THE ISOLATION AND MORPHOLOGY OF MITOCHONDRIA FROM THE MUSCLE TISSUE OF ASCARIS

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THE ISOLATION AND MORPHOLOGY OF MITOCHONDRIA

FROM THE MUSCLE TISSUE OF ASCARIS

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CHAPTER I

INTRODUCTION

The Mitochondrion

Mitochondria are subcellular particles, numbering from 500 to 2500 per cell, which have been studied for over one hundred years (Schneider, 1959). Lehninger (1964) pointed out in a recent monograph that sarcosomes of muscle cells were described and named in 1850 by Kolliker; although, Altmann in 1890 was the first to recognize these particles ("bioplass") as biologically important to all cells. The word mitochondrion was not introduced until 1898 when it was coined by Benda (Whittaker, 1966). The word was formed from the Greek roots "mitos" meaning thread and "chondros" meaning grain. It was not until 1934 that mass isolation of mitochondria was realized by Bensley and Hoerr. Soon after the advent of electron microscopy, Palade (1953) provided the classical picture of the mitochondria as it appears in a variety of cells. Studies with the electron microscope and with isolated organelles led to our current concept of the structure and function of mitochondria.

In all species studied to date, the mitochondria have certain structural features in common (i.e., double lamellar structure, and christae, etc.). However, Dempsey (1956) observed a high degree of heteromorphism between the mitochondria derived from different life forms and even between various tissues from the same animal. He also

found that an individual cell possesses mitochondria that are heteromorphic. Moreover, mitochondria which varied in morphology, also varied in their metabolic capabilities. It has been pointed out that differences in mitochondrial morphology are a reflection of differences in the enzymatic makeup of the mitochondria (Green, 1966; Kuff and Schneider, 1954).

Various investigators have determined that the mitochondria enzymes are involved in either oxidative or synthetic processes (Novikoff; 1961, Lehninger, 1964; and Whittaker, 1966).

The universal function of all mitochondria is to couple oxidation processes with the synthesis of adenosine triphosphate (ATP) (Green, 1966). The main pathway for this is the Citric Acid or Krebs' Cycle; however, the mitochondria can oxidize other substances, such as fatty acids and amino acids to give rise to members of the Citric Acid Cycle. Other substances which can be oxidized are α-glycerophosphate and β-hydroxybutyrate, though they have no direct connection with the Citric Acid Cycle. The synthetic systems include: transaminations, the synthesis of p-amino hippuric acid, citrulline, proteins, lipids and nucleic acids.

Within the last decade, various techniques have been utilized to fractionate mitochondria into particles more manageable for analytical investigation. One such technique is the use of negative staining without prior fixation (Whittaker, 1966). This causes the mitochondria to swell and burst, forming large amounts of membranous material which appeared as tubules and ribbons with spherical knobs along their borders. Fernández-Moran (1962) termed these knobs "elementary particles". Such particles were described in the mitochondria which had

been derived from various animal tissues, even from the muscle tissue of adult Ascaris lumbricoides suum, which lacked cytochromes a and c_1 (Chance, 1963). This observation was used to argue against the theory that elementary particles were the site of cytochrome function and, consequently, the site of respiratory activity.

Parsons, et al., (1967) reported a technique for separating and studying the inner and outer membranes of mitochondria derived from guinea pig liver. He found no components of the Citric Acid Cycle in the outer membrane. The results of his investigation led him to the conclusion that the outer membrane was similar to the smooth endoplasmic reticulum, while the inner membrane was similar to the plasma membrane of bacteria. This comparison remains the basis of the "endosymbiotic" theory of the evolution of mitochondria (Parsons, et al., 1967; and Roodyn and Wilkie, 1968).

The Mitochondria of Ascaris

Epithelial cells of the gut of Ascaris lumbricoides suum are large and so are the mitochondria found within these cells. With the light microscope, these mitochondria appear to be arranged in strands. Kessel, et al., (1961) reported that mitochondria were aggregated somewhat at the apical end of the gut epithelial cells, while in other cells the mitochondria were distributed more or less evenly throughout the cytoplasm. When one examines mitochondria of the gut eipithelium with the electron microscope they appear to have various forms (Sheffield, 1964). Some are seen as ovals, while others appear as elongated beaded strands. Cristae are recognized, but they are not plentiful and are not found in all mitochondrial profiles examined.

The mitochondria of ascarid muscle take on various morphological characteristics depending on their location within the cell (Reyer, 1964; and Rosenbluth, 1965a and b). The muscle cells themselves are very unusual in that they have three distinct parts, a belly, a fiber and an arm (Rosenbluth, 1965a and b). The belly, the largest portion, contains a nucleus, a great store of glycogen, and a very few mitochondria. The fiber contains the contractile apparatus and numerous mitochondria. The arm extends from the belly and makes contact with the worm's nervous system. It contains giant mitochondria, which possess large vesicular cristae.

There is strong evidence that neither a complete Citric Acid Cycle nor an electron transport system operate in adult Ascaris (Bueding, 1962 a and b; Fairbairn, 1957; and Fairbairn, 1960). Kikuchi (1961) obtained measurements with enriched preparations of Ascaris muscle mitochondria which he believed demonstrated the presence of cytochromes in the organelle. However, other investigators (Seidman and Entner, 1961; Kmetic and Bueding, 1961; and Chance, 1963) failed to confirm his observations. Lactic acid does not accumulate and is not excreted by the worm, instead various volatile acids (acetic, propionic, n-valeric, 2-methylbutyric and 2-methylvaleric acid) as well as non-volatile succinic acid and acetoin are fermentation products (Saz and Bueding, 1966). The anaerobic production of succinic acid in ascarid muscle requires a mitochondrial system which transfers electrons from a reduced pyridine nucleotide to fumaric acid (Kmetic and Bueding, 1961; and Seidman and Entner, 1961). In this process an ATP is formed from ADP and inorganic phosphate. Propionic acid is formed by the decarboxylation of succinic acid (Saz and Vidrine, 1959). Saz and Weil (1960, and

1962) showed that Ascaris muscle catalyzed the condensation of propionic acid with acetic acid or with a second propionic acid to form respectively 2-methylbutyric and 2-methylvaleric acid. Presumably these volatile acids were formed by enzymes located within the mitochondria but the reactions were described from determinations carried out with intact tissue.

Isolation of Subcellular Components

One of the objectives of cellular physiology is to determine the intracellular molecular arrangement of enzyme systems and to relate them to their function (Schnel and Anderson, 1964). This objective has been approached by morphological determinations and biochemical analysis of isolated cellular components. Many investigators have pointed out that with the latter approach it is necessary to test the purity of isolated components and to be critically aware of the limitations of the isolation method employed (Allfrey, 1959; Anderson, 1956; De Duve, 1964; Kmetic and Bueding, 1961).

In general terms, there are three steps in the isolation of cellular organelles (Grant, 1963). The first step is to convert the particular tissue under investigation into a "homogenate". That is, the cellular components are randomized. The second step is to reorder the cellular components, relying upon their differing physical characteristics (i.e., density or sedimentation coefficient). The third step is to analyze the isolated fractions. The methods used to "homogenize" and reorder the tissue are determined by the objectives of the researcher and the analyses that are to be carried out. The criteria which are employed to analyze the purity of the isolated fractions are both

morphological and biochemical. Many investigators employ only morphological criteria; but, the addition of biochemical criteria can give the investigator greater confidence in his isolation technique.

Differential Centrifugation

Differential centrifugation involves filling a centrifuge tube with homogenate and subjecting it to a constant gravitational force for a given amount of time. The resolution which may be achieved is obviously limited because small particles near the bottom of the tube will sediment out along with the larger ones near the top (Anderson, 1956). Nevertheless, satisfactory separations of various subcellular particles have been achieved and the simplicity of the procedure causes it to be used frequently.

Density Gradient Centrifugation

Density gradient centrifugation, in contrast to differential centrifugation, affords a method of separating particles on the basis of highly uniform physical characteristics (De Duve, 1965; and Thompson, 1959). The homogenate is layered on top of a stabilizing gradient made of materials suitable to the experiment. A centrifugal force is applied which is insufficient to cause complete sedimentation of the particles of the homogenate. After a period of centrifugation, the various components are dispersed linearly along the gradient. Their position is determined by their sedimentation coefficient (Anderson, 1956; and De Duve, 1965). The component layers can be removed and assayed for a particular enzyme.

There are numerous pitfalls in density gradient centrifugation

(De Duve, et al., 1959). One of the first considerations should be a possible lack of geometry in the centrifugation system. Of equal importance is variable sedimentation rates for particles of the same type. Sedimentation rate is influenced by the characteristics of the particle under consideration, the composition of the medium, the temperature, and the particle concentration. Finally, intrinsic characteristics of the centrifuge can present problems. These include swirling, vibration, and thermal convection. Most of these latter problems have been removed by the manufacturers of modern centrifuges, but their possible presence cannot be ignored.

There are other variables which also must be considered carefully. Osmotic pressures must be taken into account in the determination of particle size. This is handled by a variation of Stokes' Law. Another variable is the method of tissue homogenization. The use of the masher type tissue homogenizer has a particularly deleterious effect on mitochondria, but not apparently on the other organelles. Such homogenizers considerably alter the morphology of mitochondria, but have little effect on the rate of oxidative phosphorylation (Thompson, 1959). A more suitable homogenizer is the Potter-Elvejehm homogenizer fitted with a teflon pestle.

The most popular media for density gradient centrifugation is sucrose; but, polyvinyl pyrrolidone (PVP) also has been used (Thompson and Klipfel, 1958). One particular advantage of PVP is that it does not cause the gross morphological changes in mitochondria which are associated with the use of sucrose gradients. A particular disadvantage is its greater viscosity, which necessitates the employment of greater centrifugal forces.

Isolation of Mitochondria From *Ascaris*

Kmetic, *et al.*, (1962) used differential centrifugation to isolate mitochondria from tissues of *Ascaris*, which they then examined with the electron microscope. The mitochondria isolated from tissue that was homogenized in dialyzed body fluid showed the least morphological change. They reported that the mitochondria had a diameter of 0.33 μ and that the cristae extended only a short distance into the matrix. They failed to find lamellar cristae or the outer membrane that had been reported in other species. They suggested that the small size of the cristae was associated with the essentially anaerobic type of metabolism found in adult *Ascaris*.

Differential centrifugation does not always produce a highly purified fraction of mitochondria when it is employed with *Ascaris* tissue; yet, for determinations relating to enzyme-catalyzed-reactions, it is desirable to have mitochondria free of other subcellular material. This thesis presents a preferred method of density gradient centrifugation that isolates muscle-mitochondria of *Ascaris* in reasonable quantity and free of contaminating subcellular material.

CHAPTER II

MATERIALS AND METHODS

Tissue Preparation

Adult female Ascaris lumbricoides suum were collected at the packing house and transported to the laboratory in saline (Harpur, 1963) maintained between 35-39° C. Worms were held at 37° C and used in experiments not more than 6 hours after they were collected.

Worms were incised longitudinally with scissors, the intestine and reproductive system were removed and strips of muscle tissue were isolated from the cuticle. For the isolation of mitochondria, muscle strips were minced with scissors and homogenized in an appropriate solution with the aid of a Potter-Elvehjehm tissue grinder equipped with a teflon pestle.

Centrifugation

All centrifugation was carried out with a Sorvall RC2-B refrigerated centrifuge.

The differential centrifugation technique employed a fixed angle rotor with a radius of 4.25 inches. The following sequence of steps was carried out at 2° C.

(1) A 10% homogenate (1:9, w/v) was centrifuged for 10 min. at 128 x g (S_1).

(2) The S_1 supernatant was saved and centrifuged for 30 min. at

2,000 x g (S_2).

(3) The S_2 supernatant was saved and centrifuged for 30 min. at 9,500 x g (S_3).

(4) The S_3 pellet was saved, resuspended in the original volume of buffer and centrifuged for 30 min. at 9,500 x g (S_4).
The S_4 pellet was taken as the mitochondrial fraction. The procedure is diagrammed in Appendix A.

