this laboratory described the complete oxidation of L(+) lactate by rabbit heart muscle mitochondria in a medium supplemented with lactic dehydrogenase[1] and DPN^+ (2), and the conversion of fructose diphosphate to 3-phosphoglyceric acid under aerobic conditions in the presence of mitochondria, DPN^+, and the appropriate glycolytic enzymes (3). Aerobic oxidation of DPNH mediated by the mitochondria was necessary in these systems to regenerate the catalytic amount of added DPN^+. Although other investigators (4) have reported the oxidation of DPNH by heart muscle mitochondria, Linnane and Ziegler (5)[2] have implied that extramitochondrial DPNH is inaccessible to beef heart mitochondria and, therefore, not oxidized unless structural damage to the mitochondria has occurred. Heart muscle mitochondrial fragments have been reported to oxidize DPNH at a rapid rate (7, 8).

Further study on the ability of heart muscle

[1] The following abbreviations are used: DPN^+, diphosphopyridine nucleotide; DPNH, reduced DPN^+; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetate; Tris, tris-(hydroxymethyl)aminomethane; LDH, lactic dehydrogenase; ATPase, adenosine triphosphatase.

[2] See also reference 6.

mitochondria to oxidize cytoplasmic DPNH is needed to provide increased confidence in the use of model systems for research on regulatory mechanisms in cellular metabolism, and to clarify the route by which lactate is utilized for energy production by the intact working heart (9–11). This investigation was undertaken to study the morphology of heart muscle mitochondria before and after isolation, and the morphology of isolated mitochondria before and after measurement of their capacity to oxidize DPNH and lactate.

MATERIALS AND METHODS

Reagents were obtained from the following sources: ATP, ADP, DPN^+, and DPNH from the Pabst Laboratories; sodium pyruvate from the Nutritional Biochemicals Corporation; cytochrome *c* from the Sigma Chemical Company; bovine serum albumin from the Armour Laboratories; and antimycin A from the Wisconsin Alumni Research Foundation, Madison, Wisconsin.

Isolation of Mitochondria

The rabbits (3 to 4 lbs) were stunned by a blow on the head and the hearts were removed immediately and transferred to an ice-cold medium containing EDTA (0.01 M, pH 7.4), either sucrose or KCl, and in most cases bovine serum albumin (1 per cent), as indicated in the legends for the figures. Subsequent operations were conducted at 4° C.

After removing fat and connective tissue, the hearts were diced and passed through a pre-cooled stainless-steel tissue press. The mince was homogenized in 10 volumes of medium in a Potter-Elvehjem homogenizer. Nuclei and debris were removed by centrifugation at 600 *g* for 10 minutes and the supernatant solution centrifuged at 8500 *g* for 10 minutes to recover the mitochondria.

To remove mitochondrial fragments (12) the pellet was resuspended in 10 ml of sucrose or KCl solution containing heparin[3] (0.04 per cent) and Tris (0.025 M, pH 7.7). The suspension was centrifuged at 15,000 *g* for 15 minutes, and the loosely packed light fraction at the top of the pellet was removed by adding small quantities of the medium, tapping the tube gently, and decanting the supernatant solution. This procedure was repeated 2 or 3 times to obtain the washed mitochondrial pellet which was then suspended in the sucrose or KCl solution indicated in the legend for the figure illustrating a specific experiment. All figures of isolated mitochondria represent washed preparations.

Assay Procedures

Oxidation of lactate or pyruvate was measured at 38° C using conventional Warburg manometry. The medium contained L(+) lactate (or pyruvate), 3.3 to 6.7 mM; malate, 0.3 mM; ATP, 1.3 mM; phosphate buffer, pH 7.0, 10 mM; $MgCl_2$, 4 mM; KCl, 116 mM; and bovine serum albumin, 10 mg/ml; and mitochondrial suspension, 0.5 ml. DPN^+, 0.2 to 0.5 mM, and LDH (20 units)[4] were added when lactate was the substrate. The total volume was 3.2 ml, including 0.2 ml 4 N KOH in the center well. The flasks were equilibrated for 8 minutes before the reactions were started. The reaction was terminated with 0.09 ml of 70 per cent $HClO_4$. Mitochondrial nitrogen was determined by a micro-Kjeldahl procedure. ADP was estimated on neutral protein-free solutions by the method of Kornberg and Pricer (14).

DPNH oxidation was measured as follows: DPNH (2 to 4 μmoles) was added to a 50 ml Erlenmeyer flask and the volume made up to 5.5 ml with 0.35 M sucrose, with or without additions, as noted in Figs. 11 and 12. Water (0.5 ml.) was added to one flask to serve as a control for determination of the amount of DPNH added. Mitochondrial suspension (0.5 ml) was added to the remaining flasks, and aliquots were withdrawn immediately and centrifuged at 26,000 *g* for 5 minutes. An appropriate aliquot of the supernatant solution was diluted to 1.0 ml. with water and read at 340 mμ to furnish a zero time value. The flasks were incubated at 38° C in a Dubnoff metabolic shaker and aliquots withdrawn at 10-minute intervals. Aliquots of the supernatant solutions were diluted as above and read immediately at 340 mμ to determine residual DPNH.

Electron Microscopy

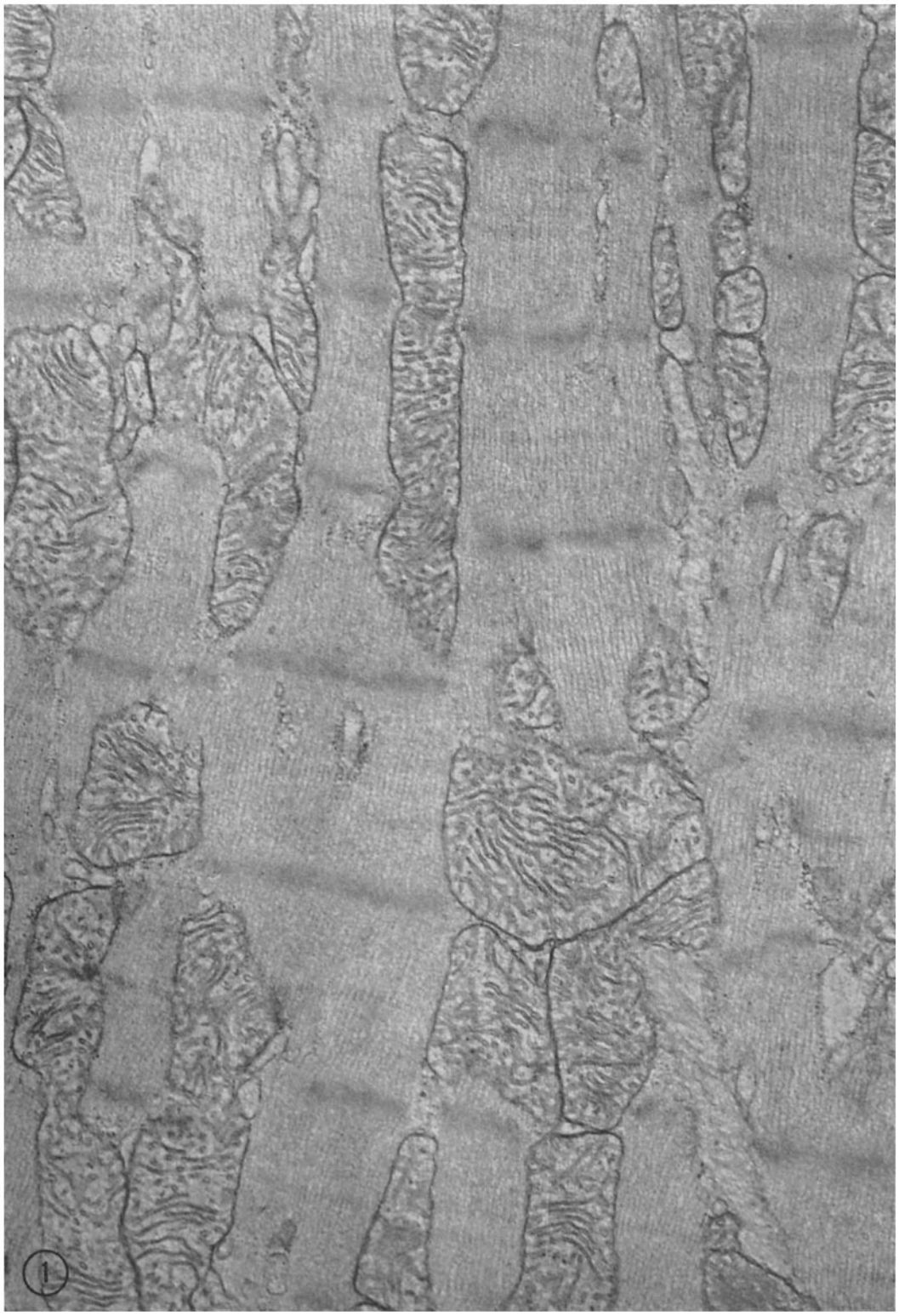
For the study of mitochondria *in situ*, small blocks of heart muscle were fixed for 2 hours at room tem-

[3] Personal communication from Dr. D. M. Ziegler.

