MORPHOLOGY AND ATP-ASE OF ISOLATED MITOCHONDRIA*

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Plates 40 to 43

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Recent studies of the ATP-ase[1] of mitochondria isolated from rat liver have shown that this enzyme is latent (1–5). Thus it does not exhibit full activity unless dinitrophenol (DNP) is present (1, 3, 5, 6), or the preparation is aged at 37°C. (1–3), or frozen and thawed (4, 7). The mitochondria used in most studies have been isolated in 0.25 M sucrose solutions (8). Such preparations differ in morphology from the mitochondria *in situ*. The former are spherical and appear to be swollen, whereas the latter are rod- or oval-shaped (9). Rod-shaped mitochondria can be isolated in 0.88 M sucrose (9) and have been found to possess much higher ATP-ase activity (10, 11) than the round type (1–5).

In view of these differences in enzymatic activity between the rod- and the round-shaped mitochondria, the possibility arises that the observed properties of mitochondria isolated in 0.25 M sucrose may be artifacts of preparation which may be correlated with changes in morphology upon isolation. This point becomes even more significant when it is realized that the round swollen mitochondria have been successfully used in the study of oxidative phosphorylation because of the very fact that their ATP-ase is latent thus permitting the measurement of maximal P/O ratios (1, 12–14). Although preliminary experiments indicate that the ATP-ase activity of mitochondria in 0.88 M sucrose may sometimes be increased by freezing and thawing (12) there are no reports of detailed experiments showing that this enzyme is latent in this preparation. Finally, the properties of the ATP-ase of mitochondria isolated in solutions of sucrose concentration other than 0.88 M or 0.25 M have not been investigated.

An attempt has been made, therefore, to correlate changes in morphology of mitochondria brought about by different methods of isolation with character-

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[1] In this study the term "ATP-ase" will be used to refer to the group of mitochondrial enzymes which is responsible for the over-all production of inorganic phosphate from ATP. This terminology has been used by others studying the dephosphorylation of ATP by mitochondria (1–5).

istics of the ATP-ase of these preparations. The activity of this enzyme was studied in: (*a*) fresh preparations upon addition of magnesium, calcium, or DNP, (*b*) preparations aged at 37°C., and (*c*) frozen and thawed preparations. Because of their small size only gross details of the morphology of mitochondria can be seen with the aid of the light microscope. Recent advances in methods of embedding (15), fixation (17), and sectioning (16–18) have made possible the preparation of electron micrographs of mitochondria fixed *in situ* which show the internal structure in great detail (19–22). A systematic application of these methods to suspensions of isolated mitochondria has not been reported although the persistence of the internal structure has been reported in preliminary form (19). Accordingly, in the present studies, mitochondria isolated in various media were fixed, embedded, and sectioned, and then examined in the electron microscope in order to determine whether a correlation exists between their fine structure and their ATP-ase activity.[2]

Materials and Methods

Chemicals.—The tris (hydroxymethyl) aminomethane (tris) buffer was obtained from Sigma Chemicals Company, the sodium salt of ATP from the Pabst Company, sodium versenate, C.P., from Bersworth Chemical Company, dichlorobenzoyl peroxide with 50 per cent dibutyl phthalate (luperco CDB) from the Lucidol Division of the Novadel Agene Corporation, butyl methacrylate monomer from Rohm and Haas Company.

ATP-ase Assay.—The ATP-ase assay was carried out by determining the micromoles of phosphate released from 3.75 micromoles of ATP in 10 minutes at 30°C. by 1 mg. dry weight of liver mitochondria. This amount was equivalent to 25 mg. of wet liver. The reaction was conducted in a total of 0.75 ml. of solution containing 0.12 M sucrose, 0.02 M "tris" buffer, pH 8.0, 0.005 M ATP adjusted to pH 8.0, and either 0.007 M magnesium chloride, 0.007 M calcium chloride, 1.3×10^{-4} M dinitrophenol, or 7×10^{-4} M versene. Most of the assays were carried out in 16 × 140 mm. test tubes, but similar results were obtained using shaken Warburg flasks filled with nitrogen.

Preparation of Mitochondria from Rat Liver.—Young fed rats were killed by decapitation, and the livers were removed. Homogenates in 45 ml. of sucrose solution were prepared with a new type of homogenizer (24) from 5 gm. of the fresh liver. The nuclear fraction was isolated by centrifugation and washed once with 30 ml. of sucrose solution. In a similar manner, the mitochondrial fraction was isolated from the combined supernatant fluids of the nuclear fraction and washed two times with 40 ml. portions of sucrose solution. The mitochondrial pellets were suspended in a total volume of 10 ml. of sucrose solution. The relative centrifugal forces and times of centrifugation are given for each type of preparation in Table I. Except where noted, both the nuclear and mitochondrial fractions were washed with sucrose solutions of the same composition as those used to make the homogenates. In the case of homogenates in 0.44 M sucrose plus either citric acid at pH 6.2, 5×10^{-4} M versene at pH 6.2, or calcium chloride, the nuclear and mitochondrial fractions were washed with 0.44 M sucrose. Further details of the preparation of mitochondria by the citrate method are given by Dounce *et al.* (24). All manipulations were carried out at 0 to +2°C. Centrifugation at low speed was conducted in 50 ml. cups of the International refrigerated centrifuge, and at high speed in 25 ml. cups with the multispeed attachment.

[2] A preliminary report of some of these results has been presented before the Federation of American Societies for Experimental Biology (23).