The density gradient centrifugation employed a swinging bucket rotor with a radius of 5.75 inches. A gradient-former manufactured by Beckman Instrument Co. was used to prepare the 14-50% gradient of sucrose in phosphate buffer, pH 7.5. It was maintained at 2^{o} C and could be held for up to 5 days in the refrigerator. A 20 cc syringe equipped with a 20 gauge 6 inch hypodermic needle with the point bent into a hook was secured in a micromanipulator apparatus and used to layer the supernatant onto the gradient column. The apparatus is diagrammed in Appendix B. After centrifugation the bands of subcellular material were removed from the gradient with the same apparatus. Absorbance of each band was determined at 260 mμ with a Cary Model 15 spectrophotometer. The following steps in the isolation prior to the gradient centrifugation were carried out at 2^{o} C.

(1) A 17% homogenate (1:5, w/v) was centrifuged for 10 min. at 500 x g (S_1).

(2) The pellet and supernatant were rehomogenized and centrifuged for 10 min. at 500 x g (S_2).

(3) Five ml. of the S_2 supernatant was layered on the sucrose gradient.

(4) The gradient was centrifuged for 90 min. at 27,000 x g.

The procedure is diagrammed in Appendix C.

Cytological Techniques

Immediately after removing the tissue, samples were sliced to an appropriate size for cytological examination. The tissue was rinsed in 0.05 M potassium phosphate buffer, pH 7.5, maintained at 2^{o} C, and fixed for one hour in 0.02 M phosphate buffer, pH 7.4, containing 1% osmium tetroxide (Palade's fixative, Appendix D (1)). The fixed tissue was washed in distilled water, dehydrated at 2^{o} C in graded dilutions of ethanol and embedded in a mixture of Epon 812 and Araldite 6005 (Appendix D (2)). Silver and gold sections were cut with glass knives and a Sorvall MT-2 microtome. In most instances, the sections were mounted on 200 mesh copper grids or 120 mesh nickel grids. When serial sections were prepared they were placed on 100 mesh parlodion (Appendix D (3)) coated grids stabilized by carbon coating. The mounted sections were stained with lead citrate (Venable and Coggeshell, 1965; Appendix D (4)) and examined with a Philips EM 200 electron microscope at 60 KV using a 25 micron objective apperture. Electron micorgraphs were taken on Eastman Fine Grain Release Positive film type 5302 developed in DK60a (Kodak).

Small bits of the subcellular fractions, isolated by the various centrifugation procedures were carried through the fixation, embedding and sectioning procedure for examination with the electron microscope.

Biochemical Assay

The colorimetric method of Ells (1959) was employed to determine dehydrogenase activity. The method measures the reduction of the dye

2,6-dichlorophenol indophenol at 600 mμ. The dye is reduced when electrons are transferred from the substrate of the experimental system. Protein was determined by the method of Lowry, et al., (1951).

CHAPTER III

RESULTS

Description of Mitochondria *in Situ*

Muscle-Mitochondria

Mitochondria appear in every region of *Ascaris* muscle cells, but they are concentrated in the cell's sarcoplasmic core. The mitochondria are quite heteromorphic as evidenced by their highly lobular to near spherical forms shown in Plate 1-1. The cross sectional diameters of these mitochondria range from about 1 μ to over 2 μ.

One of the peculiarities of ascarid mitochondria is the indistinctness of their outer double membrane (Kmetic, *et al.*, 1962). However, some mitochondria seen in this study clearly show the classical double membranous cover (Plate 1). Another peculiarity of these mitochondria is the size of the cristae. The cristae are usually small, appearing as isolated finger-like projections of the inner membrane as shown in Plate 1-2. Serial sections were prepared and the results are presented in Plate 2.

Serial sections of mitochondria (Plate 2) show that some of the apparent finger-like projections are, in fact, ridges extending along the inner membrane, that is to say, true cristae. On the other hand, the presence of isolated vesicular structures in the matrix of the cross-sectioned mitochondria could indicate the presence of finger-like

Plate 1. Electron Micrograph of Muscle-Mitochondria Fixed in Situ. 1) Typical mitochondria (m) interposed with glycogen (g) - X 15,000; 2) Large lobular mitochondrion with small finger-like cristae - X40,000; 3) Mitochondrion with an extended and looped crista. Double membranes appear at various points along the outer membrane - X40,000; Bar = 1 micron.

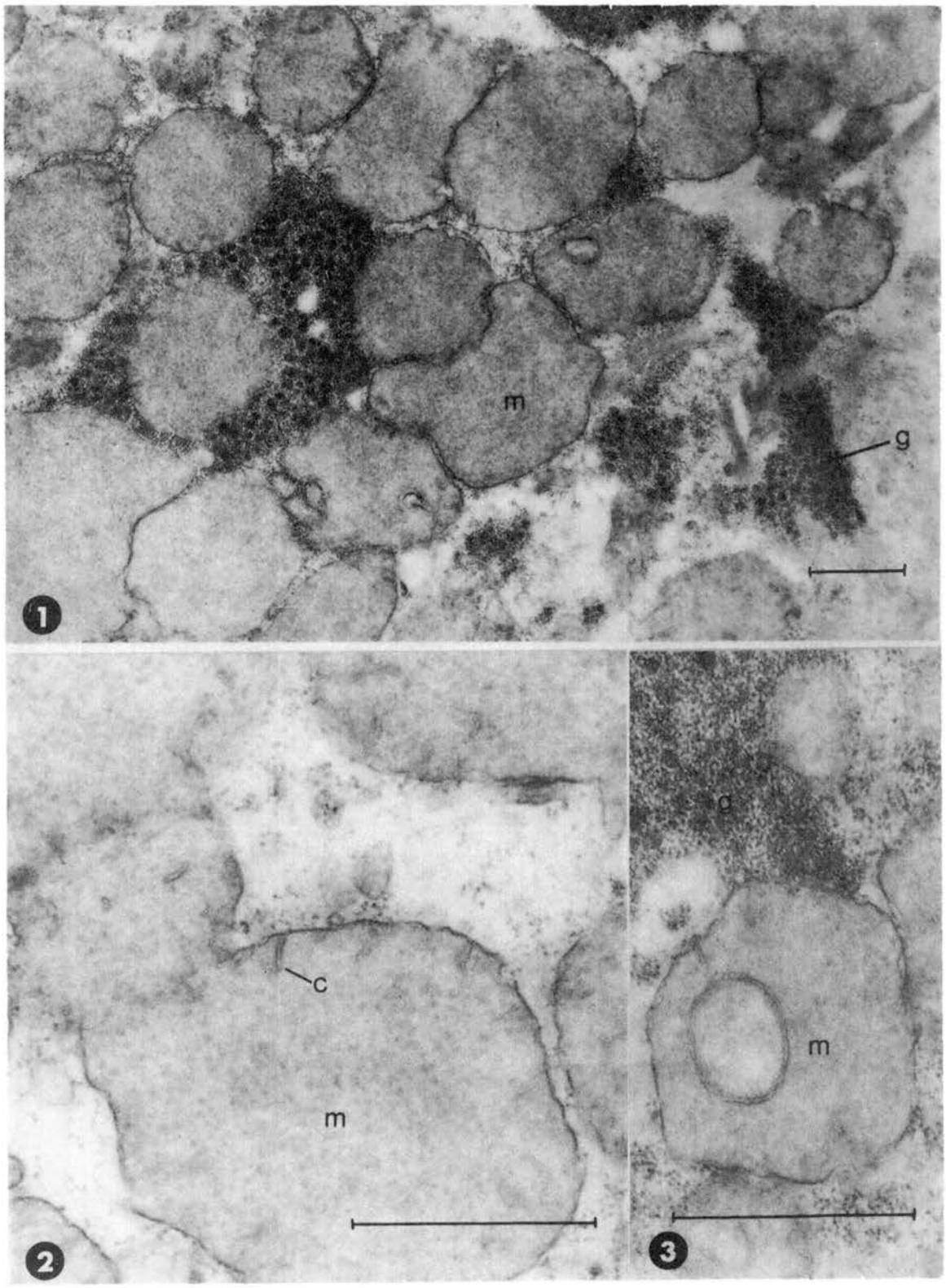
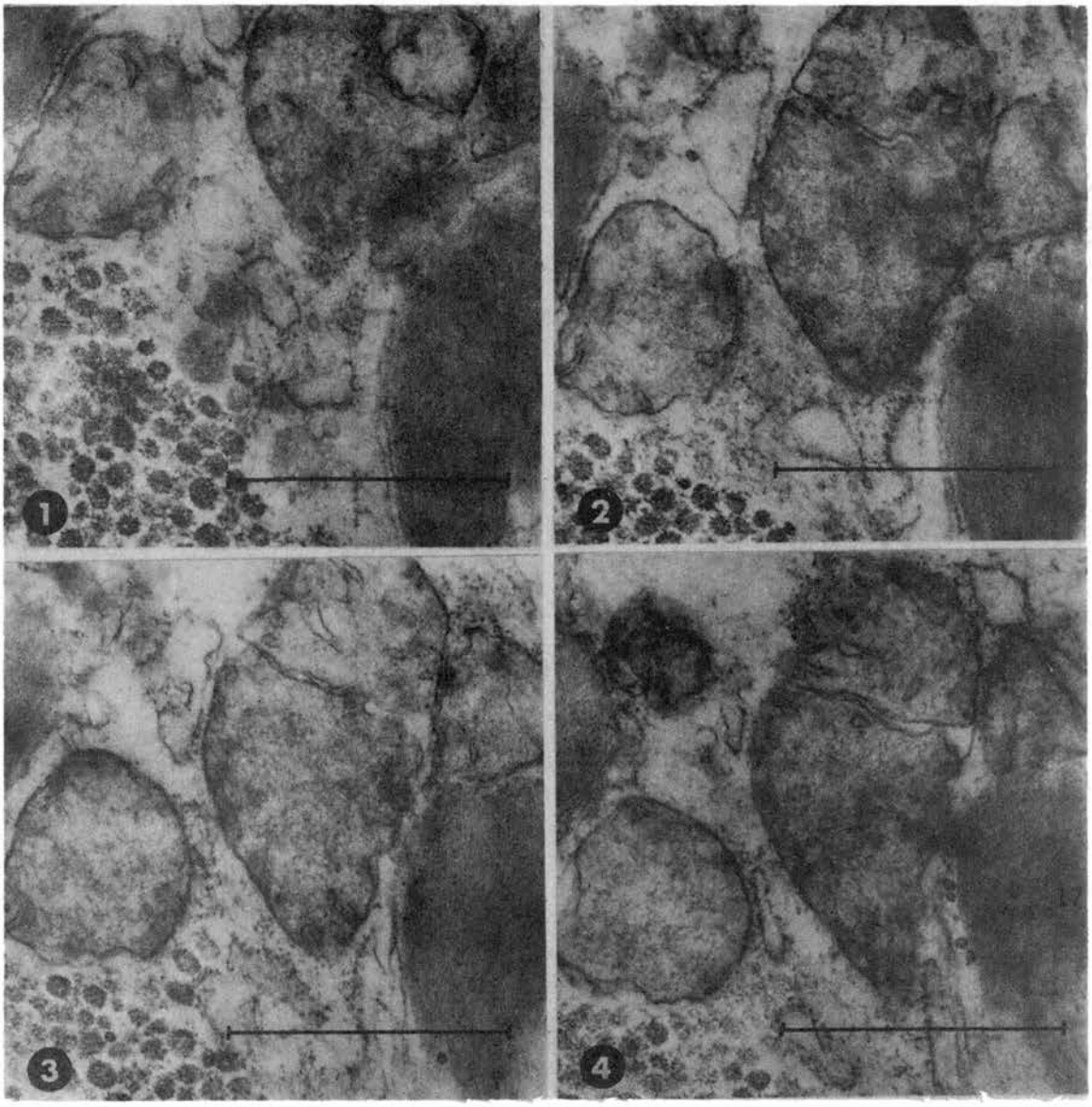

m
g
1
c
m
2
g
m
3

Plate 2. Serial Sections of Muscle-Mitochondria Fixed in Situ. A cleft-like crista bisects the larger mitochondrion and is found in all four sections. Since each section represents a thickness of 0.1 μ, the total width of the crista must equal not less than 0.5 μ. The outer membrane can be seen to be separate from the inner membrane at various points - X40,000; Bar = 1 micron.