[4] All enzyme activities are expressed as International units (13).

FIGURE 1

Section of rabbit heart muscle showing orientation of the mitochondria with respect to the myofibrils. The mitochondria are typically irregular in shape, with randomly arranged cristae. Embedded in Vestopal W. × 22,000.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perature in 1 per cent osmium tetroxide dissolved in Veronal-acetate buffer at pH 7.6 (15), and then dehydrated and embedded in Vestopal W (16) or methacrylate.

Isolated mitochondria were fixed by the addition of osmium tetroxide to the medium in which the mitochondria were suspended at the time the sample was taken for fixation. One volume of medium containing 2 per cent osmium tetroxide was added to an equal volume of mitochondrial suspension. After fixation for 2 hours at room temperature, the preparations were dehydrated in ethanol and embedded in partially prepolymerized methacrylate.

Sections were prepared with a Porter-Blum microtome and observed in an RCA EMU-3D electron microscope. To reduce sampling error, three to five different samples of each preparation of isolated mitochondria were sectioned and observed in the electron microscope.

RESULTS

Fig. 1 shows the variable mitochondrial profiles and irregularly arranged cristae commonly found in sections of rabbit heart muscle. However, an occasional atypical mitochondrion, characterized by relatively few cristae, has also been observed (Figs. 2, 3).

Mitochondria isolated in 0.25 M sucrose are shown in Fig. 4. The characteristic structure evident *in situ* has been altered. The cristae retain their random orientation, with the membranes of the cristae often folding out into the adjacent vacuoles. When the 0.25 M sucrose isolation medium is supplemented with 1 per cent serum albumin, swelling is reduced (Fig. 5), and many of the mitochondria retain the irregular form characteristic of *in situ* mitochondria. In this respect they are similar to mitochondria isolated in 0.88 M sucrose (Fig. 6), a concentration reported (17) to result in fewer morphological alterations. However, the parallel arrangement of cristae found in these two preparations is not found *in situ*.

Sucrose was replaced by KCl because mitochondria are permeable to sucrose (18, 19) and because other investigators (20, 21) have reported sucrose to be detrimental. Mitochondria isolated in KCl, 0.18 M; EDTA, 0.01 M; and serum albumin, 1 per cent; and resuspended in KCl, 0.18 M, are shown in Fig. 7. These mitochondria differ in appearance from those isolated in sucrose (Figs. 4 and 5).

The effect of adding 1 ml of the KCl suspension of mitochondria (shown in Fig. 7) to 5 ml of 0.35 M sucrose and resedimenting the mitochondria is illustrated in Fig. 8. A change to the form characteristic of mitochondria isolated in 0.25 M sucrose is evident. This structure was not altered after 20 minutes at 38° C.

DPNH as Substrate

Aliquots of the original mitochondrial suspension (Fig. 7) were incubated in various media for 20 minutes at 38°C and the mitochondria then examined in the electron microscope.

When the mitochondria are incubated in sucrose, 0.35 M; P_i, 3.3 mM, pH 7.0; and DPNH, 0.7 mM (Fig. 9), most of the mitochondria show pronounced swelling, loss of matrix, and disruption of the cristae. The presence of the small, relatively dense mitochondria in this preparation probably can be attributed to the plane of sectioning and to preferential swelling of certain areas of a mitochondrion. Loss of intramitochondrial DPN^+ and a change in structure on incubation in solutions containing P_i has been reported by Hunter *et al* (22). When incubated in the complete medium containing L(+) lactate (Fig. 10), as used in the Warburg experiments (see *Assay Procedures*), the degree of vacuolation, continuity of cristae, and retention of matrix appear to be superior to that of the original preparation (Fig. 7).

The rate of DPNH oxidation generally ranges between 4 to 9 μmoles per mg mitochondrial N per hour, and does not increase over a 30-minute period (Fig. 11). The rate is greatly increased by cytochrome *c*, while P_i and acetoacetate have negligible effects. In view of the marked structural changes induced during incubation with P_i in the

FIGURES 2 AND 3

Sections of rabbit heart muscle. Atypical mitochondria containing relatively few cristae and, especially in methacrylate-embedded material, less dense matrix are occasionally found closely associated with mitochondria of normal appearance. Fig. 2: embedded in methacrylate, × 35,000. Fig. 3: embedded in Vestopal W. × 45,000.

