ENZYME LOCALIZATION IN THE ANAEROBIC MITOCHONDRIA OF *ASCARIS LUMBRICOIDES*

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ABSTRACT

Mitochondria from the muscle of the parasitic nematode *Ascaris lumbricoides* var. *suum* function anaerobically in electron transport-associated phosphorylations under physiological conditions. These helminth organelles have been fractionated into inner and outer membrane, matrix, and intermembrane space fractions. The distributions of enzyme systems were determined and compared with corresponding distributions reported in mammalian mitochondria. Succinate and pyruvate dehydrogenases as well as NADH oxidase, Mg^{++}-dependent ATPase, adenylate kinase, citrate synthase, and cytochrome *c* reductases were determined to be distributed as in mammalian mitochondria. In contrast with the mammalian systems, fumarase and NAD-linked "malic" enzyme were isolated primarily from the intermembrane space fraction of the worm mitochondria. These enzymes are required for the anaerobic energy-generating system in *Ascaris* and would be expected to give rise to NADH in the intermembrane space. The need for and possible mechanism of a proton translocation system to obtain energy generation is suggested.

The adult stage of the parasitic roundworm *Ascaris lumbricoides* resides in the small intestine where the oxygen tension is low. Accordingly, this nematode possesses a specific type of anaerobic energy metabolism, which is outlined in Fig. 1, and which subsequently has been found to occur in a relatively large number of other parasitic helminths (Scheibel and Saz, 1966; Saz, 1972), as well as in molluscs (Hammen, 1969; Hochachka and Mustafa, 1972), turtle liver (Penney, 1974), and possibly to some extent in anoxic mammalian heart (Cascarano et al., 1968; Wilson and Cascarano, 1970).

Pyruvate kinase activity is barely detectable in *Ascaris* muscle (Bueding and Saz, 1968). Therefore, rather than giving rise to cytoplasmic pyruvate, CO_2 is fixed into phosphoenolpyruvate, resulting in the formation of oxalacetate which, in turn, is reduced by NADH to form malate thereby regenerating glycolytic NAD (Saz and Lescure, 1969). *Ascaris* muscle mitochondria then utilize this malate anaerobically as their major substrate by means of a dismutation reaction. Within the mitochondrion, the "malic" enzyme catalyzes the oxidation of malate to form pyruvate, CO_2, and NADH. This reaction serves to generate intramitochondrial reducing power in the form of NADH. Concomitantly, fumarase catalyzes the dehydration of an equivalent amount of malate to form fumarate which, in turn, is reduced by an NADH-linked fumarate reductase to succinate. The flavin-linked fumarate reductase reaction results in a site I electron transport-associated phosphorylation of ADP, giving rise to ATP (Kmetec and Bueding, 1961; Seidman and Entner, 1961; Saz, 1971).

In an attempt to better understand how the invertebrate mitochondrion carries out these reac-

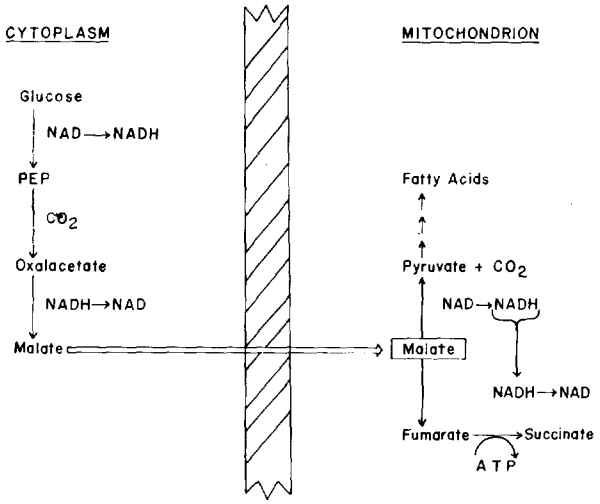


FIGURE 1 Pathway of carbohydrate dissimilation in *Ascaris* muscle.

tions, membranous and soluble submitochondrial fractions were examined from the point of view of morphological features as well as enzyme localization, and compared with the corresponding mammalian organelles. Important differences in the localization of at least two of the enzymes of the energy-generating pathway were found, giving rise to a number of questions concerning the transport of reducing power across the inner mitochondrial membrane. Preliminary findings from these studies have been reported (Rew et al., 1973).

MATERIALS AND METHODS

Preparation of Submitochondrial Fractions

Adult female *A. lumbricoides* var. *suum* were obtained from a local slaughter house. Muscle strips were removed by dissection in the cold (Laser, 1944), and washed *Ascaris* muscle mitochondria were prepared as previously described employing a medium containing 0.24 M sucrose, 0.005 M ethylenediaminetetraacetate (pH 7.4), and 0.15% crystalline bovine serum albumin (Saz and Lescure, 1969). Rat liver mitochondria were prepared in a similar manner.