Morphological Examination.—Preparations were examined unstained under oil immersion with the light microscope. The mitochondria were subjected to the following treatment before they were studied with the electron microscope. The isolated mitochondria were centrifuged sufficiently to form a loose pellet. They were then resuspended in sucrose at the concentration used in the isolation and containing 2 per cent OsO_4, and were left in this fixation medium for 16 hours at room temperature. They were then centrifuged at low speed and resuspended in water, after which they were immediately dehydrated by being passed through 40 per cent, 60 per cent, 80 per cent, 90 per cent, 95 per cent, and absolute ethyl alcohol solutions, with ¼ to 1 hour per step, the longer times being used at the higher concentrations. Centrifugation at low speed was used when necessary to remove the mitochondria from the dehydrating medium. The dehydrated pellet finally was placed in a 1:1 mixture of ethyl alcohol and butyl methacrylate monomer and was allowed to remain there for 1 hour. It was then allowed to remain overnight at 5°C. in 90 per cent butyl methacrylate plus 10 per cent methyl methacrylate with

TABLE I

Conditions of Centrifugation for the Isolation of Mitochondria

The relative centrifugal forces are calculated to the middle of the centrifuge cup.

Sucrose solution used for the preparation of mitochondria	Nuclear fraction		Mitochondrial fraction	
	Time of centrifugation	Relative centrifugal force	Time of centrifugation	Relative centrifugal force
	min.	*g*	*min.*	*g*
0.25 M sucrose	13	450	7	11,000
0.35 M sucrose	15	520	10	13,000
0.44 M sucrose	20	675	10	13,000
0.44 M sucrose + citrate pH 6.2	20	675	10	13,000
0.44 M sucrose + 0.005 M $CaCl_2$	22	675	10	13,000
0.6 M sucrose	20	800	10	18,000
0.88 M sucrose	20	800	5	18,000

2 per cent luperco CDB catalyst added. Polymerization followed in fresh methacrylate at 45°C. for 24 hours. Sections were cut with a special microtome (25) equipped with a glass knife and were collected from the surface of a solution of 10 per cent acetone. Sections were examined in an RCA EMU-2A microscope with a 0.001 inch platinum objective aperture.

The procedures of preparation outlined were designed to minimize extraction of partially soluble material present particularly within the matrix of the mitochondria. For this reason, the fixative solution was not buffered as in the current technique for fixation of tissue specimens, because, in our experience, the addition of veronal-acetate buffer (pH 7.0 to 7.5) to the OsO_4 solution increased to a marked degree the extraction of isolated mitochondria.

The mitochondrial isolate was centrifuged before fixation to form a thick suspension from which the supernatant was decanted. In this form, the mass of mitochondria was porous enough to permit easy penetration of the fixative, but at the same time dense enough to form a loose pellet which retained its identity throughout the following procedures. Initial fixation proceeded very rapidly because of the small particle size and the absence of barriers such as cell membranes. Under these conditions, the acidification which precedes the fixation of the preparation is apparently minimized and the presence of a buffer in the fixative solution seems unnecessary.

The criterion of satisfactory fixation in evaluating the various procedures of preparation

tried (*i.e.*, buffering of fixative, long *vs.* short fixation, pellet *vs.* suspension, etc.) was the degree of resemblance to mitochondria seen in sections of intact liver. The procedures used gave the isolated mitochondria an appearance most nearly resembling that seen in intact cells and in addition were adequate to reveal substantial differences in the appearance of mitochondria isolated by different procedures.

Any given section showed considerable variation in apparent degree of preservation of morphological detail. It was considered that some destruction of fine detail occurred during the embedding procedures rather than during isolation and that this destruction was unevenly distributed throughout the sample. This inhomogeneity may be related to clumping of portions of the pellet during fixation and to the possibility that mitochondria on the surface of a clump would receive different treatment from those within. Production of such inhomogeneity during the original isolation seems unlikely. On this basis, it is felt justifiable to consider only the best preserved mitochondria as being representative of the original isolated mitochondria.

EXPERIMENTAL

Morphology

It was found by optical examination that mitochondria isolated in 0.25 M sucrose were round and swollen, those isolated in 0.35 M sucrose were mixtures of round and rod types, whereas those isolated in 0.44 M, 0.6 M, or 0.88 M sucrose were rod-shaped. Essentially the same results were obtained by Hogeboom, Schneider, and Palade (9).

The principal effects observed with the electron microscope on the structure of the isolated mitochondria were those ascribable to the tonicity of the isolating medium. By comparison with liver mitochondria examined *in situ*, mitochondria isolated at high tonicities appeared shrunken and presumably dehydrated as suggested by their higher degree of electron scattering, whereas, those isolated at low tonicities were swollen and apparently more hydrated.

Mitochondria isolated in 0.88 M sucrose (Fig. 3) are irregular in shape and small in size. Their contents, including the *cristae mitochondriales* (20), and the matrix, are highly condensed and in places appear to have pulled away from the outer mitochondrial membrane. The cristae have opened up in such a way as to present the appearance of cracks in the matrix. The matrix is nearly homogeneous and is in contact with the walls of the cristae and the inner mitochondrial membrane.

Mitochondria isolated in 0.44 M sucrose (Fig. 2) are larger and are round or oval rather than irregular in shape and their internal structure appears satisfactorily preserved. The presence of citrate in this isolating medium (0.44 M sucrose) appeared to the authors to aid in preserving the distinctness of the membranes. When citrate is present, the walls of the cristae run closely parallel. The matrix is not highly condensed and appears to have an ill defined structure. The inner mitochondrial membrane and the walls of the cristae are in contact with the matrix. The inner and outer mitochondrial membranes lie closely parallel.