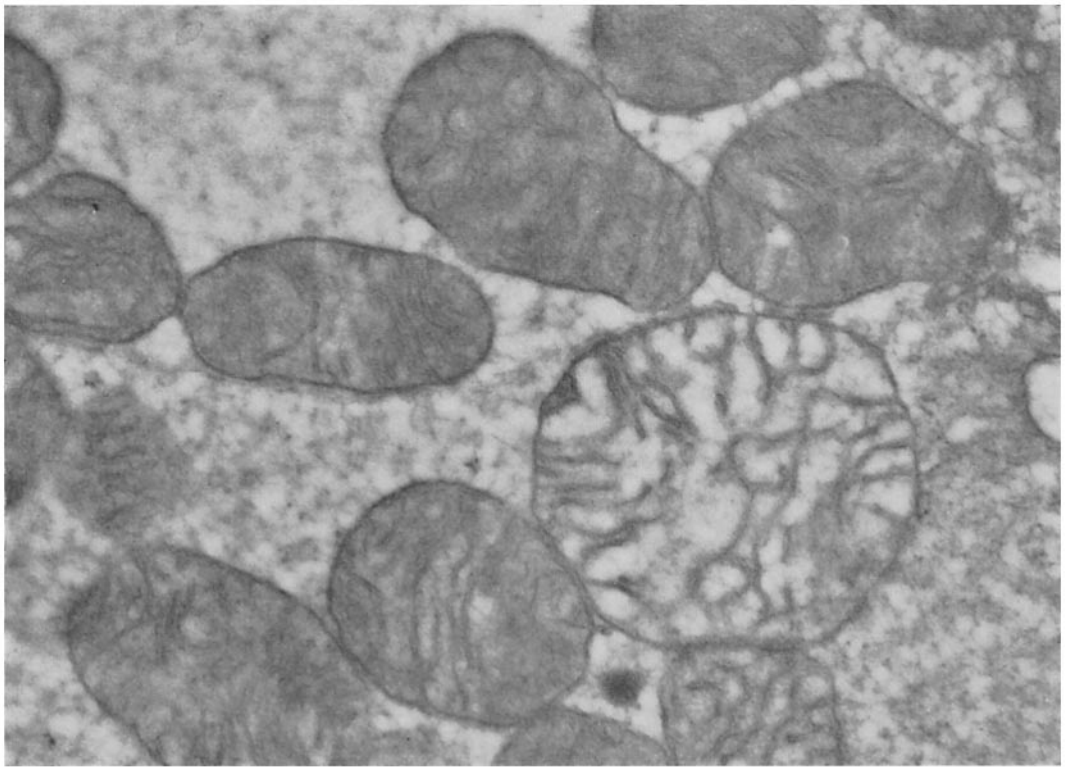

FIGURE 2

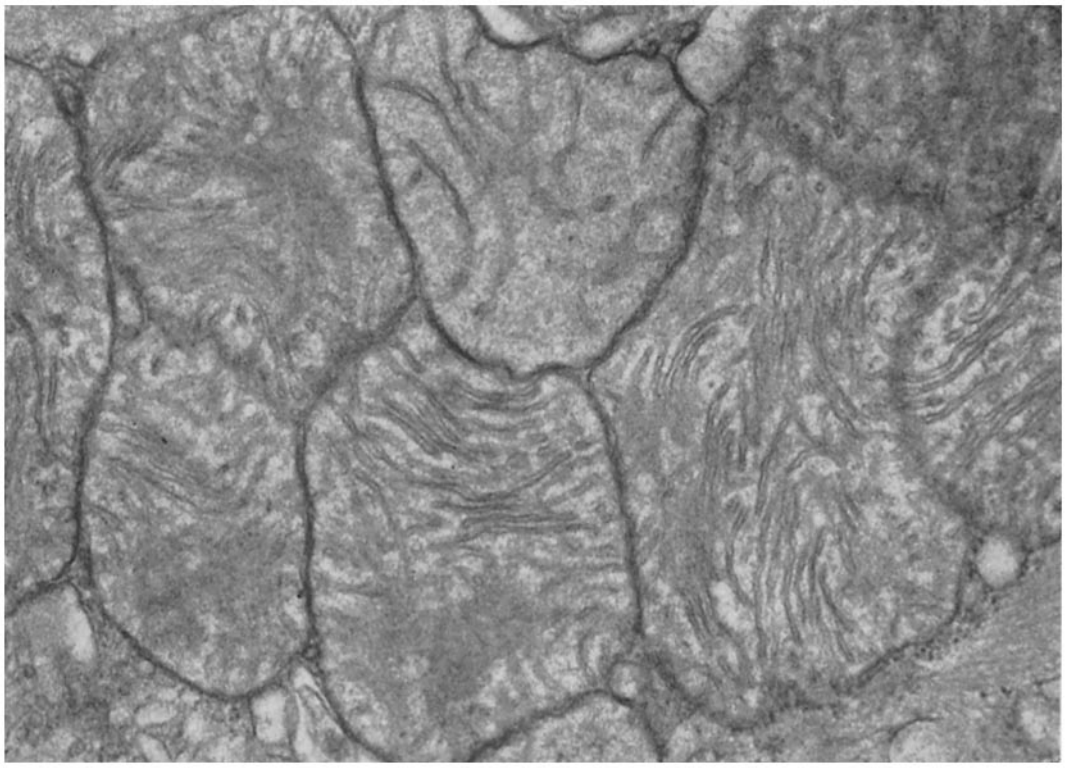

FIGURE 3

absence of oxidizable components (Fig. 9), it is surprising that a marked increase in the DPNH oxidation rate does not occur. The lowest rates, however, generally are observed in a complete medium containing KCl.

The effects of cytochrome *c* and antimycin A on the rate of DPNH oxidation are shown in Fig. 12. In the absence of cytochrome *c*, the oxidation of DPNH is almost completely inhibited by antimycin A, while in the presence of cytochrome *c* the inhibition by antimycin is markedly reduced. Similar findings have been reported for heart muscle mitochondria by Avi-Dor *et al* (4). Baltscheffsky and Baltscheffsky (8), in studies with a submitochondrial DPNH oxidase, found complete inhibition by antimycin A even in the presence of cytochrome *c*.

When DPNH oxidation by 0.02 ml (105 μg protein) of a mitochondrial suspension was followed in a cuvette (3 ml volume), using an equivalent concentration in the blank cell to correct for turbidity, the optical density change amounted to only 0.06 in 10 minutes. When calculated on a protein-N basis, this rate is 10 μmoles DPNH/mg protein-N/hour, an amount greater than that shown in the experiments of Figs. 11 and 12, and adequate to explain the rate of lactate oxidation by mitochondria isolated as described.

Mitochondrial Morphology Before and After Respiration in vitro

Mitochondria isolated in KCl-EDTA-serum albumin and added to a medium containing pyruvate, 3.3 mM; $MgCl_2$, 4 mM; KCl, 116 mM; malate, 0.3 mM; phosphate, pH 7.0, 20 mM; and serum albumin, 1 per cent, are shown in Fig. 13. Aliquots (3 ml) of the mitochondrial suspension in the latter medium were equilibrated in Warburg flasks for 8 minutes. Oxygen consumption was measured in duplicate for 15 minutes after tipping in the following additives: ATP-8-C^{14}, 4 μmoles (Fig. 14); ADP, 4 μmoles (Fig. 15); ATP-8-C^{14}, 4 μmoles, plus hexokinase, 9 units (Fig. 16). Aliquots of the mitochondria were fixed for electron microscopy at the end of this incubation period. The mitochondria, prior to incubation in the original medium (Fig. 13), exhibited less vacuolation, a more delicate structure of the cristae, and better retention of the matrix than was evident after incubation (Figs. 14 to 16).

TABLE I

Assay of Adenine Nucleotide Composition after Measurement of Respiratory Rate and Control Ratio

See text, pp. 82, 87, and Figs. 14 to 16.

Nucleotide added	Nucleotide found (per cent present as) ATP-C^{14}	ADP-C^{14}	AMP-C^{14}	ADP*
ATP-8-C^{14}	100	0	0	0
ATP-8-C^{14} + hexokinase	33	38	29	42
ADP	—	—	—	12

* Enzymic assay (14).

After the above incubation, samples of each medium were assayed enzymically for ADP (14) and, where C^{14}-nucleotides were present, for ATP, ADP, and AMP after paper electrophoresis by a strip-scanning procedure.[5] These results are given in Table I.

Average oxygen consumption for the three pairs of flasks was 93, 101, and 183 μl for additions of ATP, ADP, and ATP + hexokinase, respectively. Respiratory control

$$\frac{\Delta O_2, \text{ with ATP + hexokinase}}{\Delta O_2, \text{ with ATP}}$$

over the intervals 0 to 5, 5 to 10, and 10 to 15 minutes, was 1.6, 2.1, and 2.3, respectively. The increase in control ratio with time is due to a

[5] Reference 23 and to be published.

FIGURE 4

Mitochondria isolated in 0.25 M sucrose containing 0.01 M EDTA. × 25,000.

FIGURE 5

Mitochondria isolated in 0.25 M sucrose containing 0.01 M EDTA and 1 per cent bovine serum albumin. × 25,000.