1
2
3
4

infoldings as well. Evidence from the serial sections of Plate 2 indicates that the width of individual cristae can exceed 0.5 μ.

Gut-Mitochondria

As in the muscle cell, mitochondria of the gut-epithelium are found in every region of the cell; however, most appear in the central portion of the cell. Moreover, their size relates to their location. Those near the center are larger than those near the periphery of the cell.

Serial sections of the gut-epithelium (Plate 3) indicate that gut-mitochondria are highly lobular and their cristae form both ridges and finger-like projections. Like the muscle-mitochondria, gut-mitochondria vary extensively in their morphology. Some appear lobular; others more spherical as shown in Plate 3. The size of gut-mitochondria varies more than that of muscle-mitochondria. They ranged from less than 1 μ to over 3 μ in diameter. In many instances the double membrane structure is well delineated in the electron micrographs. Furthermore, the double membrane structure is less difficult to demonstrate than in the muscle-mitochondria. Gut-mitochondria contain longer and more vesicular cristae than muscle-mitochondria. Some of these cristae extend into the matrix and swell to hugh proportions. Structures at first seemingly similar to vesicular cristae are actually involuted portions of the outer membrane, as indicated by their double walls.

Ovary-Oviduct-Mitochondria

Electron micrographs of mitochondria of ovary tissue are presented in Plate 4. Although their size is similar to that of muscle-

Plate 3. Serial Sections of Gut-Mitochondria Fixed in Situ. 1 - 3 demonstrate that the mitochondria are highly lobular and that their cristae form both ridges and finger-like projections - X40,000; Bar = 1 micron.

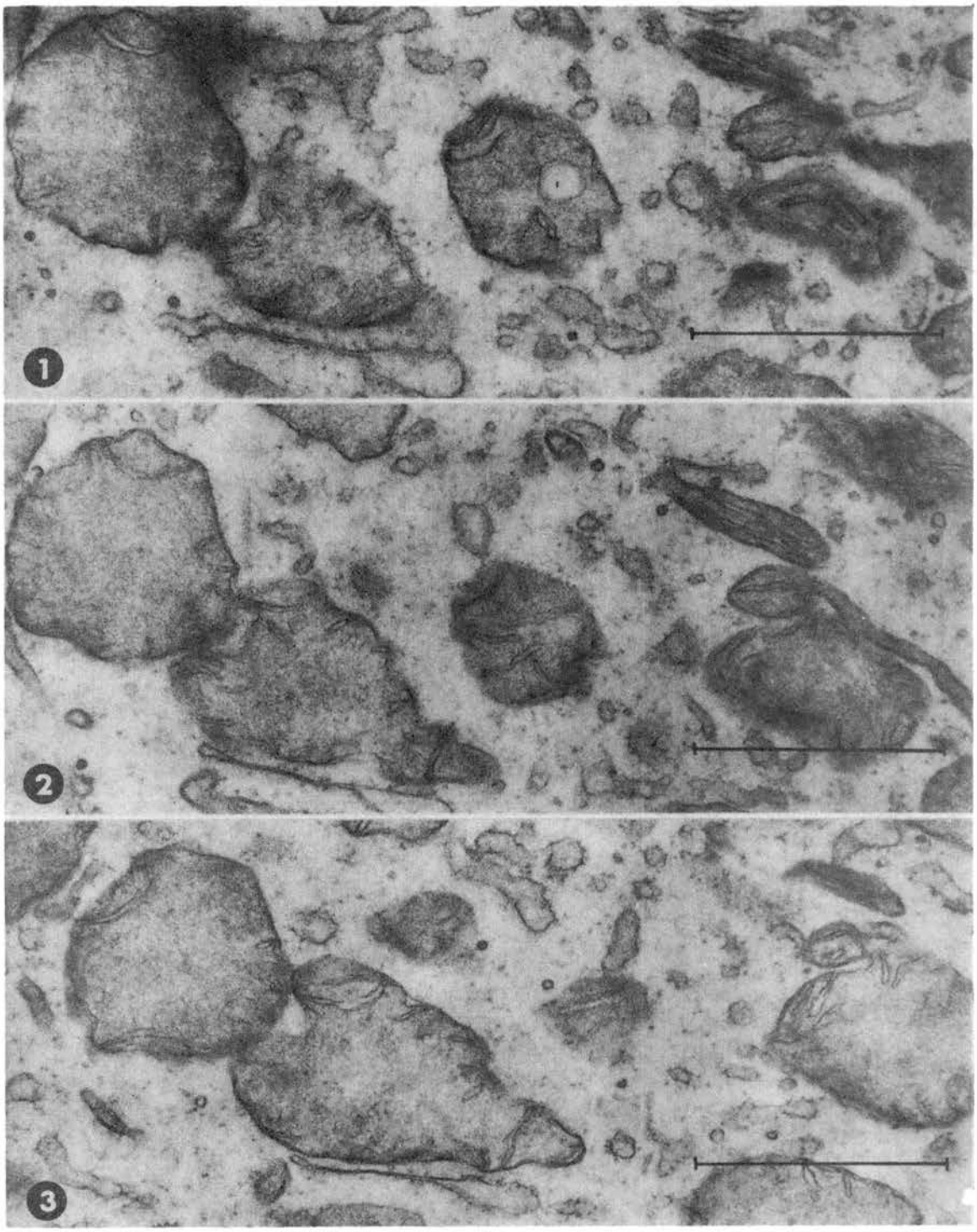
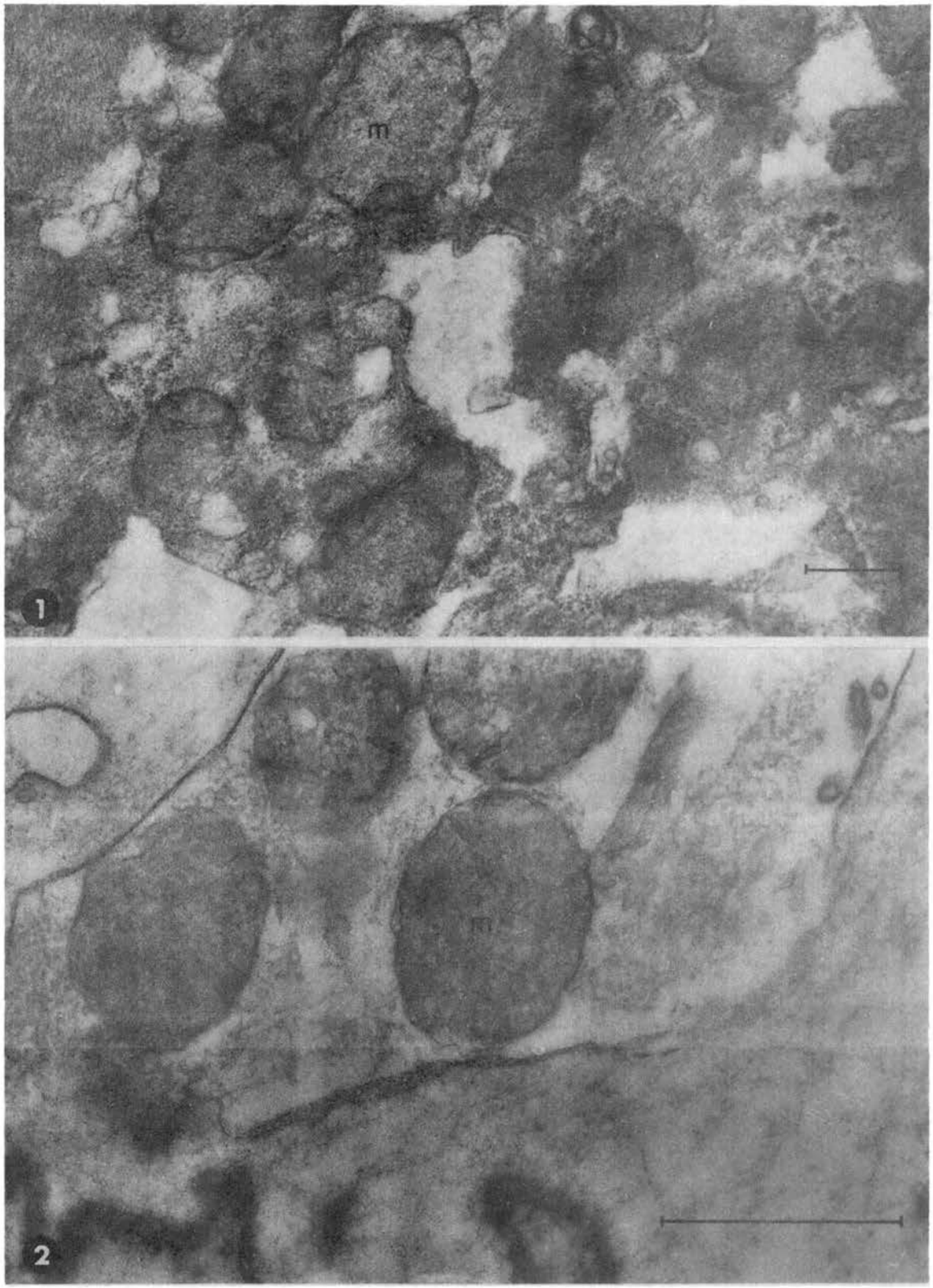

1
2
3

Plate 4. Ovarian-Mitochondria Fixed in Situ. 1) Ovarian-mitochondria resemble muscle-mitochondria except in the former the cristae appear to be larger and more numerous - X15,000; 2) Higher magnification reveals the presence of double membranes - X40,000; Bar = 1 micron.


m
1
m
2

mitochondria, the morphology of their cristae and outer double membrane are more like those of gut-mitochondria.

Isolation of Mitochondria

Differential Centrifugation

Following the approach of Kmetec, et al., (1962), uncontaminated mitochondrial fractions from muscle have been attempted by differential centrifugation. Five homogenization solutions have been tested. The composition of these solutions is presented in Table I. Some of the solutions used by Kmetec, et al., (1962) have been reinvestigated.

TABLE I

SOLUTIONS EMPLOYED TO HOMOGENIZE TISSUE FOR THE ISOLATION OF MITOCHONDRIA BY DIFFERENTIAL CENTRIFUGATION

	Material Added					
Solution No.[1]	Potassium Phosphate Buffer	Sucrose	Bovine Albumin	EDTA[2]	Hemolymph	Dialyzed Hemolymph
1	0.05 M	-	-	-	-	-
2	0.05 M	0.24 M	-	-	-	-
3	-	0.24 M	0.15%	0.005 M	-	-
4	-	-	-	-	+	-
5	-	-	-	-	-	+

[1]All solutions were adjusted to pH 7.5.

[2]EDTA = Ethylene Diamine Tetra-acetic acid.

A pellet isolated from potassium phosphate buffer (Solution No. 1; Table I) contains mitochondria which are slightly swollen (Plate 5); yet their outer membranes are not as scalloped and their general appearance is more uniform than muscle-mitochondria in Situ. The outer two membranes can be recognized in some, while in others this characteristic is totally obscured, possibly due to the swollen state of the matrix. These mitochondria present few cristae. Those seen are likely to have a vesicular form and are usually smaller than the cristae of mitochondria fixed in Situ. Glycogen and assorted unit membrane structures contaminate pellets derived from this solution.

When ascarid muscle is homogenized with phosphate buffer-sucrose solution (Solution No. 2; Table I) the isolated pellet contains mitochondria with more of the morphological characteristics of muscle-mitochondria fixed in Situ (Plate 6). Some swelling was apparent but there were numerous cristae. The outer two membranes rarely are defined. Glycogen granules and various membranous structures contaminate this pellet heavily.