Isolation of mitochondria in sucrose solutions of low tonicity (0.25 M)

(Fig. 1) results in some swelling. In 60 to 70 per cent of such mitochondria the outer membrane seems intact and many of the cristae appear more or less normal, that is, with parallel walls; other cristae, however, have a circular outline which may indicate a vesicular type of swelling. In most of these mitochondria, the matrix, although present, shows a noticeably low density due presumably to dilution. In 30 to 40 per cent of mitochondria isolated in 0.25 M sucrose nearly all the cristae have a circular outline and the matrix appears to have leached out completely.

Thus, proceeding from an isolating medium of high tonicity to one of low tonicity, the structure of the isolated mitochondria is altered in a number of recognizable ways. The average diameter increases, being about 0.8 μ in 0.88 M sucrose, 1.1 μ in 0.44 M sucrose, and 1.5 μ in 0.25 M sucrose. The matrix is highly condensed in the 0.88 M sucrose preparations and is highly attenuated in the 0.25 M sucrose preparations in which the matrix of 30 to 40 per cent of the mitochondria appears to have leached out completely. The cristae and the inner membrane of the mitochondria adhere, in general, to the matrix. In the preparations at high tonicity (0.88 M sucrose), the shrinkage of the matrix separates the inner and outer walls of the mitochondria and pulls apart the walls of the cristae making them appear as cracks in a dense mass. At low tonicity, on the other hand, many of the cristae present a circular appearance which may indicate vesiculation. The preparations which most nearly resemble in structure the mitochondria of intact cells are those isolated in a medium containing sucrose at a concentration of 0.44 M together with citrate. It is probable, however, that the concentration of sucrose is not critical so far as the appearance of the mitochondria in the electron microscope is concerned and that a favorable range of concentrations around 0.44 M exists. The vesicular transformation of the cristae and the leaching of the matrix observed in the 0.25 M preparations appear undesirable on the one hand, while the highly condensed matrix, the separation of the inner and outer mitochondrial membranes, and the "cracking" of the cristae in the 0.88 M preparations are not satisfactory at the other extreme. It thus appears desirable to isolate liver mitochondria at intermediate concentrations of sucrose in order to preserve their structure in so far as possible.

Despite the fact that the "fluffy" layer was removed during the isolation, the preparations isolated in 0.25 M sucrose were contaminated with "microsomes" apparently derived from the endoplasmic reticulum (26) and its associated particles (27). Such contamination was not obvious in the mitochondria isolated at higher sucrose concentrations. Some authors (28, 29) have concluded from studies with 0.25 M sucrose preparations that several enzymatically distinct types of mitochondria may exist. Such conclusions are in doubt unless the possibility of contamination is ruled out by checking the purity of fractions with the electron microscope.

A few experiments were carried out to determine whether citrate could be replaced by other agents reported to preserve the structure of isolated cell constituents. Mitochondria prepared in 0.44 M sucrose plus 0.0005 M sodium versenate at pH 6.2 were clumped and appeared to be abnormal, curled, rod-shaped bodies under the light microscope. Those prepared in 0.44 M sucrose containing 0.005 M calcium chloride were similar except that they were not clumped. Thus, these two agents do not satisfactorily replace citric acid.

In confirmation of earlier work with the light microscope (9), it was found with the electron microscope that the nature of the initial homogenizing medium had a permanent effect on the morphology of the isolated mitochondria. Thus, mitochondria isolated in 0.88 M sucrose did not swell and become hydrated when suspended in 0.25 M sucrose for 2 hours at 0°C., although some distortion was produced (Fig. 4); whereas mitochondria isolated initially in 0.25 M sucrose at 0°C. were always swollen (Fig. 1). Evidently, either the mitochondria were altered in the course of isolation or some constituent of the whole tissue which is not present in the purified suspension of mitochondria is responsible for the swelling in the low concentrations of sucrose. These possibilities are under investigation at the present time by one of us.

ATP-ase Activity

The possibility of correlation between the morphology of the isolated mitochondria and the properties of the ATP-ase present in them was investigated using a variety of preparations. In Table II are shown the effects of freezing and thawing, aging at 37°C., and of the presence of DNP, calcium, or magnesium on the ATP-ase of fresh mitochondria prepared by various methods. In these experiments, calcium or magnesium when added were at 0.007 M concentrations, but similar results were obtained when 0.0014 M solutions of these salts were used. Calcium appeared to be a more effective activator than magnesium in fresh preparations, but the greatest effect was obtained with DNP. The magnesium-activated ATP-ase of each preparation was latent since the activity of the enzyme was increased by aging at 37°C. or by freezing and thawing (Table II). Similar results, not illustrated in Table II, were obtained by aging or freezing and thawing with 0.007 M calcium as the activator; the activating effect of DNP was destroyed by these processes. Others (1, 3) have obtained similar results with mitochondria from 0.25 M sucrose. A detailed account of the effects of aging on the ATP-ase of mitochondria will be presented elsewhere.