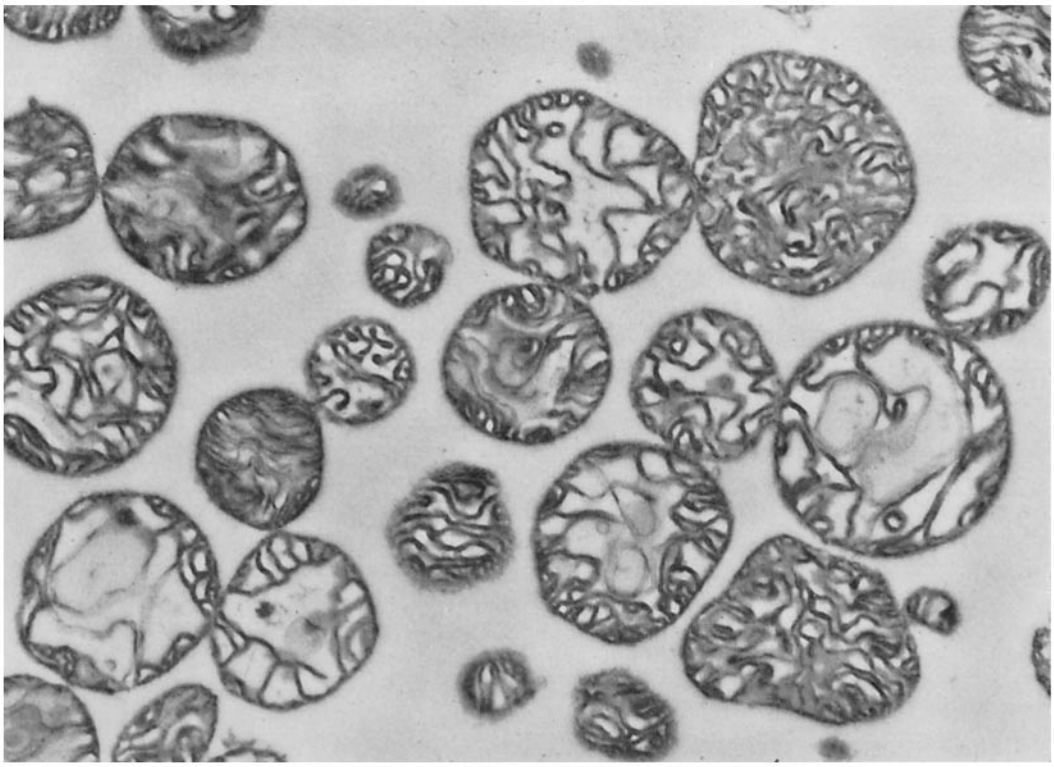

FIGURE 4

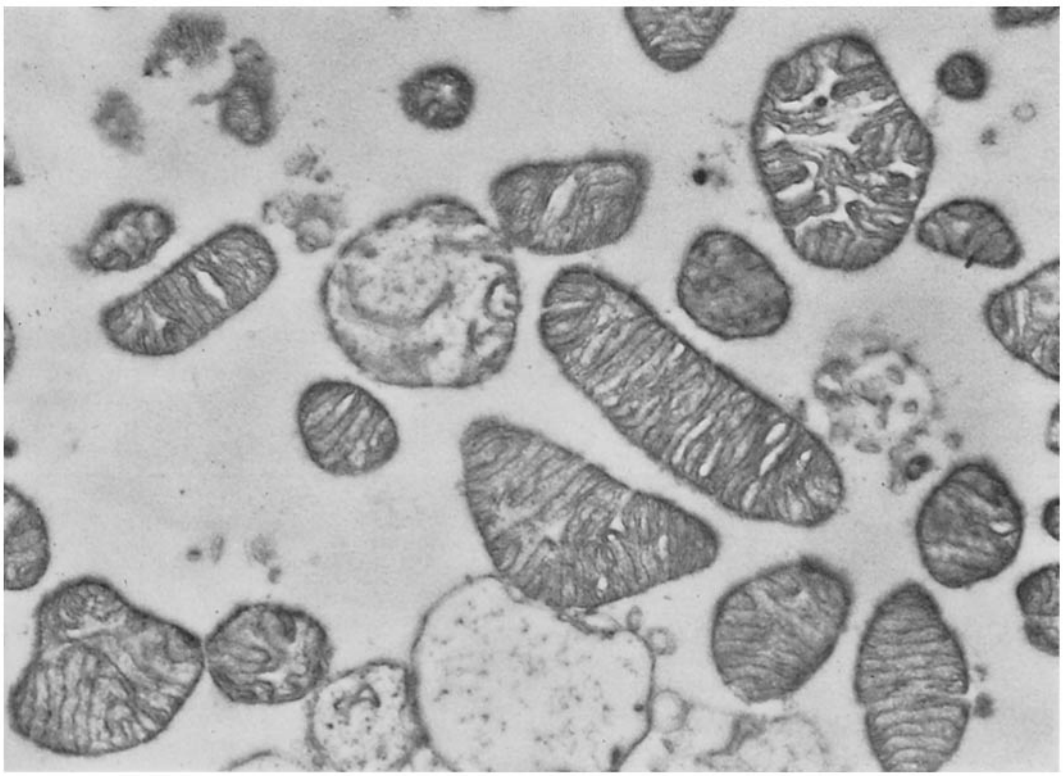

FIGURE 5

slowly increasing rate of respiration observed for the flasks containing hexokinase.

Oxidation of L(+) lactate by the fractionated mitochondria was similar to that reported in an earlier paper (2) describing results with unfractionated mitochondria. In a typical experiment, after a 30-minute incubation of a complete system containing L(+) lactate-1-C^{14} (specific activity 4170 CPM/μmole)[6], 12.5 μatoms of oxygen was consumed, and 12,050 CPM as $C^{14}O_2$ were recovered in the center well. When DPN^+ (1.5 μmoles) was omitted, the oxygen consumption was only 1.3 μatoms and 1,725 CPM were recovered. Omission of LDH resulted in a gas pressure change equivalent to +0.4 μatoms oxygen (gas evolution or inadequate equilibration) and a recovery of 2700 CPM. The added DPN^+ could be reduced to 0.3 μmole with little loss in ability to oxidize lactate.

DISCUSSION

None of the preparations of isolated mitochondria presented here have retained all of the morphological characteristics of the *in situ* mitochondria shown in Fig. 1. A change to spherical forms and sometimes swelling, accompanied by vacuolation, loss of matrix, and rearrangement of the cristae, was the most frequently encountered alteration. The addition of serum albumin to 0.25 M sucrose (Fig. 5) afforded as much protection against these changes as did 0.88 M sucrose alone (Fig. 6).

Rearrangements of the cristae occurred in all preparations of isolated mitochondria, with the type of rearrangement depending on the suspending medium. Thus, the cristae varied from numerous (Fig. 6) to few (Fig. 5), from narrow (Figs. 4, 6) to broad (Figs. 5, 14), from relatively high density (Figs. 4, 6) to relatively low density (Figs. 10, 13), from a random orientation (Figs. 4, 10) to a parallel orientation (Figs. 5, 6). Further evidence of the morphological inconstancy of isolated mitochondria may be seen in Fig. 8. Although these mitochondria were originally isolated in KCl-EDTA-serum albumin, and then transferred to KCl (Fig. 7), upon subsequent transfer to 0.35 M sucrose they exhibited essentially the same structure as mitochondria originally isolated in 0.25 M sucrose (Fig. 4).

The apparent heterogeneity of isolated mitochondria in a single preparation may be accounted for in several ways. Undoubtedly, there is incomplete elimination of damaged mitochondria and other tissue fragments during the isolation and washing procedures. The absence of such fragments in some of the figures presented here, however, shows that this has been minimized. It is considered more likely that all portions of an individual mitochondrion do not react equally, so that one region of a mitochondrion may swell while adjacent regions within the same mitochondrion would show little or no swelling (Figs. 9, 10, 14, 16). Sections through the swollen portion would present an entirely different picture from sections through the unswollen portions. On this basis, it must be assumed that preferential swelling must be more pronounced in some instances (Figs. 9, 10, 14 to 16) than in others (Figs. 4, 7, 8, 13). The atypical forms of mitochondria observed *in situ* (Figs. 2, 3) have not been observed in sufficient numbers to account for the variations found in the isolated mitochondrial preparations.

Electron microscopic observations on the original preparations of isolated mitochondria serve as a valuable initial check on the morphology of the mitochondria and on the amount of extraneous material present in the preparation. However, during subsequent manipulations of the preparations, further modifications in mitochondrial morphology are likely to occur. In attempts to correlate the morphological and biochemical

[6] Prepared by reduction of pyruvate-1-C^{14} with alcohol, alcohol dehydrogenase and DPN^+ followed by purification on a Dowex-1 (Cl^-) column.

FIGURE 6

Mitochondria isolated in 0.88 M sucrose, 0.01 M EDTA. × 25,000.

FIGURE 7

Mitochondria isolated in 0.18 M KCl, 0.01 M EDTA, pH 7.4, and 1 per cent bovine serum albumin, sedimented and resuspended in KCl. (See also Figs. 10 and 13.) × 25,000.