Saz and Lescure (1969) isolated mitochondria from ascarid muscle homogenized in sucrose-bovine albumin-EDTA solution. Hence this solution (Solution No. 3, Table I) was employed for comparison and the results are presented in Plate 7. The morphological integrity of mitochondria is well preserved in the isolated pellet (Plate 7). The mitochondria vary in size and shape and frequently the double membrane structure can be recognized. Cristae are reasonably numerous; however, they are more vesicular than those in mitochondria isolated from homogenates with Solution No. 1 or 2, Table I. Although the mitochondria appear better preserved, there are more glycogen granules and membranous

Plate 5. Electron Micrographs of Muscle-Mitochondria Isolated by Differential Centrifugation, Using 0.05 M Phosphate Buffer (pH 7.5). 1) Mitochondria appear swollen and few cristae are present. Contamination consisted of glycogen and assorted unit membrane structures - X15,000; 2) In this electron micrograph, double membranes can be seen - X40,000; Bar = 1 micron.

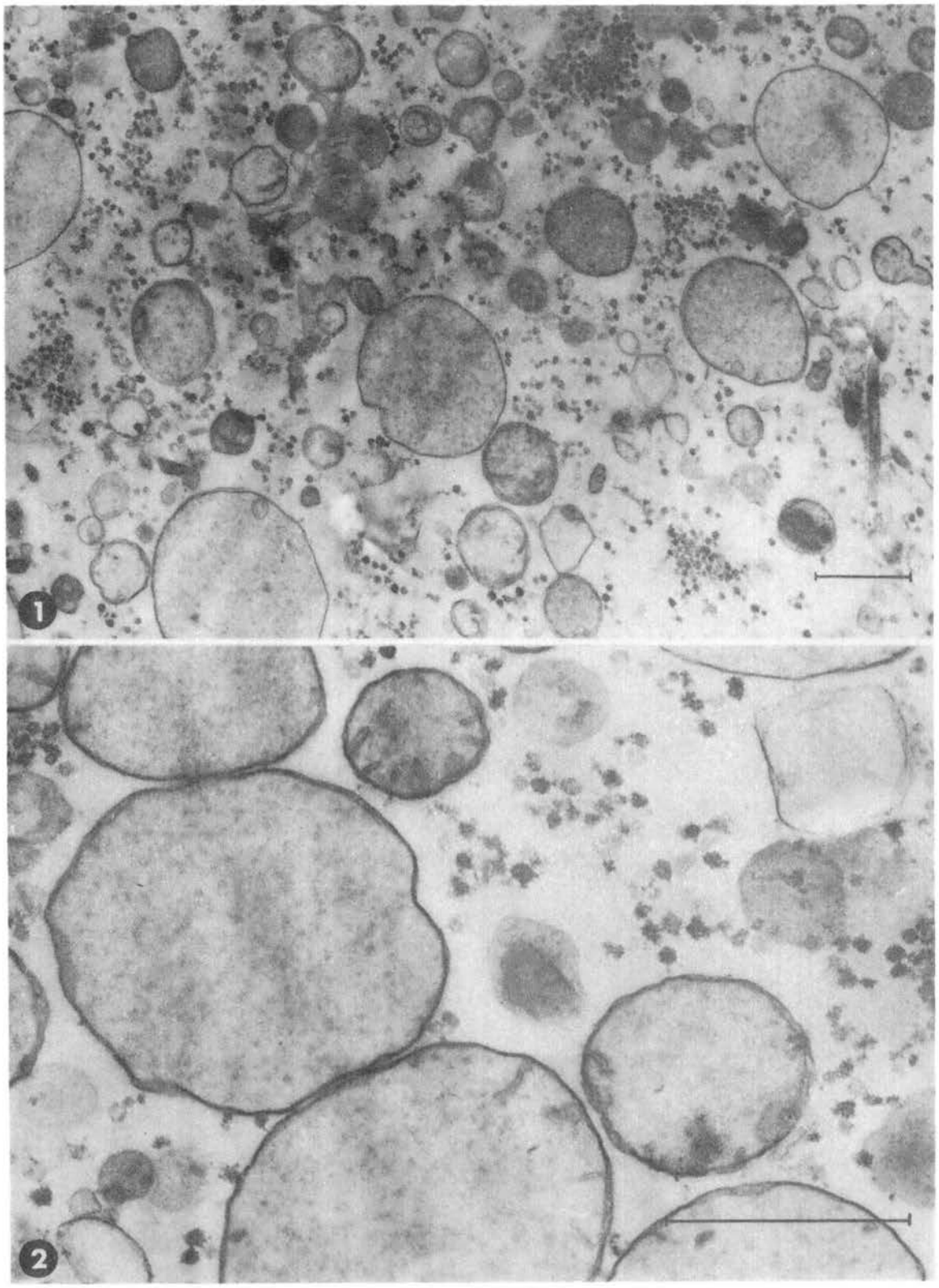

1
2

Plate 6. Electron Micrograph of Muscle-Mitochondria Isolated by Differential Centrifugation Using 0.05 M Phosphate Buffer and 0.24 M Sucrose. 1) Mitochondria are swollen. Cristae are few in number. Contamination consisted of glycogen and membranous structures - X15,000; 2) Double membranes are present as are vesicular cristae - X40,000; Bar = 1 micron.

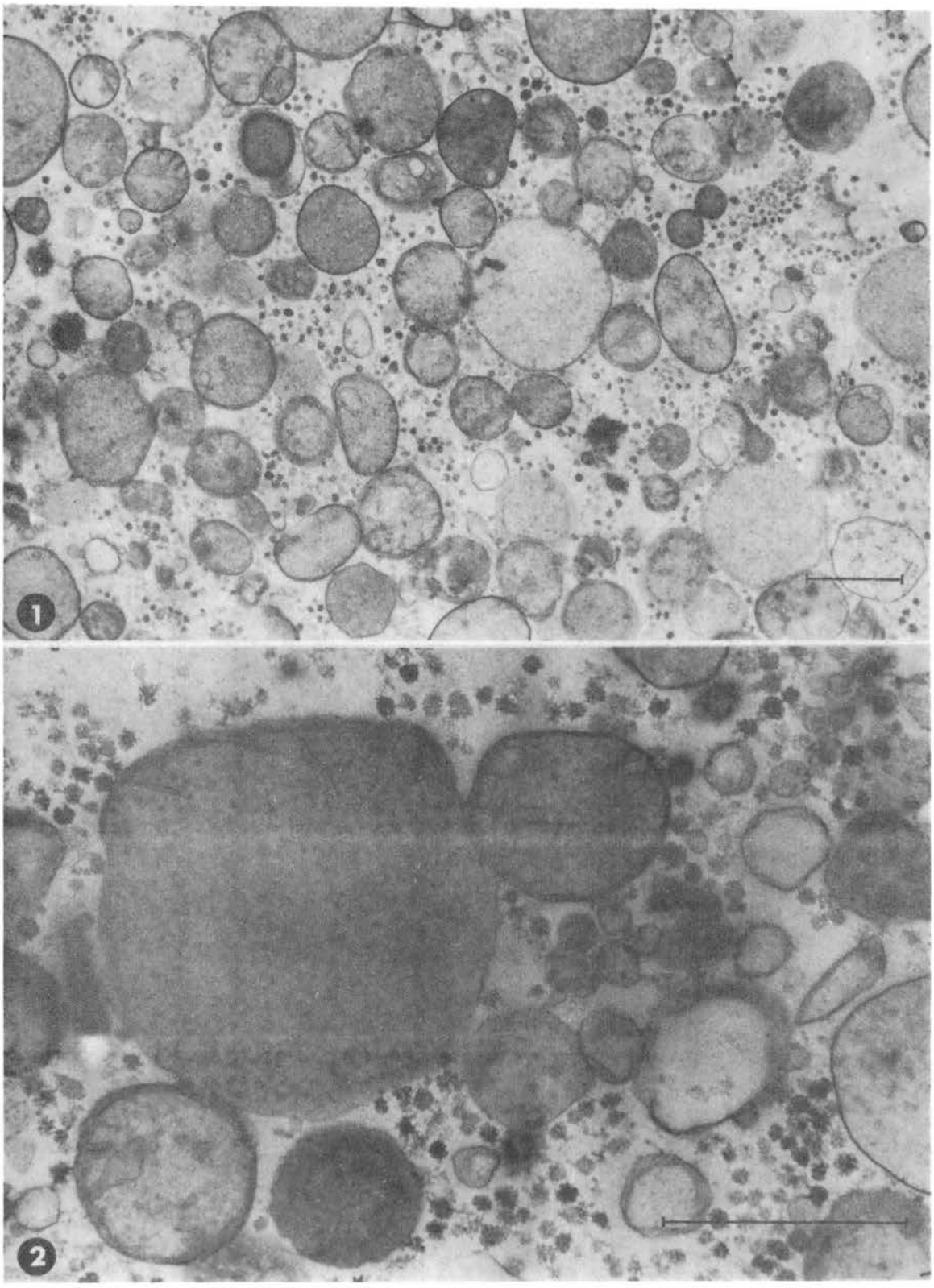
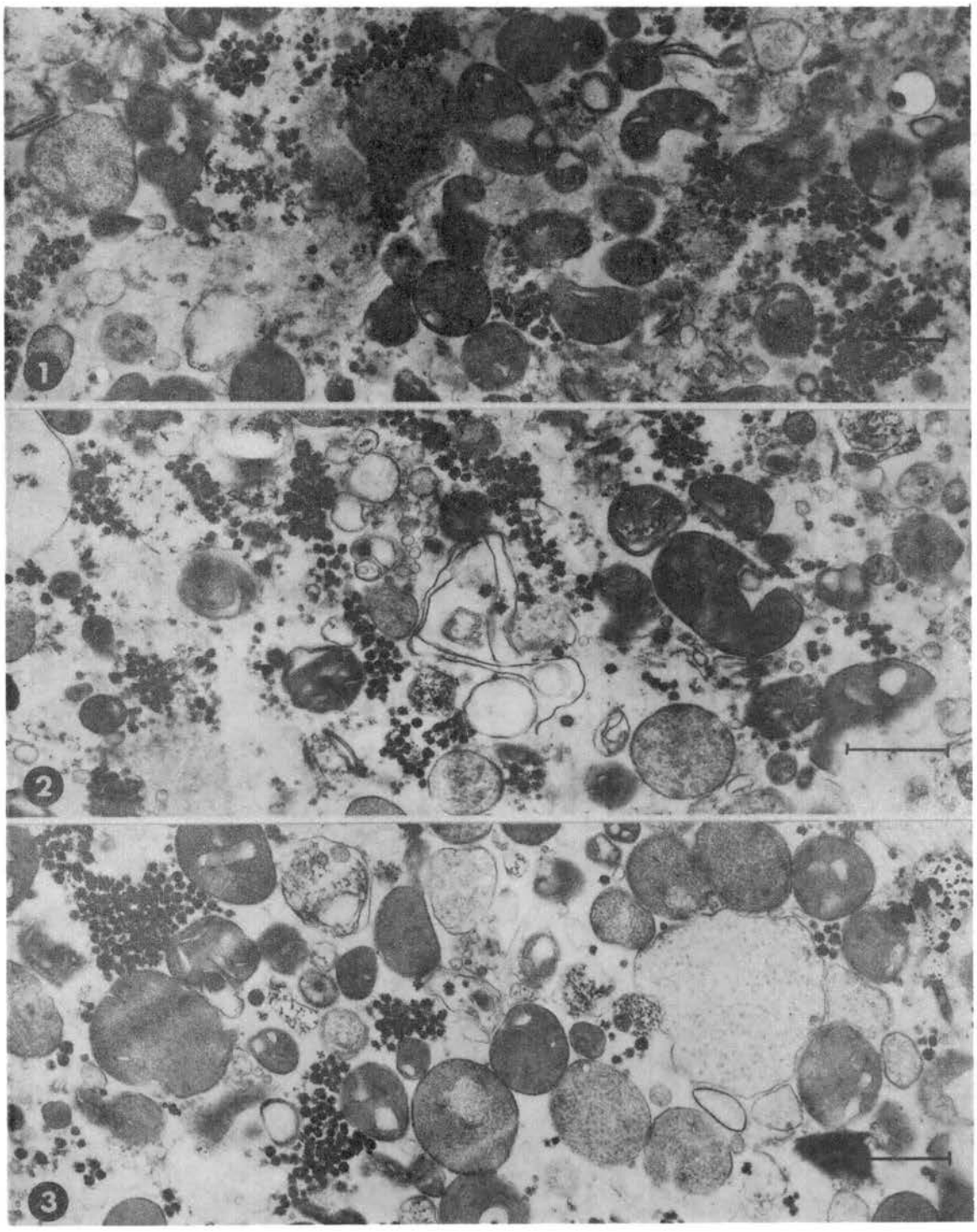

1
2

Plate 7. Electron Micrograph of Muscle-Mitochondria Isolated by Differential Centrifugation Using 0.24 M Sucrose, 0.15% Bovine Albumin, and 0.005 M E.D.T.A. 1) After one centrifugation, the mitochondria contained large vesicular cristae. Contamination consisted of glycogen and various membranous structures - X15,000; 2) The first pellet was resuspended and centrifuged a second time. No apparent reduction in the amount of contamination was observed - X15,000; 3) The pellet was resuspended and centrifuged for a third time. No apparent improvement in the isolation was observed - X15,000; Bar = 1 micron.