In the next series of experiments factors influencing the activation of the mitochondrial ATP-ase by DNP were investigated. With mitochondria isolated in 0.25 M, 0.44 M, or 0.88 M sucrose, it was found that calcium, magnesium, or higher concentrations of sucrose repress the activating effect of

DNP. The data for preparations isolated in 0.44 M sucrose are given in Table III. Similar results have been reported by Lardy and Wellman (2) with mitochondria from 0.25 M sucrose. Also, the addition of versene had no effect on the ATP-ase activity, either in the presence or absence of DNP. Since versene is a complexing agent for polyvalent cations (30), these results indicate that dinitrophenol does not act by catalyzing the release of some metal

TABLE II

Activation of ATP-ase of Isolated Liver Mitochondria by Mg, Ca, DNP, Aging, or Freezing and Thawing

Mitochondrial suspensions were kept at 0° and if used within 1 hour were considered fresh. In the aging experiments the mitochondrial suspensions in the appropriate sucrose solution were heated at 37° for 30 minutes. Fresh mitochondrial suspensions in sucrose were frozen at −20° overnight and thawed at 5°. The conditions of the ATP-ase assay are given in the section entitled "Materials and Methods."

Preparation of mitochondria	Phosphate per mg. of dry mitochondria					
	Fresh mitochondria plus				Aged mitochondria plus	Frozen, thawed mitochondria plus
	Blank	Mg	Ca	DNP	Mg	Mg
	μM	μM	μM	μM	μM	μM
0.25 M sucrose	0.06	0.08	0.94	2.6	1.2	1.8
0.25 M sucrose	0.20	0.29	1.3	1.9	1.0	1.3
0.44 M sucrose	0.13	0.20	0.50	2.3	1.1	1.2
0.44 M sucrose	0.22	0.24	0.80	2.0	1.4	1.3
0.60 M sucrose	—	0.60	—	1.5	—	1.5
0.88 M sucrose	0.6	1.1	1.6	1.4	1.5	1.8
0.44 M sucrose + 0.005 M $CaCl_2$	—	1.2	—	1.3	2.2	1.9
0.44 M sucrose + 0.0005 M versene pH 6.2	0.46	0.48	0.92	1.5	—	1.0
0.44 M sucrose + citrate pH 6.2	0.34	0.30	1.0	2.1	0.9	1.0
0.44 M sucrose + citrate pH 6.2	0.20	0.23	0.8	1.8	1.1	1.3
0.25 M sucrose + citrate pH 6.2	0.18	0.20	0.8	1.4	1.0	1.2

activator from the mitochondria. These results with mitochondria of liver are in contrast to those obtained with heart homogenates in which versene was found to be a good activator of ATP-ase (31). The results of the two series of experiments in Tables II and III indicate that ATP-ase activity of essentially the same character was present whether the mitochondria were round and swollen or rod-shaped. Evidently the ATP-ase activity of these isolated mitochondria is not related to changes in morphology brought about by different methods of isolation.

However, mitochondria isolated in 0.44 M sucrose plus calcium chloride or in 0.88 M sucrose are different enzymatically from the other preparations

TABLE III

Activation of ATP-ase of Fresh Mitochondria from 0.44 M Sucrose by Dinitrophenol

The conditions of these experiments are given in the description of the ATP-ase assay in the section entitled "Materials and Methods."

Addition	DNP	Phosphate per mg.
		μM
0.12 M sucrose	−	0.21
0.12 M sucrose	+	1.9
0.12 M sucrose + $CaCl_2$	−	0.80
0.12 M sucrose + $CaCl_2$	+	1.2
0.12 M sucrose + $MgCl_2$	−	0.33
0.12 M sucrose + $MgCl_2$	+	1.3
0.12 M sucrose + versene	−	0.2
0.12 M sucrose + versene	+	2.1
0.44 M sucrose	−	0.25
0.44 M sucrose	+	0.35

TABLE IV

Effects of DNP and Potassium Chloride on the Hydrolysis of ATP by Liver Mitochondria from 0.88 M, 0.44 M, or 0.25 M Sucrose

The conditions of these experiments are given in the description of the ATP-ase assay in the section entitled "Materials and Methods." When added, the concentration of potassium chloride was 0.09 M.

Preparation of mitochondria	DNP	KCl	Phosphate per mg.
			μM
0.88 M sucrose	−	−	0.71
	+	−	1.90
	−	+	0.66
	+	+	0.92
0.44 M sucrose	−	−	0.20
	+	−	3.1
	−	+	0.53
	+	+	3.4
0.25 M sucrose	−	−	0.13
	+	−	2.85
	−	+	0.30
	+	+	3.0

reported here. First of all, in Table II it can be seen that these two preparations, unlike the others, had, when fresh, relatively high ATP-ase activity which was activated by magnesium. Further illustration of the differences found when 0.88 M sucrose is used is given in Table IV. It was found that the addition of 0.09 M potassium chloride repressed the activation of ATP-

ase by DNP in mitochondria prepared with 0.88 M sucrose but had no effect on the system in mitochondria made with 0.44 M or 0.25 M sucrose. Lardy and Wellman (2) found that potassium chloride activated this enzyme in mitochondria from 0.25 M sucrose. The reason for this discrepancy between their results and ours is unknown but may be related to the differences in assay systems.

The differences between the ATP-ase activity of mitochondria isolated in 0.88 M sucrose and of those isolated in 0.44 M or 0.25 M sucrose solution depend on the conditions of homogenization and cannot be reversed merely by resuspending isolated mitochondria in a medium different in concentration from that in which the mitochondria were isolated. Thus, resuspension in 0.25 M sucrose at 0°C. of the mitochondria originally isolated in 0.88 M sucrose or the reverse of this did not result in a change in the ATP-ase activated by magnesium or DNP. The calcium-activated ATP-ase was not tested in this experiment. These results appear to eliminate the possibility that the low initial ATP-ase activity of mitochondria from 0.25 M sucrose is due to the extraction of an activator which is soluble in 0.25 M but not in 0.88 M sucrose. In this connection, it will be recalled that the morphology of the mitochondria also did not change significantly when the mitochondria were transferred from 0.88 M sucrose to 0.25 M sucrose solution.