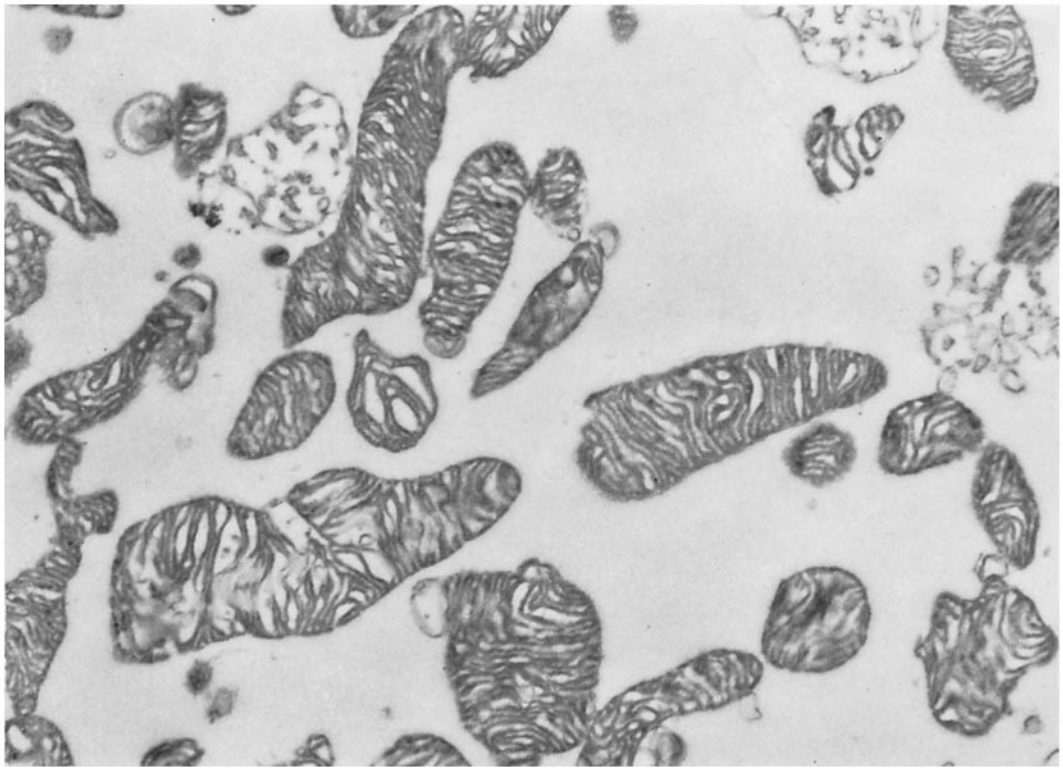

FIGURE 6

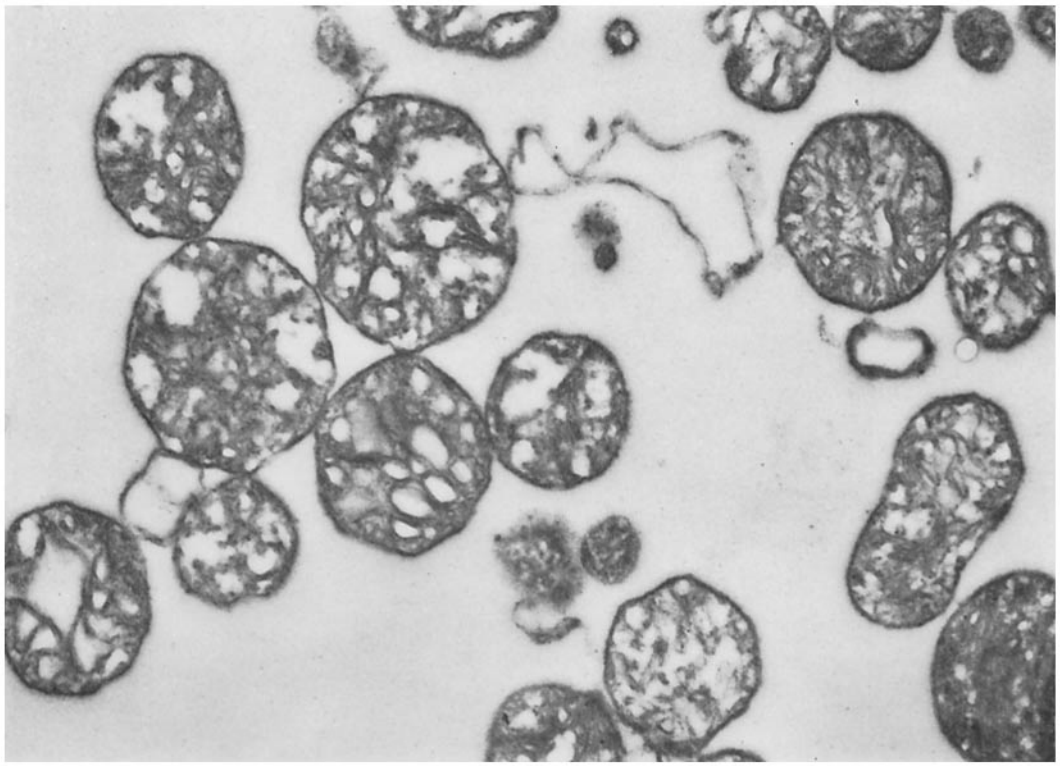

FIGURE 7

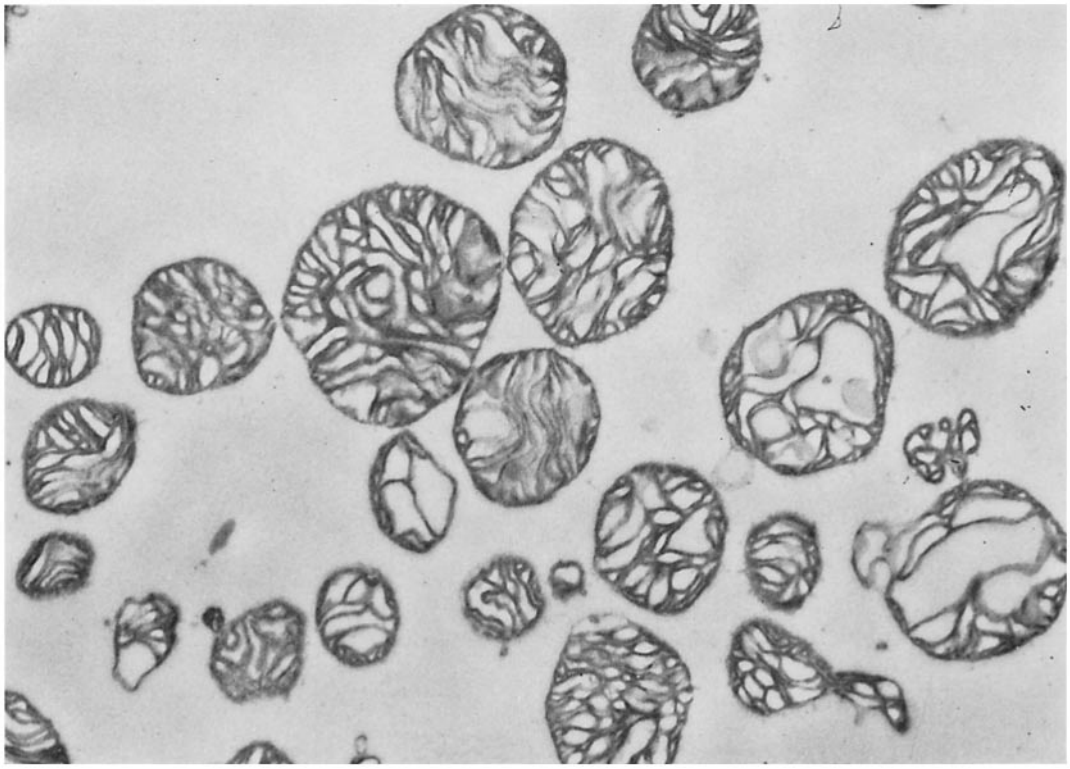

FIGURE 8

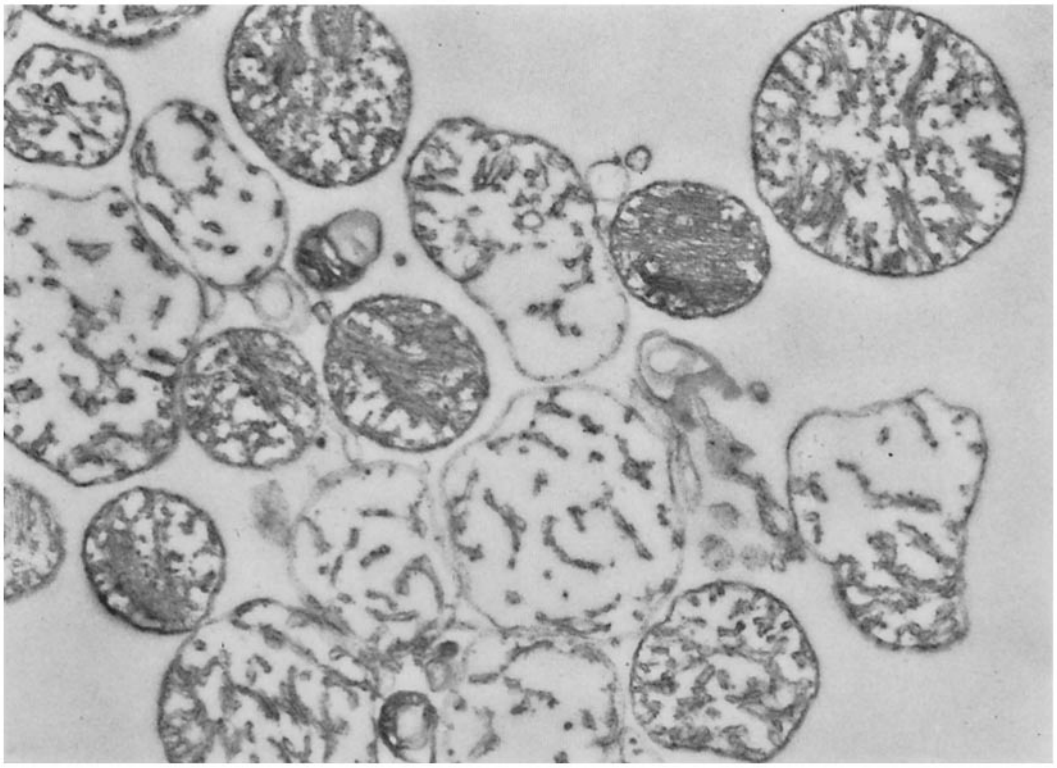

FIGURE 9

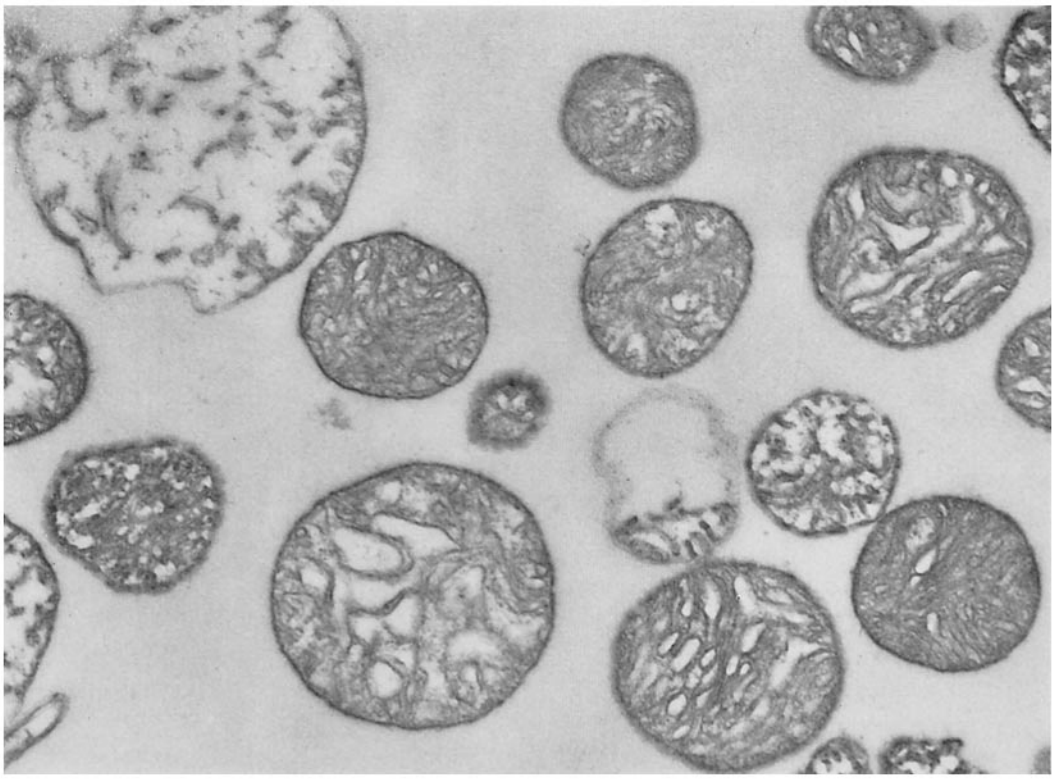

FIGURE 10

Mitochondria of Fig. 7 following incubation for 20 minutes in a medium containing L(+) lactate. × 25,000.

properties of isolated mitochondria with those of mitochondria *in situ*, it will be necessary to follow each step of the entire procedure by electron microscopy to assure that morphological integrity is maintained throughout the experiment. Since the nature and extent of intracellular metabolic controls is unknown, a comparison between the activity of mitochondria *in vivo* and *in vitro* can not be made at the present time. The fact that disrupted mitochondria and mitochondrial fragments retain varying degrees of biochemical activity does not negate the necessity of maintaining morphological integrity in the isolated preparation.

The data of Table I show that whereas addition of hexokinase markedly alters the partition of adenine nucleotides during measurement of respiratory control (and at the time the mitochondria are fixed for electron microscopy), it is relatively immaterial whether the nucleotide added initially is ATP or ADP. In the absence of

FIGURE 8

Mitochondria of Fig. 7 after adding 1 ml of the KCl suspension to 5 ml of 0.35 M sucrose. Note the appearance is now characteristic of mitochondria isolated in sucrose. × 25,000.

FIGURE 9

Mitochondria of Fig. 7 following incubation for 20 minutes in 0.35 M sucrose containing 3.3 mM phosphate, pH 7.0, and DPNH, 0.7 mM. × 25,000.

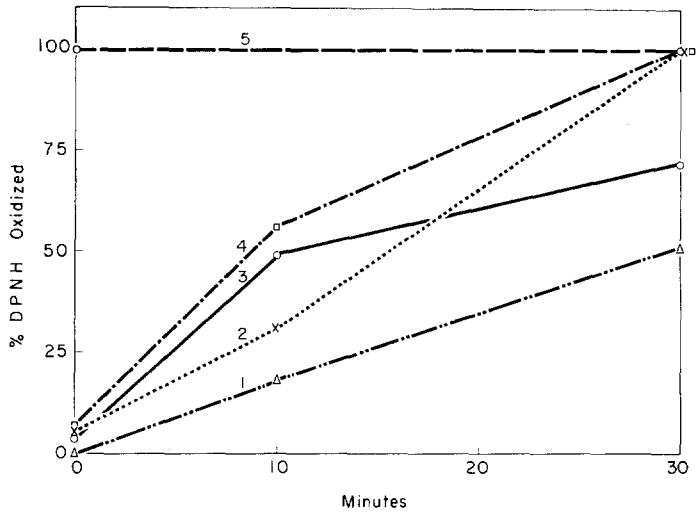

FIGURE 11