1
2
3

structures than are observed in the other pellets. In an attempt to remove more of the non-mitochondrial material, the pellet was resuspended and centrifuged. This process did not remove much of the material that contaminated the mitochondrial fraction (Plate 7-2). The procedure was repeated a second time; however, there is little change in the composition of the isolated pellet (Plate 7-3).

Very poor results (Plate 8) are obtained when attempts are made to isolate mitochondria from tissue homogenized with hemolymph (Solution No. 4, Table I). The mitochondria are swollen and in most instances the outer membrane is poorly defined. The pellet appears to be composed in large part of glycogen granules.

The structure of the mitochondria is very well preserved (Plate 9) when they are isolated from tissue homogenized with dialyzed hemolymph (Solution No. 5, Table I). The size of the mitochondria is near the ones fixed in Situ. The double membrane can be recognized, and the outer membrane appears to be slightly scalloped as found after in Situ fixation. The cristae are very similar to those of "normal" mitochondria. This agrees with previous reports by Kmetec, et al., (1962). However, the pellet contains many subcellular particles (glycogen granules, membrane structures and what appears to be intracellular skeletal fibers or muscle fibrils) in addition to the mitochondria.

The results of differential centrifugation indicate that it would be very difficult to isolate highly purified mitochondria from Ascaris muscle by this method.

Plate 8. Electron Micrograph of Muscle-Mitochondria Isolated by Differential Centrifugation Using Ascaris Hemolymph. 1) Sections showed few mitochondria but an abundance of glycogen - X15,000; 2) Greater magnification revealed tubular shaped cristae - X40,000; Bar = 1 micron.

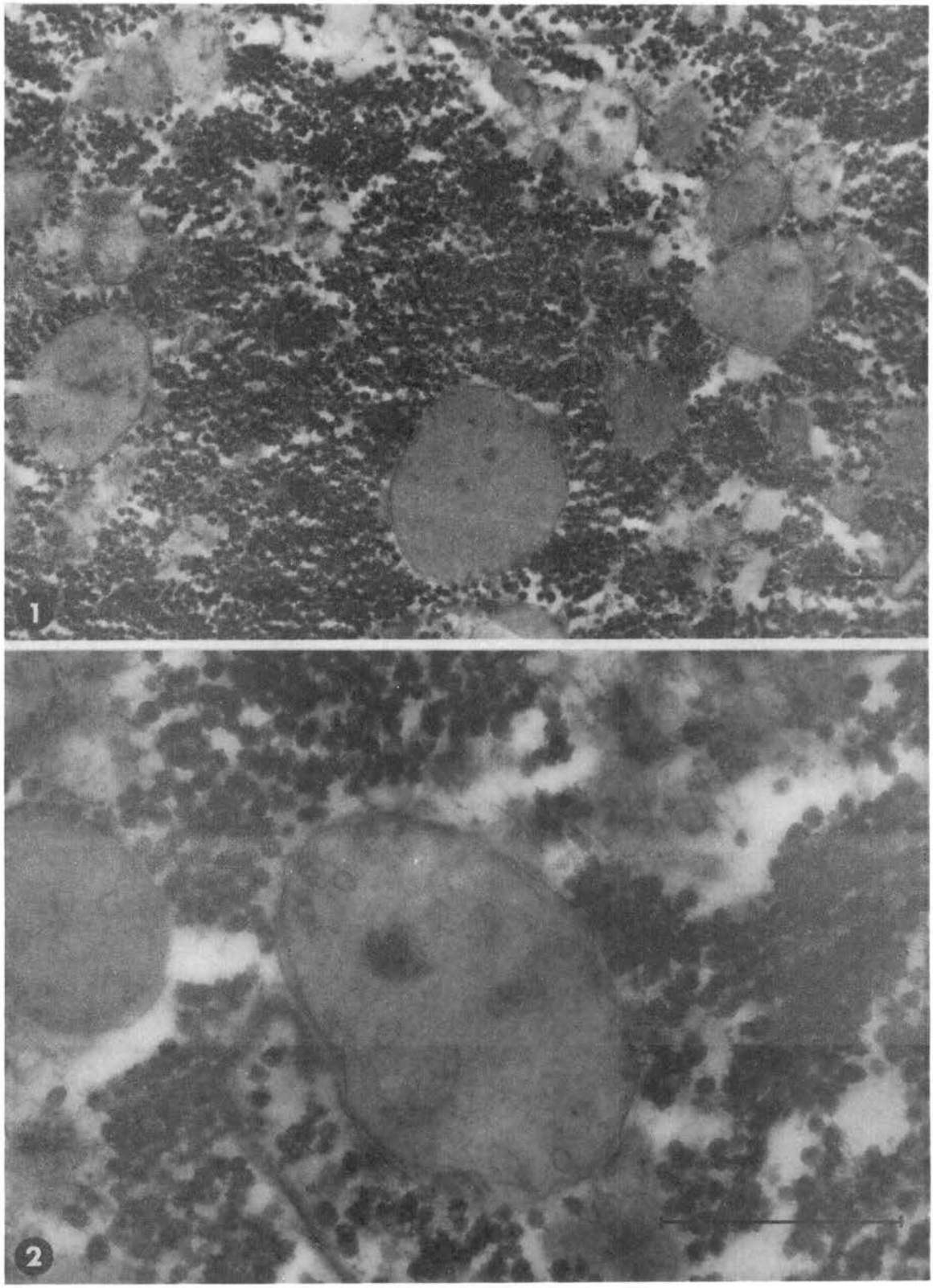

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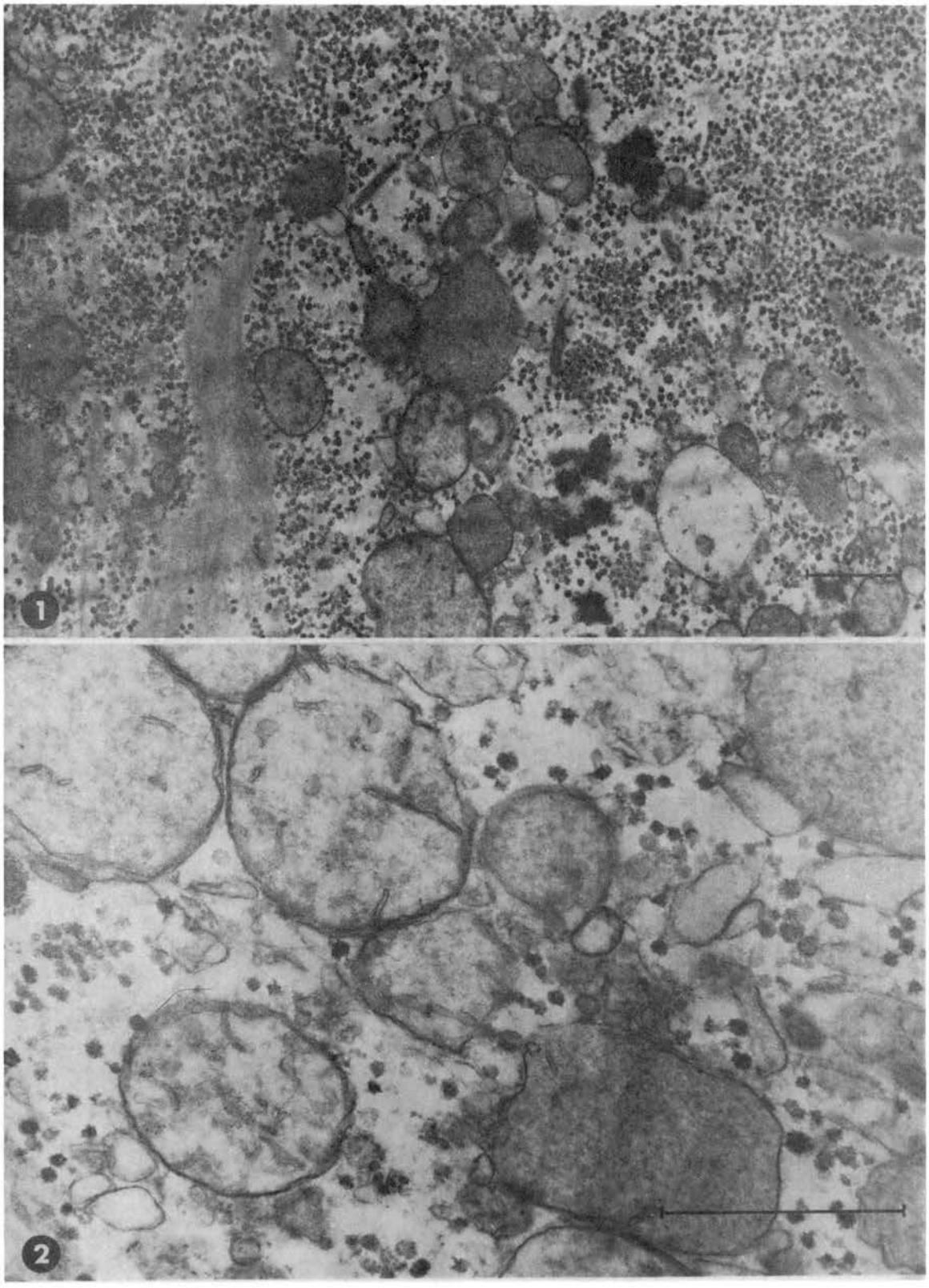
Plate 9. Electron Micrograph of Muscle-Mitochondria Isolated by Differential Centrifugation, Using Dialyzed Ascaris Hemolymph. 1) The dimensions of the mitochondria are near that of the mitochondria in Situ. Contamination consisted of glycogen, intracellular skeletal fibers, and assorted membranes - X15,000; 2) Double membrane structures can be recognized and the cristae appear similar to those of mitochondria in Situ - X40,000.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Density Gradient Centrifugation

Only with 14-50% sucrose gradient was good fractionation of the supernatant accomplished. Due to differing refactive characteristics of various bands, they could be observed visually. The banding in the centrifuge tube after centrifugation is diagrammed in Figure 1a. The gradient was divided into nine fractions as shown in Figure 1a and the ultraviolet absorption of each determined at 260 mμ. The first and largest peak (Fractions 1, 2, 3 and 4 in Figure 1a) was examined with the electron microscope (Plate 10) and was found to contain mainly microsomes. It also contained a few small mitochondria and other unidentified structures. The mitochondria in this fraction did not appear normal. They seemed either highly vesicular or crenulated. The second peak (Fraction 7, Figure 1a) which absorbed at much lower intensity than the first, corresponded to the mitochondrial fraction. When this fraction was examined with the electron microscope it was found to contain numerous mitochondria as shown in Plate 11. The mitochondria resembled those isolated by differential centrifugation using sucrose-bovine albumin-EDTA. The size of the mitochondria was very nearly the same as that of those isolated by differential centrifugation. The double membrane structure of the mitochondrial wall could be recognized in most of the organelles. Cristae were well exemplified; however, many were greatly enlarged. In some mitochondria, the cristae were so large as to appear as involutions of the outer membrane. Cross- and longitudinal-sections give the cristae the appearance of finger-like projections. Many of the alterations which some mitochondria undergo, are not readily explainable. Contamination of this fraction was minimal. It consists mainly of a few membranous loops, which seem to