DISCUSSION

The results of studies of oxidative phosphorylation on the swollen mitochondria from 0.25 M sucrose provide one of the bases of our present concepts of the role of "high energy" phosphate in the utilization of the oxidative energy of the cell (1, 13, 14). These experiments have been possible because of the properties of the ATP-ase and oxidative enzymes of the mitochondria so isolated. The present studies with ATP-ase and the previous ones with oxidative enzymes (24) have shown that, with the exception of mitochondria isolated in 0.88 M sucrose (8), the properties of these enzymes are indistinguishable regardless of whether the isolated mitochondria appear well preserved or swollen and partially extracted. As a corollary, the ATP-ase and oxidase activities are not very sensitive enzymatic criteria of the changes in morphology discussed here. Previous investigators (1–3, 6) have felt that possession of latent ATP-ase was characteristic of "undamaged" mitochondria in spite of the fact that such mitochondria were always morphologically distorted by swelling. This view was based on data indicating that processes known to damage isolated mitochondria such as aging (1–3) or freezing and thawing (4, 7) activate this enzyme. In this connection it should be pointed out that the damage to morphology caused by these treatments is much more extensive than that which may occur during the isolation procedures used in this study. This is shown by the marked decrease in turbidity of suspen-

sions of mitochondria in sucrose solutions and the extensive damage visible in the light microscope after aging and after freezing and thawing.

The results reported in this study show that by using an intermediate concentration of sucrose (0.44 M) supplemented with citric acid it is possible to isolate mitochondria which bear a closer resemblance to mitochondria *in situ* than do preparations at lower (0.25 M) or higher (0.88 M) sucrose concentrations. Such mitochondria have high oxidase (24) and low ATP-ase activities which indicates that latency of ATP-ase and high oxidase activity do not necessarily have to be accompanied by drastic alterations in morphology.

Previous investigators observed latent ATP-ase and high oxidase activity only in the morphologically altered mitochondria from 0.25 M sucrose because they failed to study the enzymatic activity of preparations made in solutions containing intermediate concentrations of sucrose between 0.25 and 0.88 M. The present results show that increasing the concentration of sucrose used in isolation of mitochondria from 0.25 to 0.44 M reduces morphological alterations ascribable to swelling and extraction of the mitochondria without change in their enzymatic activity. A further increase to 0.88 M, however, introduces morphological alterations ascribable to dehydration, and results also in an alteration in enzymatic activity. Previous investigators (6, 9) using the light microscope were unable to detect these alterations in morphology in the mitochondria from 0.88 M sucrose and concluded that such preparations resembled those fixed *in situ*.

Since mitochondria with high oxidase and latent ATP-ase should be more efficient in capturing as ATP the energy of oxidative processes, biochemists (1–3, 6) have considered that mitochondria with such enzymatic activity are enzymatically in better condition than those with high ATP-ase and low oxidase activity. By the addition of citric acid to 0.44 M sucrose, it has been found possible to prepare mitochondria which resemble those sectioned *in situ* more closely than do the mitochondria of other preparations, and furthermore without change in the properties of the oxidative enzymes (24) and ATP-ase that are observed in the absence of citrate. Thus, according to the previously mentioned criteria, the mitochondria prepared by the sucrose-citrate method not only are morphologically well preserved but also appear to be enzymatically intact.

It is possible that more than one type of ATP-ase may be present in rat liver mitochondria, since this enzyme was activated in fresh preparations by calcium, or DNP and after aging by calcium or magnesium (3). ATP-ases activated specifically by magnesium (32) or by DNP and magnesium (2) have been separated from these organelles. The possibility that one of the activators may be acting on an enzyme present in contaminating particles is probably eliminated by the fact that these same results were obtained with mitochondria prepared in a variety of ways and known to be relatively free

of contamination. Whether or not there are classes of mitochondria which contain only one of these enzymes is not known at the present time and remains a subject for further investigation. In such studies, the new method for the preparation of morphologically well preserved mitochondria should be of great value.

SUMMARY

Changes in the morphology of rat liver mitochondria brought about by different methods of isolation and the concomitant changes in ATP-ase activity were studied. The morphology was investigated with the electron microscope. It was found that the ATP-ase activity of the isolated mitochondria cannot be readily correlated with the morphology of the mitochondria. The ATP-ase found in these preparations was latent, resembling the enzyme described in mitochondria prepared in 0.25 M sucrose. In confirmation of earlier results the use of 0.88 M sucrose yielded preparations with a higher initial ATP-ase than did other methods. Preparation in 0.25 M sucrose resulted in round, swollen mitochondria of which 30 to 40 per cent appeared to have lost a substantial part of the mitochondrial matrix. Preparations in 0.44 to 0.88 M sucrose contained mainly rod-shaped mitochondria plus a small amount of another type of swollen mitochondria. The matrix of mitochondria isolated in 0.88 M sucrose was highly condensed. By the use of 0.44 M sucrose adjusted to pH 6.2 with citric acid, it was possible to isolate, for the first time, mitochondria closely resembling those *in situ* and containing latent ATP-ase.