DPNH oxidation by mitochondria isolated in 0.18 M KCl, 1 per cent serum albumin, and 0.01 M EDTA, then resuspended in 0.18 M KCl. Rates measured as Δ Log I_0/I at 340 mμ on the supernatant solutions obtained at 26,000 *g* from the following incubation media: DPNH added, 4.4 μmoles/mg mitochondrial N. Curve No. *1*: lactate, 3.3 mM; malate, 0.1 mM; ATP, 1.3 mM; K phosphate, pH 7.0, 10 mM; $MgCl_2$, 4 mM; KCl, 116 mM; bovine serum albumin, 1 per cent; Curve No. *2*: sucrose, 0.35 M, plus P_i (3.3 mM); Curve No. *3*: sucrose, 0.35 M, only; Curve No. *4*: sucrose, 0.35 M, plus acetoacetate (1.7 mM); Curve No. *5*: sucrose, 0.35 M, plus cytochrome *c* (1.2×10^{-5} M).

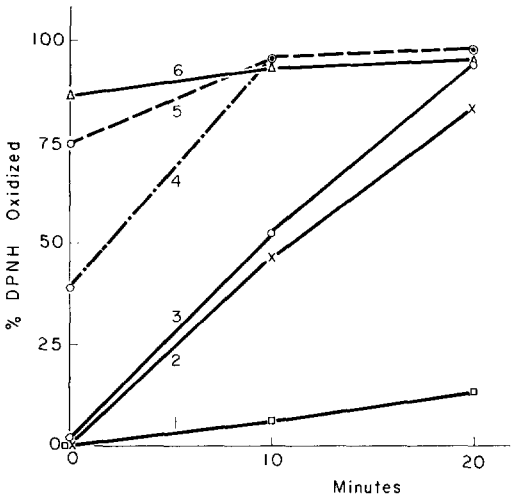

FIGURE 12

Effects of cytochrome *c*, antimycin A, and cytochrome *c* plus antimycin A on the rate of DPNH oxidation by mitochondria isolated in 0.18 M KCl, 1 per cent serum albumin, and 0.01 M EDTA. Rates measured as for experiment of Fig. 11. DPNH added, 2.3 μmoles/mg mitochondrial N. Curve No. *1*: sucrose (0.35 M) plus antimycin A (2×10^{-7} M); Curve No. *2*: The medium as used for curve No. *1*, Fig. 11; Curve No. *3*: sucrose (0.35 M) only; Curve No. *4* sucrose (0.35 M) plus cytochrome *c* (6×10^{-6} M) plus antimycin A (2×10^{-6} M); Curve No. *5*: sucrose (0.35 M) plus cytochrome *c* (6×10^{-6} M) plus antimycin A (2×10^{-7} M); Curve No. *6*: sucrose (0.35 M) plus cytochrome *c* (6×10^{-6} M).

a rapid dephosphorylation system the added ADP is rapidly phosphorylated to ATP. Comparison of the nucleotide composition with the mitochondrial morphology shown in Figs. 14 to 16 does not suggest marked differences in mitochondrial size cited by numerous studies on the so called "swelling phenomenon" (24). The morphology illustrated in Fig. 16 suggests, however, that considerably more vacuolation may be present when AMP and ADP are present in the medium.