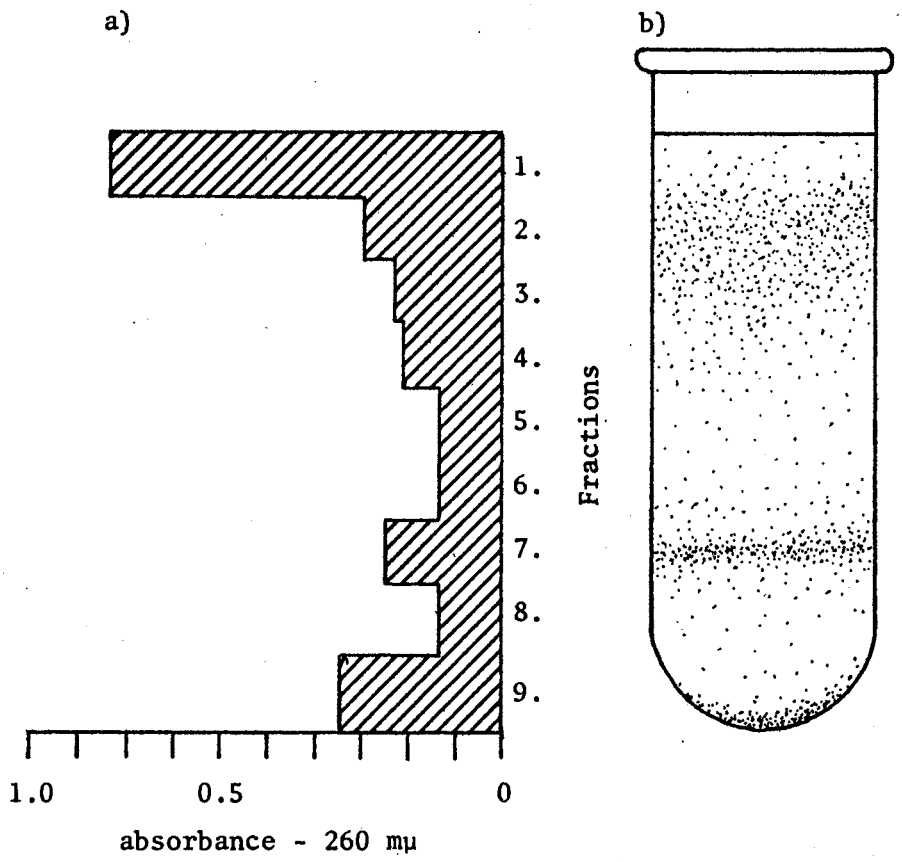

Figure 1. a) Absorbancy of the Various Density Gradient Fractions Following Centrifugation of Ascaris Muscle "Supernatant" at 27,000 x g; b) Illustration of Bands Observed in the Column Following Centrifugation at 27,000 x g.

Plate 10. Electron Micrograph of Muscle Organelles Isolated in the Upper Refractive Band After Density Gradient Centrifugation. 1) Low magnification reveals many small particles, microsomes, small mitochondria, and other structures - X15,000; 2) Higher magnification shows that the mitochondria are highly vesicular or crenulated - X40,000.

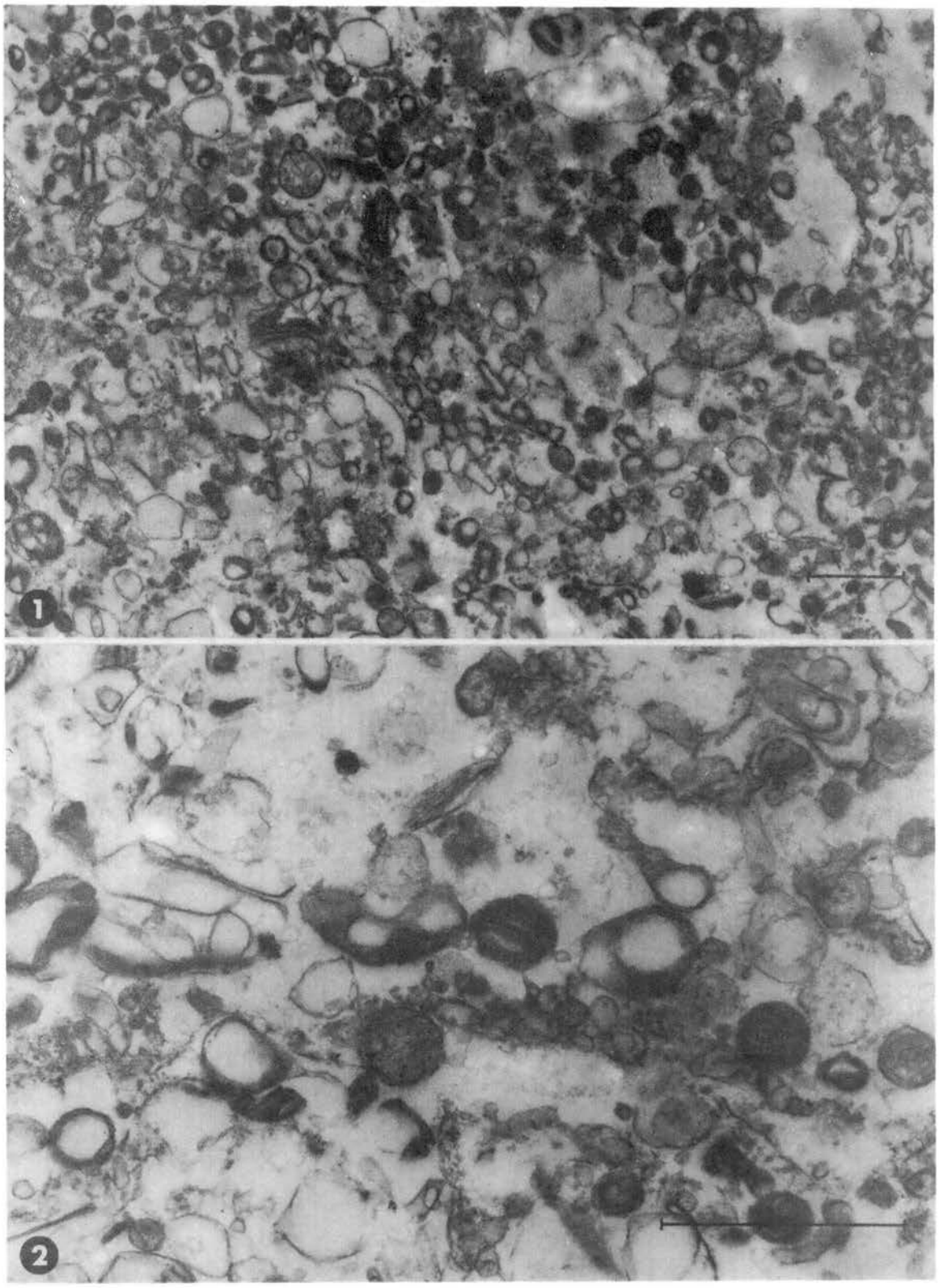
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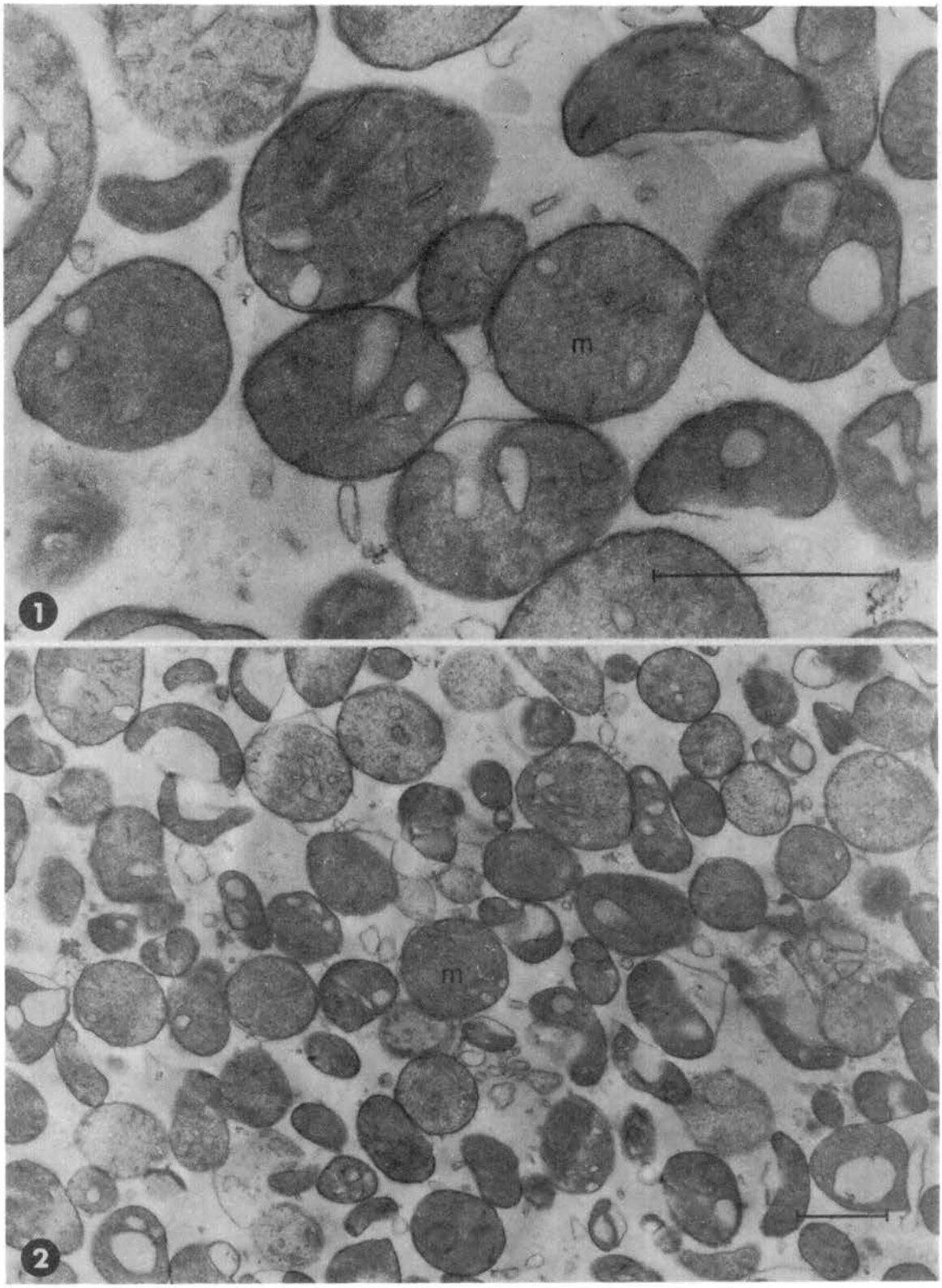
Plate 11. Electron Micrograph of the Second Visible Band of Muscle-Mitochondria Isolated in the Middle Refractive Band by Density Gradient Centrifugation. 1) The dimensions of the mitochondria are very near the size of the mitochondria fixed in Situ. Double membranes are apparent in several mitochondria. Cristae are plainly visible but many are greatly enlarged - X40,000; 2) The minimal contamination consists of membranous loops adherent to the mitochondria - X15,000; Bar = 1 micron.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adhear to the mitochondria. Although these structures possibly may be microsomes, they are not readily identifiable with particles found in the microsomal fraction (Fraction 1, Figure 1a). They may be parts of the limited smooth endoplasmic reticulum or other membranous cellular components. The third peak (Fraction 9, Figure 1a) was composed of nuclei, cells, and particles which had agglutinated.

Biochemical Assays

Ascaris muscle mitochondria are known to contain a number of active dehydrogenases (Kmetec and Bueding, 1961; Seidman and Entner, 1961; and Saz and Lescure, 1969).