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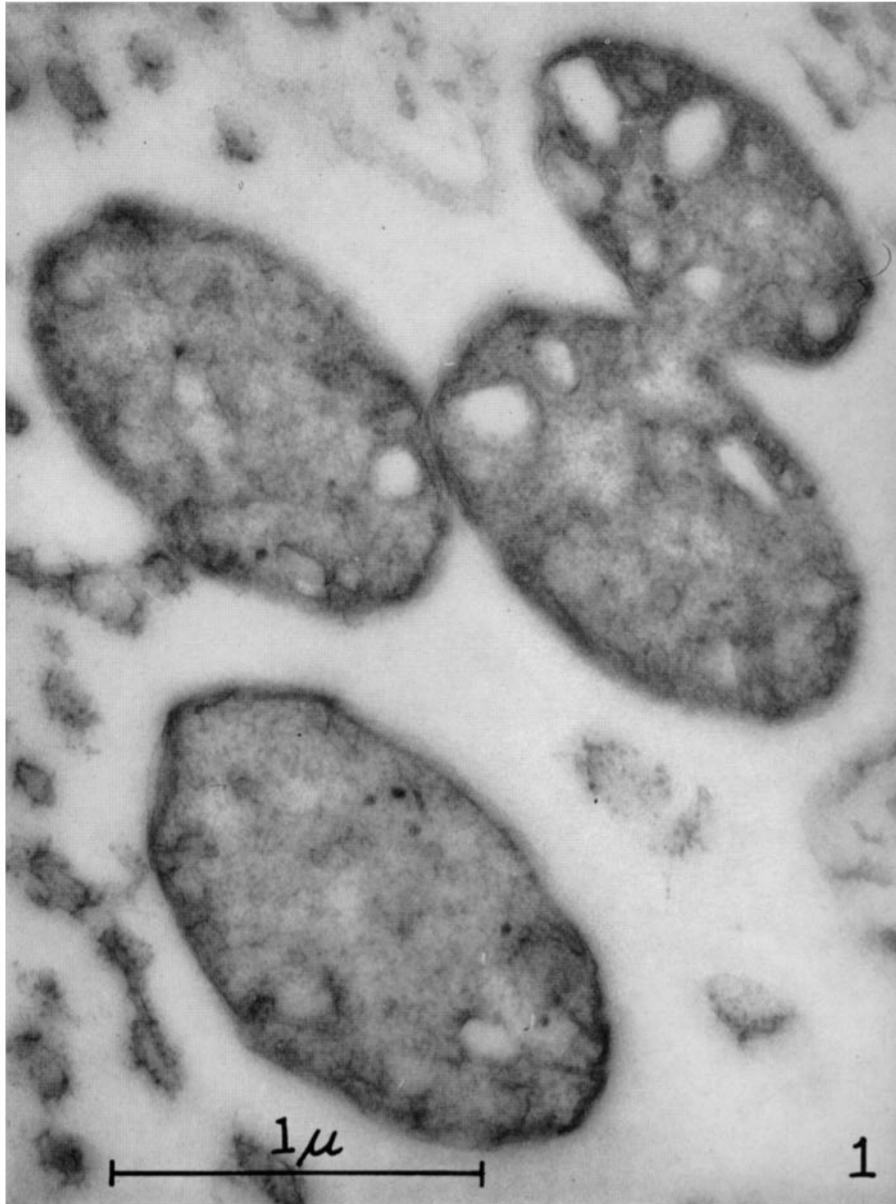
EXPLANATION OF PLATES

PLATE 40

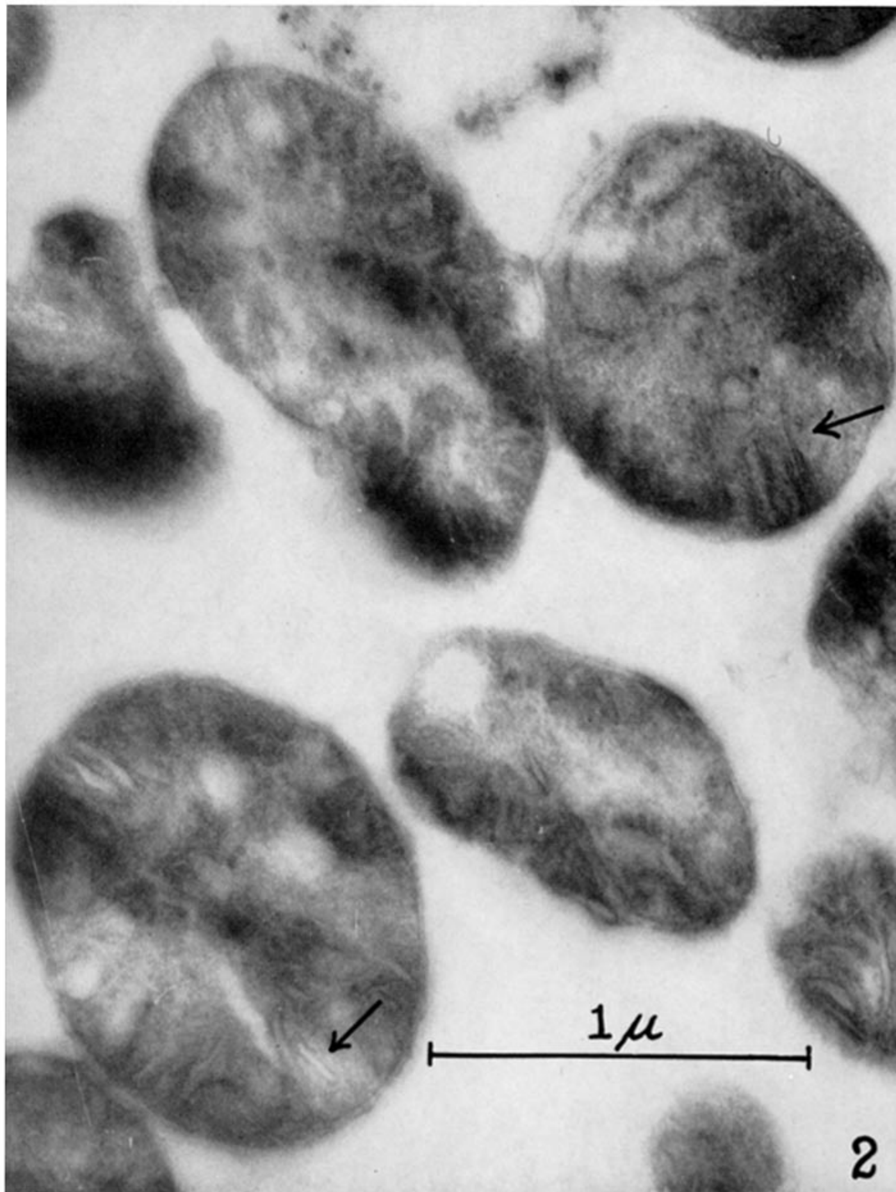
FIG. 1. Electron micrograph of thin sections of rat liver mitochondria which were isolated in 0.25 molar sucrose. They are considerably swollen and show little of their native internal structure. Light areas which seem to be associated with the cristae mitochondriales probably result from a general vesiculation of the organelles characteristic of this method of isolation. Other extramitochondrial structures in the micrograph represent elements of the endoplasmic reticulum present as contaminants in the original preparation. × 50,000.