Prior to the reports by Linnane and Ziegler (5), Green (6), and Green *et al* (25, 26), it was generally stated that isolated mitochondria are capable of catalyzing a slow oxidation of extramitochondrial DPNH (27–29, 4). The rate is greatly stimulated by hypotonic treatment of the mitochondria (27) or by cytochrome *c* (28, 29). The observations of Linnane and Ziegler (5) led them to state, "If we may assume that functionally and morphologically intact mitochondrial suspensions do not oxidize external DPNH, then such activity as is manifested

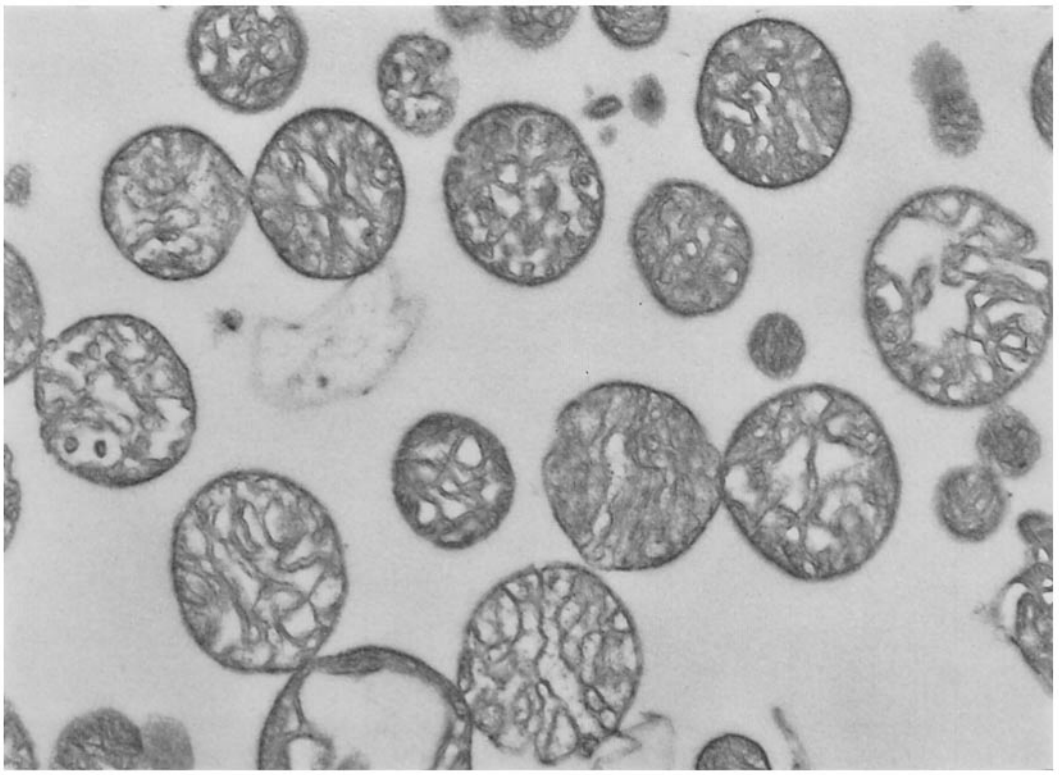

FIGURE 13

Mitochondria isolated in 0.18 M KCl, 0.01 M EDTA, 1 per cent bovine serum albumin. After washing, the mitochondria were suspended in 0.18 M KCl and added to a cold medium containing KCl, 116 mM; $MgCl_2$, 4 mM; malate, 0.3 mM; pyruvate, 3.3 mM; phosphate, 20 mM, pH 7.0; and serum albumin, 1 per cent. × 25,000.

by mitochondrial suspensions becomes a measure of the degree of damage sustained by the preparation." Electron micrographs were presented earlier showing damage resulting from the usual procedures of isolation (17). Data on the rate of development of DPNH oxidizing activity do not appear in the published reports (5, 25).

While the present work offers no evidence that mitochondria *in situ* are capable of oxidizing extramitochondrial DPNH, a slow rate of extramitochondrial DPNH oxidation is established for mitochondria essentially free of fragments. This rate is sufficient to account for the oxidation of lactate observed *in vitro* when catalytic amounts of DPNH are present. It should be noted that, in experiments not reported here, we have observed that the light fraction removed by heparin exhibits not only a higher rate of DPNH oxidation (as measured with an oxygen electrode), but also an ATPase activity 2 to 4 times greater, on a nitrogen basis, than that of the heavy fraction.

In vivo, an aerobic oxidation of DPNH is necessary to regenerate DPN^+ from extramitochondrial DPNH produced in aerobic glycolysis and in the oxidative removal of lactate. As Chance and Thorell (30) have noted, the interrelationship of cytoplasmic DPNH and oxidative metabolism of mitochondria is of importance in the study of metabolic controls and of the interactions of intracellular enzyme systems. While Chance (31) finds that DPNH formed by the oxidation of 3-phosphoglyceraldehyde in yeast cells is reoxidized by mitochondrial enzymes, Chance and Thorell (30) observe that cytoplasmic DPNH in the grasshopper spermatid apparently is inaccessible to the oxidation enzymes of the mitochondria.