Succinic dehydrogenase activity was determined for each of the nine fractions of the gradient. Change in optical density per unit time and per unit mass was plotted for the various fractions and the results are presented in Figure 2a and 2b. The greatest specific activity is found in Fraction 4 (Figure 2a). However, when the change in optical density per unit time is plotted, Fractions 2 and 3 show the greatest activity (Figure 2b). As pointed out above, Fractions 2, 3, and 4 contain an assortment of small mitochondria, unit membrane structures, and microsomes. It is possible that many of the membranous structures were mitochondrial membrane fragments and that they accounted for the high activity in these fractions.

The mitochondrial rich fraction (Fraction 7, Figure 1a) contains moderate activity with both plots. A series of additional determinations were designed to measure dehydrogenase activity of this fraction and the results are presented in Table II. Succinic dehydrogenase activity is in the range reported previously for *Ascaris* muscle-

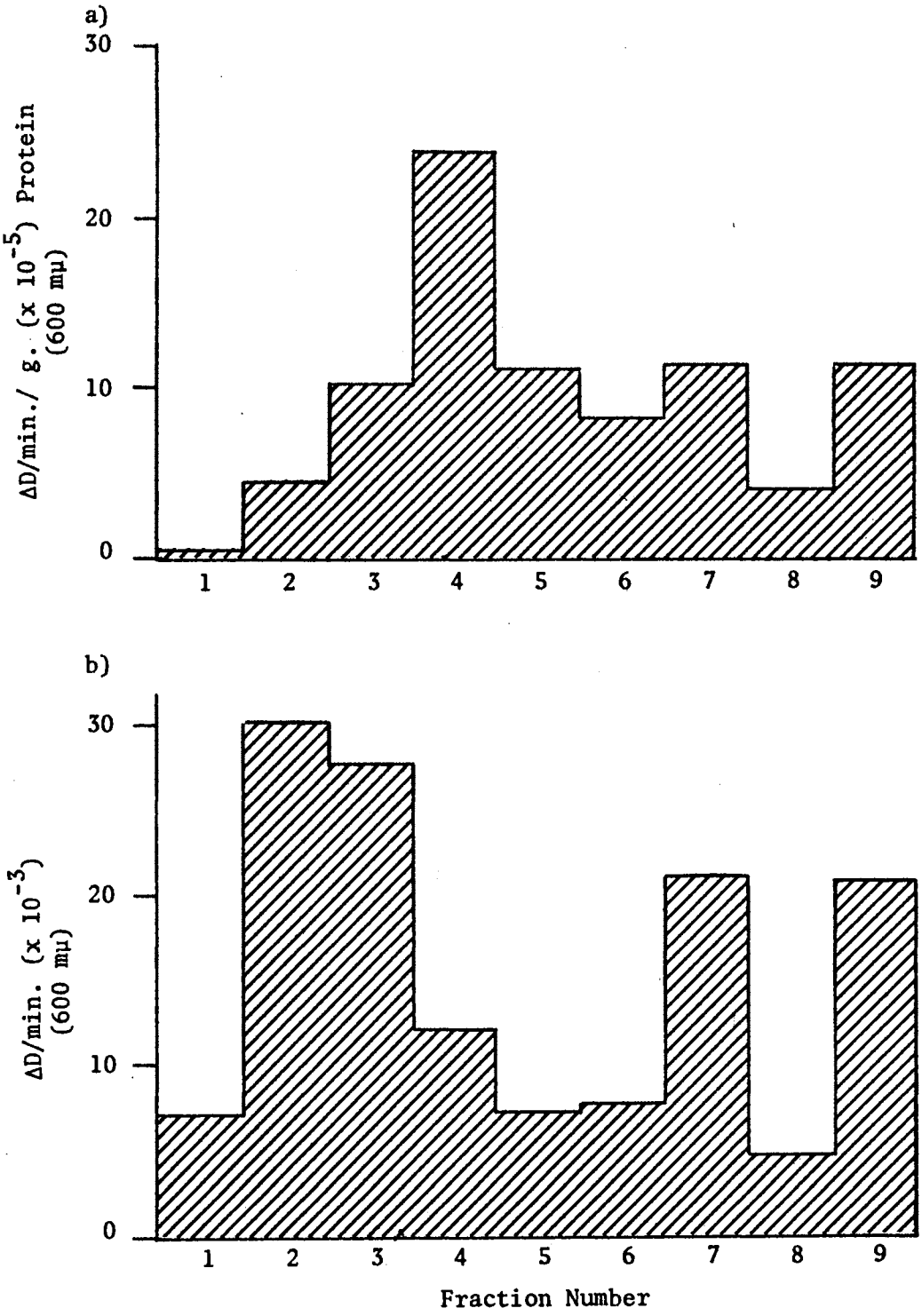

Figure 2. a) Change of Optical Density per Minute per μg. Protein; b) Change of Optical Density per Minute due to the Addition of Succinate to Various Fractions of the Density Gradient.

TABLE II

DEHYDROGENASE ACTIVITY IN *ASCARIS* MUSCLE-MITOCHONDRIA

Substrate[1]	Activity μ moles/mg protein/min.
Succinic Acid	3.6
Propionic Acid	0.0
Acetic Acid	0.7
Malic Acid	2.1
Tiglic Acid	2.1

[1]Incubation medium contained: 60 μ moles phosphate buffer, pH 7.4; 10 μ moles $MgCl_2$; 10 μ moles KCN; 9.18×10^{-5} μ moles 2,6-dichlorophenol indophenol; 1.15 mg phenazine methosulfate; 4.0 μ moles of substrate. Reaction started by add mitochondrial solution (146 μg). Final volume 6.0 ml. Temp. 30° C.

mitochondria (Seidman and Entner, 1961). Neither propionic nor acetic acid stimulate any appreciable dye reduction. Malic and tiglic acid, however, both cause a relative rapid rate of reduction. Malic dehydrogenase has been demonstrated in *Ascaris* muscle-mitochondria (Seidman and Entner, 1961). Tiglic acid presumably is an intermediate in the formation of α-methylbutyric acid (Saz and Weil, 1960). It is probably formed by the reduction of methylacetoacetic acid and the subsequent dehydration of β-hydroxy-α-methylbutyric acid. This system is apprently in the mitochondria. Hence, it is reasonable to expect that tiglic acid would cause the reverse reaction. The results in Table II suggest that the mitochondria isolated by density gradient centrifugation were functionally intact.

CHAPTER IV

DISCUSSION

As is the case in other species, the mitochondria taken from different tissues of Ascaris are heteromorphic, however the extreme morphological differences found in the mitochondria taken from the same tissue were not entirely expected. The great morphological differences between these mitochondria probably indicated that their physical characteristics would vary also. Subsequent differential centrifugation of Ascaris tissue homogenates substantiate this suspicion. Moreover, Kemetic, et al. (1962) found contamination in their attempts to isolate mitochondria. These findings suggested that the sedimentation coefficients of mitochondria vary over a wide range and/or the contaminating particles have sedimentation coefficients similar to those of the mitochondria.

Saz and Lescure (1969) suggested a method of differential centrifugation for the isolation of Ascaris mitochondria in large quantities. This technique was found to be reasonably satisfactory in retaining the morphological integrity of the mitochondria, however it does not provide a highly purified fraction. Initially, I thought that a medium could be developed for differential centrifugation that would produce an optimum yield of mitochondria with little or no contamination and minimum structural change. As compensations for pH, osmolarity, agglutination, and swelling were made in the medium, improved mitochondrial

preservation was obtained but there was greater contamination in the isolate. These results suggested that the optimum yield of mitochondria could only be obtained by forcing the mitochondria to become more uniform in structure as a result of non-physiological osmotic force. As the electron micorgraphs show, the differential centrifugation technique which provides the least contamination yields mitochondria that were distended, forming near identical, spherical bodies. It seemed probable that any further increase in osmotic pressure would decrease the metabolic efficacy of the mitochondria. The alternative to the employment of adverse osmotic force in differential centrifugation would have been to extend the number of isolation steps, use a combination of media, chemically remove contaminates, or lengthen the centrifugation time, etc. For these reasons a more satisfactory isolation technique was sought.

Density gradient centrifugation had been used previously by many researchers to isolate relatively pure mitochondria from the tissues of various species. This technique proved to be satisfactory for isolating mitochondria from Ascaris muscle also. The one disadvantage encountered was the small amount of material obtained. However, additional steps, such as increasing homogenate concentration, rehomogenizing, and multiple gradient runs, effectively increased the yield.

Ultraviolet absorption of gradient fractions can be used to locate various organelles and indicate the presence of nucleic acids (Kuff, et al., 1956). With Ascaris muscle homogenate the pattern of distribution of organelles in the gradient resemble those determined by Anderson (1962) for rat liver brei. The upper absorption peak most likely represents the combined absorption due to microsomal nucleic acids (ribosomal

RNA) and soluble RNA while the second peak represents absorption due to mitochondrial nucleic acids (mitochondrial DNA). Quantitatively, the microsomal fraction is expected to be more absorbant than the mitochondrial fraction. This is reflected in the present measurements.

Electron micrographs reveal that the mitochondria isolated by density gradient centrifugation are relatively free of contamination. The morphology of these mitochondria differ from those fixed in situ, but biochemical assays suggest that they are functionally intact.

The rate of succinic dehydrogenase activity of the mitochondrial fraction agrees with previously reported values (Seidman and Entner, 1961). As mentioned before, proprionate and acetate elicited little or no dye reduction. On the other hand, malic and tiglic acids caused measurable dye reduction. Malate is presumably reduced by a mitochondrial malate dehydrogenase which has been described recently by Saz and Lescure (1969). Saz and Weil (1960) report that tiglic acid is probably an intermediate in the formation of 2-methylbutyric acid. The present observations strongly suggest that the enzyme systems for the formation of the branched chain volatile fatty acid excreted by Ascaris are associated with the mitochondria in muscle tissue. The density gradient technique described herein seems well suited for providing a highly purified, functionally intact mitochondria.

CHAPTER V

SUMMARY AND CONCLUSIONS

Several conclusions have been made in the consideration of the use of differential centrifugation in the isolation of Ascaris muscle-mitochondria. They are:

(1) As compared to other differential centrifugation techniques the general scheme suggested by Saz and Lescure (1969) for isolating mitochondria gives an optimum yield of mitochondria.

(2) Dialized hemolymph as a homogenizing medium helps preserve essential morphological characteristics of the mitochondria. However, the final isolate is excessively contaminated with glycogen, microsomes, and other membranous structures.

In the light of the second conclusion, a method of density gradient centrifugation that reduces particle contamination was developed.

The conclusions concerning density gradient centrifugation are as follows:

(1) Contamination is satisfactorily reduced.

(2) Morphological changes are greater than when the tissue was homogenized in dialyzed hemolymph.

(3) The quantity of mitochondria is reduced.

(4) Dehydrogenase activity is preserved and physiological integrity is apparently maintained.

In evaluating the suitability of the density gradient

centrifugation technique to a research situation, an awareness of the limitations of the technique with the aim of the investigation must be considered. With these considerations in mind, the advantage of a relatively contamination free isolate out weighs the disadvantages in many research situations where contamination could lead to erroneous results. It is therefore concluded that the density gradient technique investigated in this report is a useful tool for further research.