(Witter *et al.*: Morphology and ATP-ase of isolated mitochondria)

PLATE 41

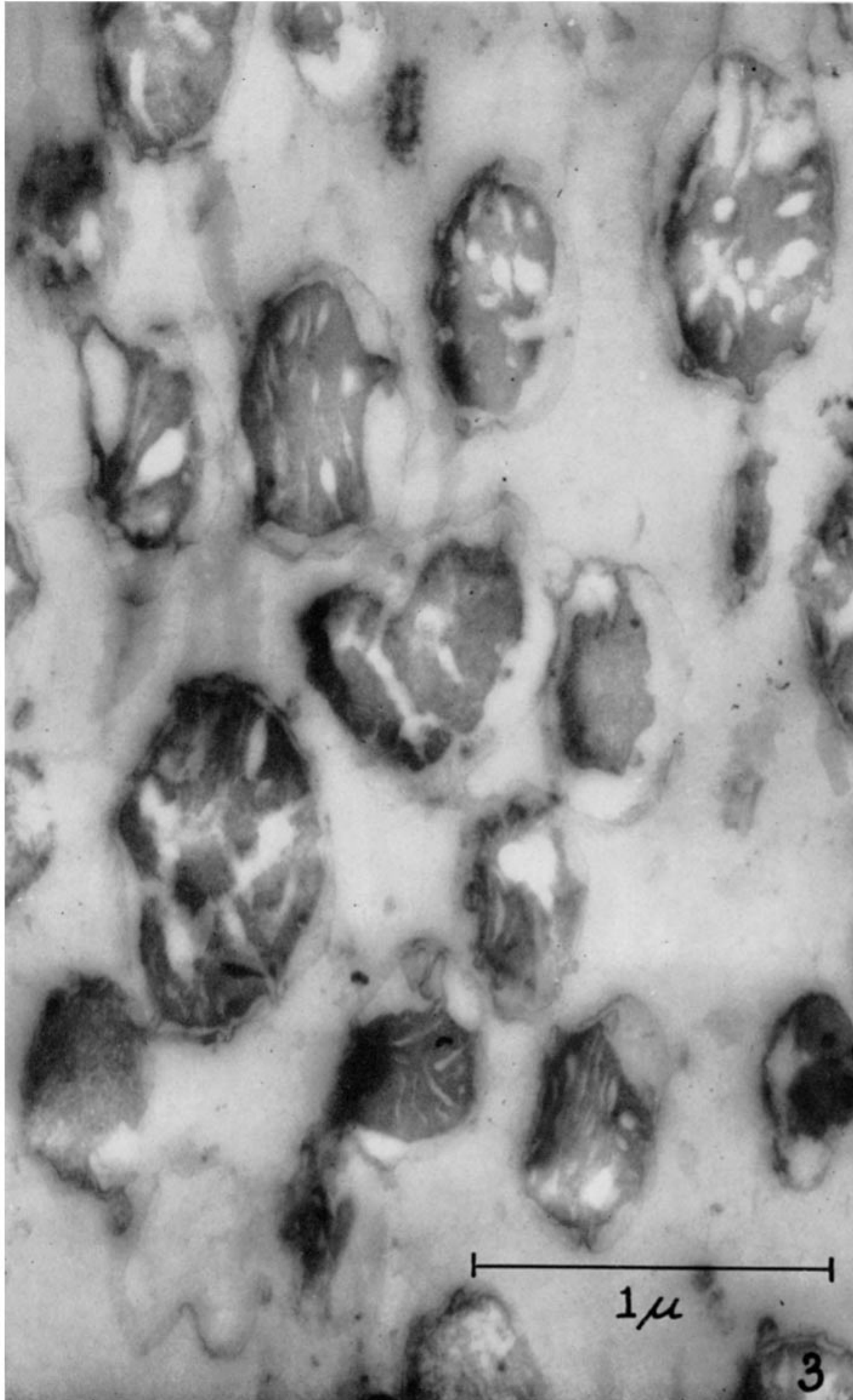
FIG. 2. Rat liver mitochondria isolated in 0.44 molar sucrose adjusted to pH 6.2 with citric acid. Mitochondria prepared in this manner most closely resembled in fine structure the appearance of mitochondria sectioned *in situ*. The cristae mitochondriales are visible (arrows) and relatively well preserved, and the characteristic double structure of the mitochondrial membrane or wall is clearly evident in places. × 50,000.