Other routes for reoxidation of cytoplasmic

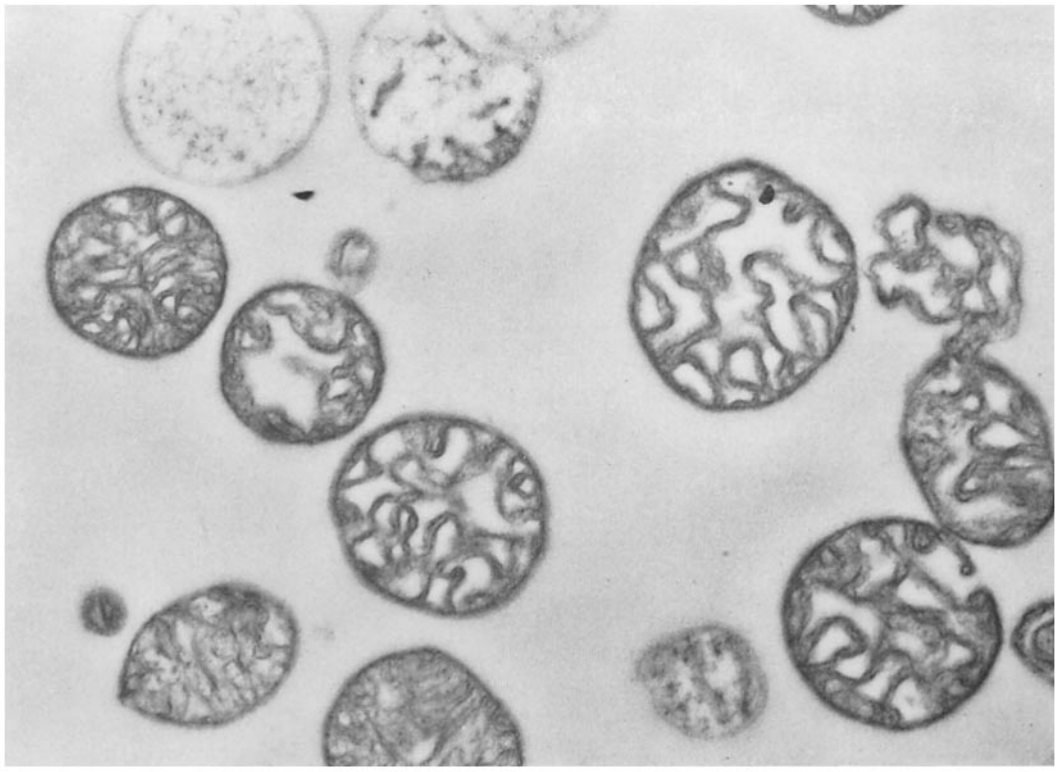

FIGURE 14

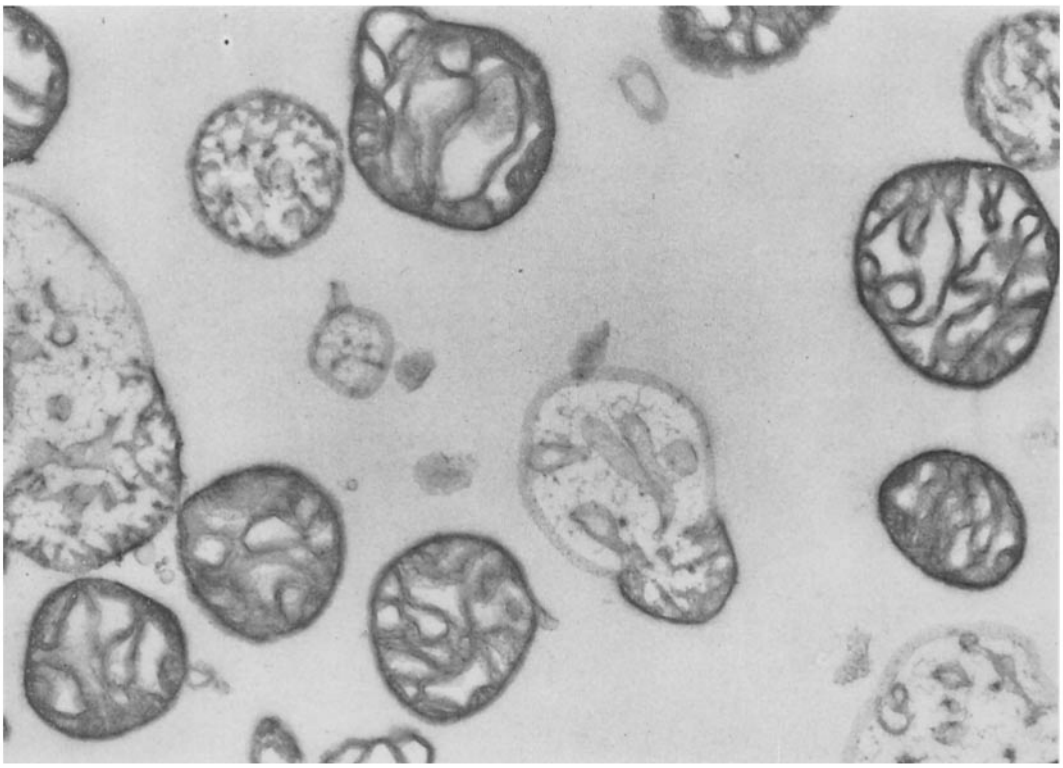

FIGURE 15

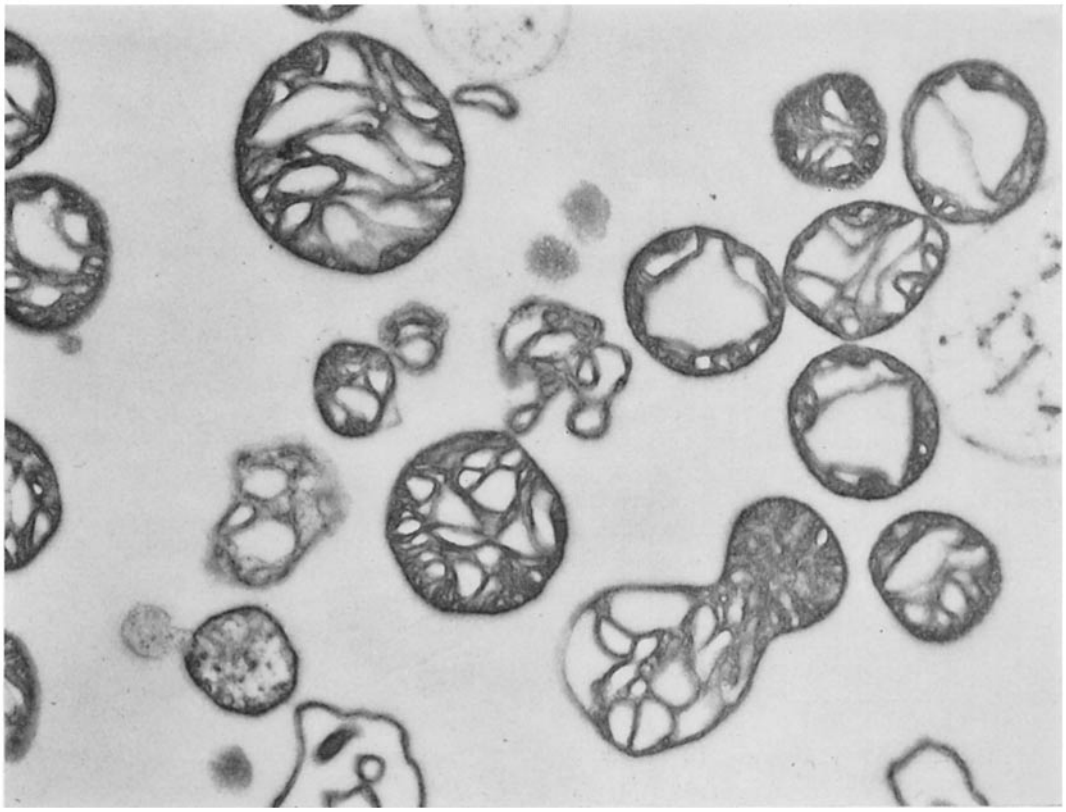

FIGURE 16

Mitochondria of Fig. 13 incubated as for Fig. 14 with hexokinase added in addition to ATP. × 25,000.

DPNH appear to be inoperative or inadequate to explain lactate oxidation by heart muscle. The α-glycerophosphate cycle (32) appears to be an unlikely route because only a small amount of mitochondrial α-glycerophosphate dehydrogenase is found in this tissue (33, 34). Bücher and Klingenberg (32) comment that it is noteworthy that efforts to detect the enzyme in heart muscle mitochondria have been unsuccessful. We have observed little or no oxygen consumption and the formation of only traces of dihydroxyacetone phosphate when heart muscle mitochondria are incubated with α-glycerophosphate.

The acetoacetate cycle described by Devlin and Bedell (35) as functioning in isolated rat liver mitochondria does not appear to be operative in the isolated rabbit heart muscle mitochondria. As noted above, we have observed only minimal

FIGURE 14

Mitochondria of Fig. 13 incubated at 38°C in the medium given for Fig. 13. After equilibration for 8 minutes, 4 μmoles of ATP was added and respiration measured for 15 minutes. The mitochondria were then examined by electron microscopy. × 25,000.

FIGURE 15

Mitochondria of Fig. 13 incubated as for Fig. 14 with ADP added in place of ATP. × 25,000.

increases in the rate of DPNH oxidation on addition of acetoacetate to the medium. Lehninger *et al* (36), in a study of liver mitochondria, find that external DPNH is not oxidized by intact liver mitochondria in the presence of acetoacetate when respiration is blocked. They conclude that the "shuttle" proposed by Devlin and Bedell may require some additional change or swelling and may not proceed with completely intact mitochondria.

The possibility that DPNH bound to 3-phosphoglyceraldehyde dehydrogenase may be more readily oxidized than free DPNH also seems unlikely. In this case, the bound DPN^+ would be reduced by interaction of the two enzymes during the conversion of lactate to pyruvate. Nygaard and Rutter (37) have observed that free DPN^+ reacts more rapidly in lactate oxidation than does bound DPN^+. In addition, our results indicate that free DPNH and bound DPNH are oxidized at the same rate by mitochondrial suspensions.

The possibility that some unknown route may exist for reoxidation of cytoplasmic DPNH remains. In view of uncertainties as to the structural and biochemical integrities of mitochondria after isolation, it would appear to be unjustified to state that heart muscle mitochondria are incapable of catalyzing the oxidation of cytoplasmic DPNH.

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