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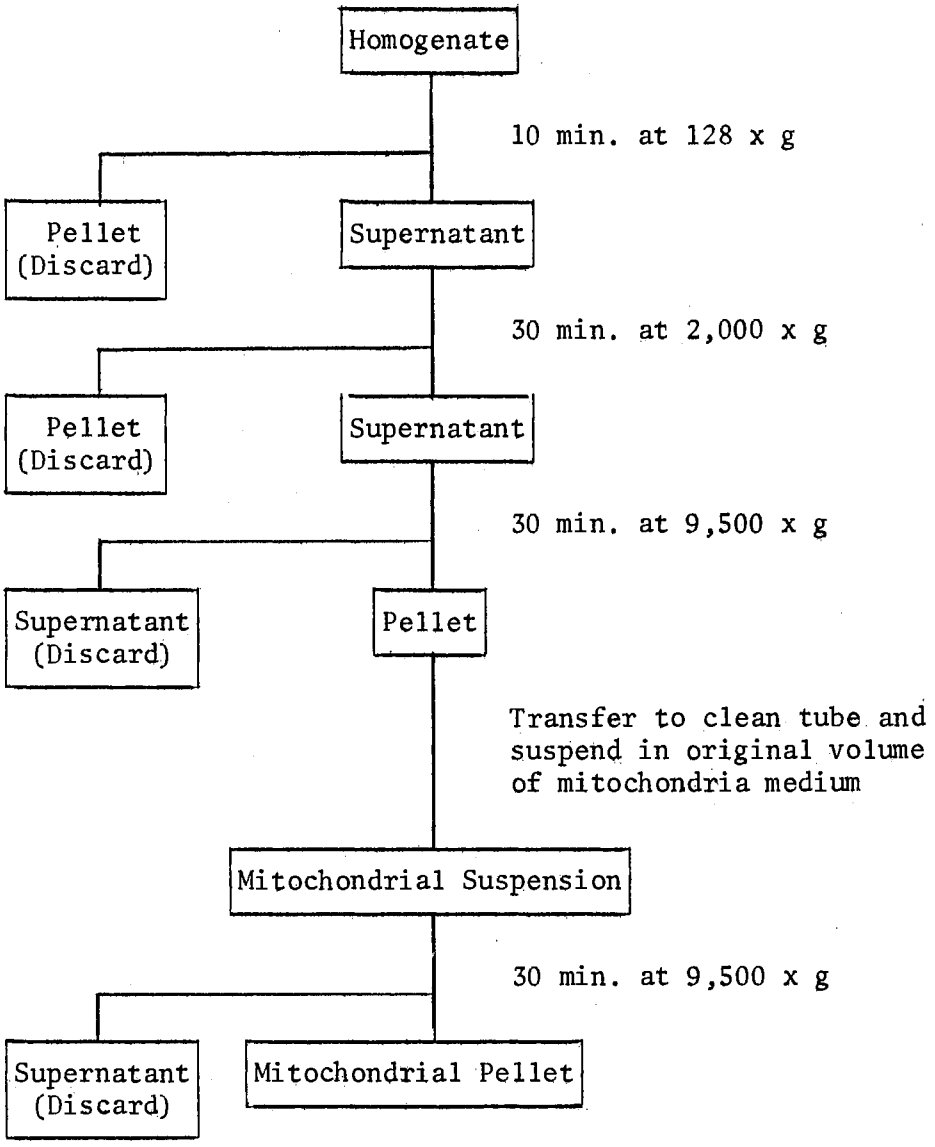
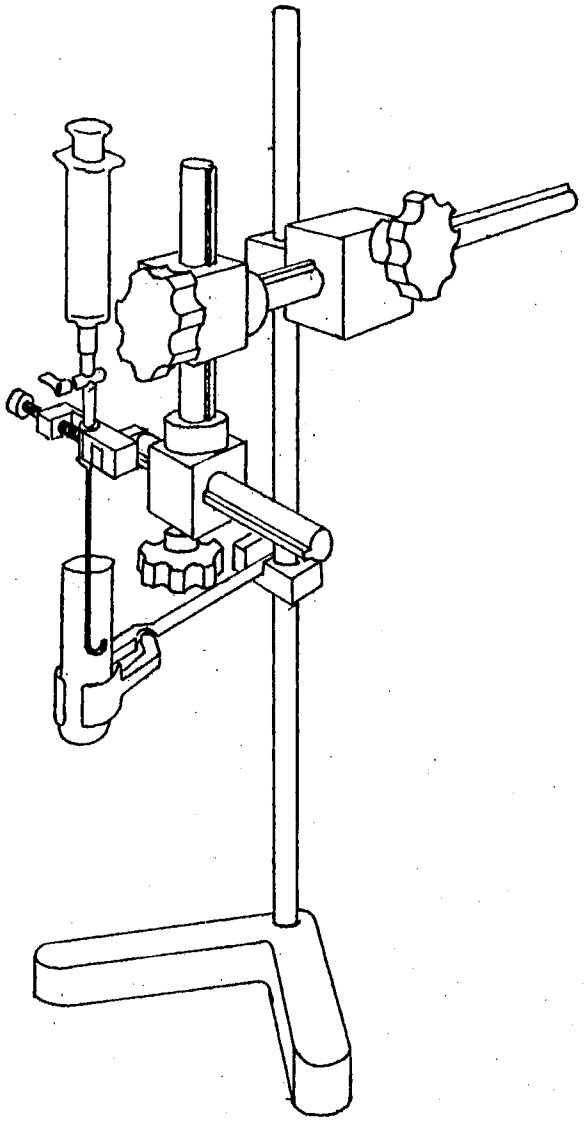
APPENDIX A

PROCEDURE FOR ISOLATING MITOCHONDRIA FROM ASCARIS MUSCLE BY DIFFERENTIAL CENTRIFUGATION



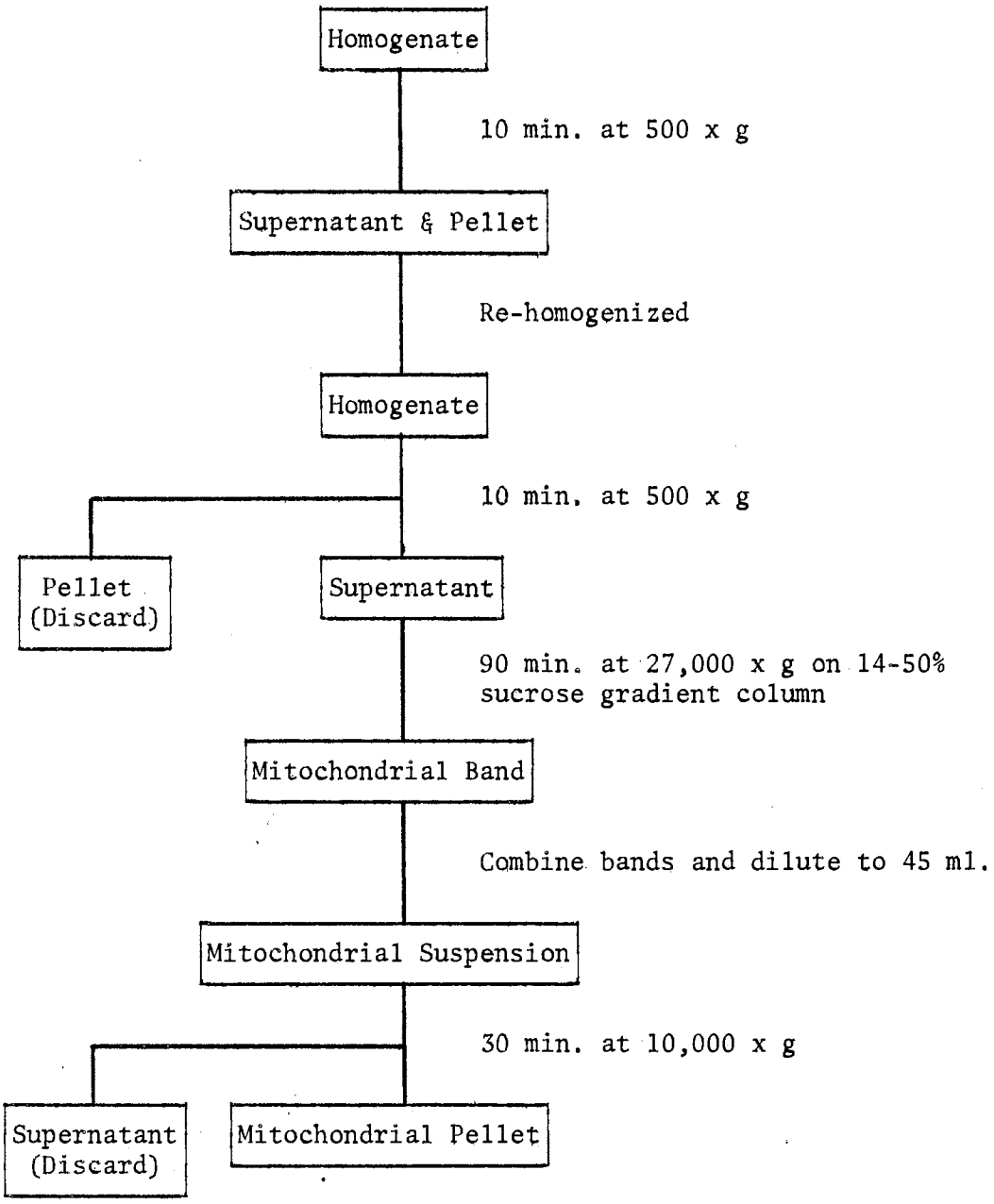
APPENDIX B

APPARATUS FOR APPLYING AND REMOVING MATERIAL FROM THE SUCROSE GRADIENT COLUMN



APPENDIX C

PROCEDURE FOR ISOLATING MITOCHONDRIA FROM ASCARIS MUSCLE BY DENSITY GRADIENT CENTRIFUGATION



APPENDIX D

SOLUTIONS FOR EMBEDDING AND STAINING

1) Stock phosphate buffer solution:

Solution 1: 27.22 g/1 KH_2PO_4

Solution 2: 28.40 g/1 $NaHPO_4$

Solutions 1 and 2 were combined in the ratio of 13.2:86.8 to give a pH 7.4.

Two per cent osmium tetroxide solution:

Equal volumes of the stock phosphate buffer solution and 2% osmium tetroxide were mixed to give the final fixitive solution.

2) Epon/Araldite mixture: J. H. Venable (unpublished)

Epon 812.	4.00 g
Araldite 6005	12.00 g
DDSA.	15.00 g
Dibutylpthalate	0.15 ml
DMP-30.	0.75 ml

Ingredients supplied through Ladd Research Industries, Inc., P.O. Box 901, Burlington, New York.

3) Five per cent nitrocellulose in butylacetate.

4) Mounted sections were stained using the following procedure: (Venable & Coggeshall, 1965)

To make 10 ml of lead citrate solution combine the following:
1) 0.01-0.04 g lead citrate
2) 10 ml triple distilled water
3) 0.1 ml 10 N sodium hydroxide.

To "stain" sections:
1) Grids were placed in a drope of lead citrate solution
2) Grids were washed with distilled water
3) Sections were then air dried.

APPENDIX E

SOLUTIONS FOR BIOASSAYS

Solution 1:

$MgCl_2$	97.6 mg.
KCN	31.2 mg.
2,6 dichlorophenoindophenol	15.2 mg.
0.05 M sodium phosphate buffer (pH 7.5)	172.0 ml.

Solution 2:

Phenazine methosulfate	4.5 mg.
0.05 M sodium phosphate buffer (pH 7.5)	4.5 ml.

Solution 3:

0.05 M sodium phosphate buffer (pH 7.5)	35.0 ml.
Substrate	0.02 M

The substrate used had the following values at 0.02 M:

Succinic acid	0.1666 g.
Proprionic acid	0.0672 g.
Acetic acid	0.09386 g.
Malic acid	0.09386 g.
Tiglic acid	0.07007 g.

Each of the above solutions were combined with a given amount of material from each of the nine fractions of the gradient according to the following table:

	Blank	Control	Substrate
Solution 1	-	4.0 ml.	4.0 ml.
Solution 2	0.5 ml.	0.5 ml.	0.5 ml.
Solution 3	-	-	0.5 ml.
Buffer	5.5 ml.	1.0 ml.	0.5 ml.
Bands (1-9)	-	0.5 ml.	0.5 ml.

APPENDIX F

CHANGE IN OPTICAL DENSITY PER UNIT TIME PER FRACTION

Data used to calculate the change of absorbance per minute per μg. of protein following the addition of succinate to various bands separated by density gradient after centrifugation

Band Number	μg./ml.	ΔD./min.	ΔD./min/μg. (x10)
1	1279	0.00738	0.57701
2	616	0.03062	4.97077
3	272	0.02813	10.34191
4	51	0.01250	24.50980
5	66	0.00750	11.36630
6	93	0.00812	8.73118
7	192	0.00500	11.30962
8	116	0.00500	4.31034
9	183	0.02125	11.61202

VITA

2

Jerry McCormick Merz

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Master of Science

Thesis: THE ISOLATION AND MORPHOLOGY OF MITOCHONDRIA FROM THE MUSCLE TISSUE OF ASCARIS

Major Field: Physiology

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