(Witter *et al.*: Morphology and ATP-ase of isolated mitochondria)

PLATE 42

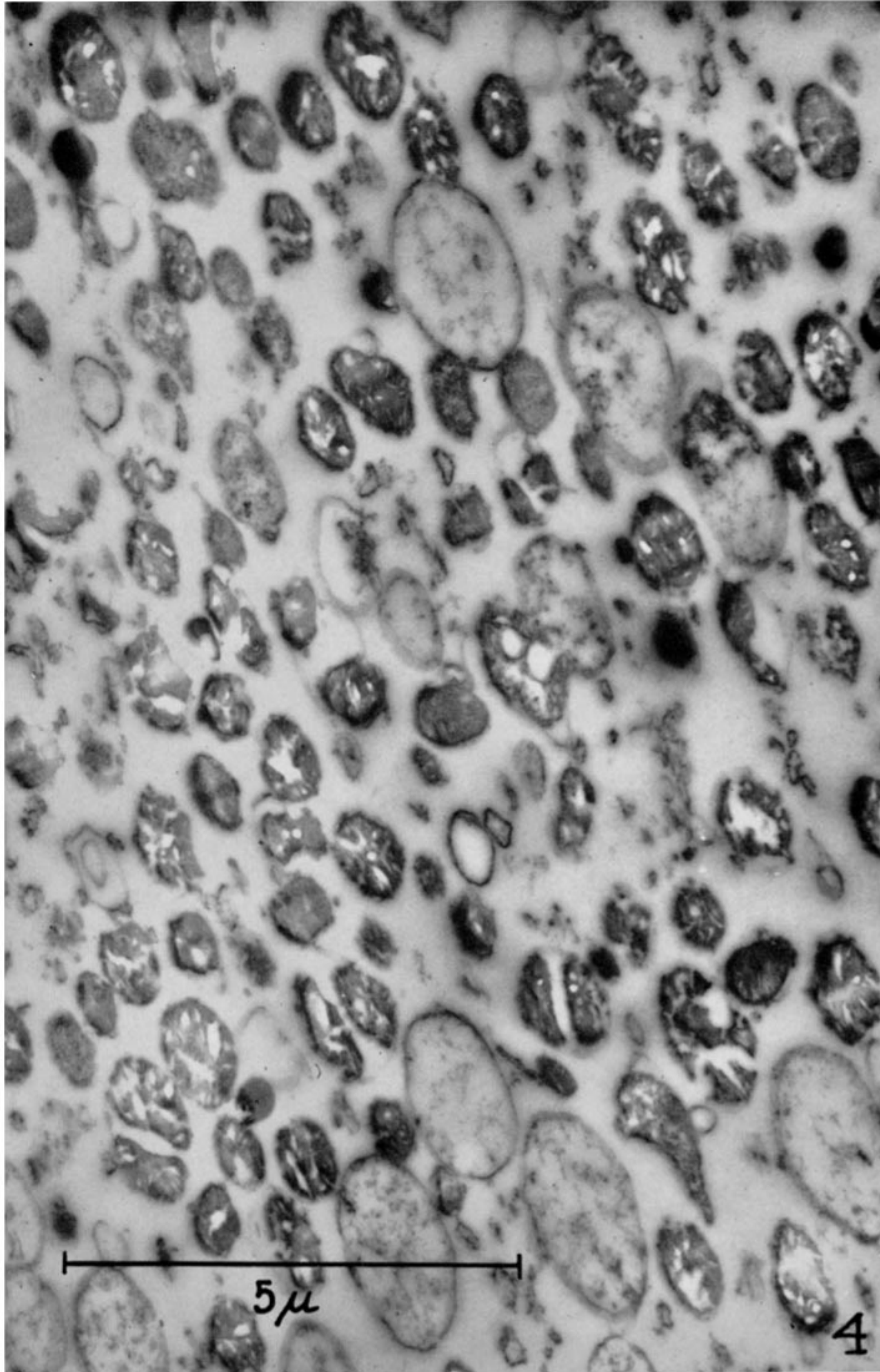
FIG. 3. Micrograph of rat liver mitochondria isolated in 0.88 molar sucrose. The matrix material appears condensed and the membranes of the cristae are pulled apart to form elongate vesicles or cracks. The matrix has separated from the outer mitochondrial membrane which is seen as a loosely applied sheath around each mitochondrion. × 50,000.



(Witter *et al.*: Morphology and ATP-ase of isolated mitochondria)

PLATE 43

FIG. 4. Lower power micrograph of mitochondria isolated in 0.88 molar sucrose and resuspended in 0.25 molar sucrose. There is little difference between these mitochondria and others similarly isolated, but not resuspended in sucrose of low tonicity. $\times$ 12,500.



(Witter *et al.*: Morphology and ATP-ase of isolated mitochondria)