Submitochondrial fractions consisting of outer membrane (OM), intermembrane space (IMS), and inner membrane plus matrix (IMM) were separated essentially according to Sottocasa et al. (1967). This procedure entailed mitochondrial swelling in 10 mM Tris-phosphate buffer (pH 7.5) followed by contraction of the IMM upon the addition of ATP and $MgSO_4$, sonication, and finally centrifugation on a discontinuous sucrose gradient. In these studies, 5 ml of the sonicate were placed on each gradient, which was comprised of 3 ml of 0.76 M sucrose layered on 5 ml of 1.32 M sucrose, and centrifuged in a Beckman Spinco rotor SW-40 (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 30,000 rpm (111,700 *g*) for 2 h. The pellet (IMM) was suspended in mitochondrial medium, 2 ml/10 g of original muscle. Inner membrane and matrix fractions were separated by the addition of one-sixth volume of a Lubrol-WX solution (10 mg/ml in mitochodrial medium). After a 15-min incubation period in an ice bath, the mixture was diluted with mitochondrial medium to twice the volume of the original IMM suspension. Inner membranes were separated from soluble matrix components by centrifugation at 269,000 *g*. The membrane fraction was washed with mitochondrial media. Rat liver mitochondrial fractions were prepared by the same procedures employed for obtaining the corresponding *Ascaris* fractions.

Glycogen was a contaminant of particulate fractions. The presence of glycogen, however, had no effect upon the enzyme assays employed in these studies. Where indicated, glycogen was removed by slow shaking of the particulate fraction concerned in a minimal volume of mitochondrial medium (0.2–0.3 ml) at 30°C after the addition of 24 U of phosphorylase b, 1.5 μmol of AMP, and 40 μmol of potassium phosphate buffer (pH 6.9). After 1 h, the suspension was diluted by the addition of 8 ml of mitochondrial medium and the particulate fraction was recovered by centrifugation.

The effects of increasing concentrations of digitonin on *Ascaris* mitochondria were examined according to the procedure of Schnaitman et al. (1967). Before use, digitonin was recrystallized from hot absolute ethanol. Suspensions of mitochondria were incubated in ice with the indicated concentrations of digitonin for 10 min before diluting 17.2 times with the mitochondrial me-

dium. Diluted suspensions were centrifuged at 27,000 *g* for 25 min and the percentages of the enzyme activities which remained in the particulate fractions were determined.

Enzyme Assays

Succinate dehydrogenase was determined spectrophotometrically by measuring the rate of reduction of ferricyanide at 410 nm essentially according to Kmetec and Bueding (1961). The micromoles of each constituent in a a total volume of 1 ml were as follows: Tris-HCl buffer, pH 8.5, 80; potassium ferricyanide, 0.8; sodium succinate, 8. In addition, cuvettes contained 0.5 mg of bovine serum albumin and an appropriately diluted enzyme fraction. Corrections were made by subtracting values obtained from control cuvettes which contained all components except the enzyme.

Malic enzyme activities (1.1.1.39 L-malate:NAD oxidoreductase [decarboxylating]) were examined spectrophotometrically by following nucleotide reduction at 340 nm (Saz and Hubbard, 1957; Saz et al., 1972). In addition to enzyme, each milliliter of the complete system contained (in micromoles): imidazole buffer, pH 6.6, 40; NAD, 1; $MnCl_2$, 2; L-malate, 20. In the absence of manganese, no malic enzyme activity was detectable in any of the fractions.

Fumarase (4.2.1.2 L-malate hydrolyase) was assayed by following the disappearance of fumarate at 240 nm (Racker, 1950). In addition to suitably diluted enzyme fractions, cuvettes contained 0.6 μmol of sodium fumarate and 40 μmol of acetate buffer, pH 5.7. The final volume was 1.0 ml.

Adenylate kinase activity (2.7.4.3 ATP:AMP phosphotransferase) was measured by coupling ADP formation with NADH oxidation by means of pyruvate kinase and lactate dehydrogenase (Adam, 1963). In addition to the enzyme source, the complete system contained the following (in micromoles per milliliter): triethanolamine buffer, pH 7.6, 70; disodium AMP, 1.2; disodium ATP, 1.2; phosphoenolpyruvate (tricyclohexylammonium salt), 0.24; $MgSO_4$, 0.96; KCl, 120; NADH, 0.12. Also present were 18 and 3 U of lactate dehydrogenase and pyruvate kinase, respectively.

Approximations of pyruvate dehydrogenase activities were made by following the reduction of NAD upon incubation of enzyme in a total volume of 1.0 ml with the following (in micromoles): potassium phosphate buffer, pH 8.0, 50; $MgCl_2$, 1; NAD, 2; cysteine, 3; thiamine pyrophosphate, 1; coenzyme A, 0.06; sodium pyruvate, 1. Values obtained were approximations, since corrections for NADH oxidase activities were not made. Employing initial rates, however, these approximations were sufficient to allow for the determination of the site of pyruvate dehydrogenase within *Ascaris* mitochondria.

NADH oxidase was examined by measuring the rate of oxidation of the nucleotide when 0.12 μmol was incubated with enzyme, 80 μmol Tris-HCl buffer (pH 7.4), and 5 mg of bovine serum albumin in a final volume of 1.0 ml.

Magnesium-dependent ATPase activity was estimated by quantitating the inorganic phosphate liberated from ATP. The complete system in a total volume of 1.0 ml contained (in micromoles): Tris-HCl buffer, pH 7.4, 40; $MgSO_4$, 8; potassium ATP, 2.4; tricyclohexylammonium salt of *P*-enolpyruvate, 5. In addition, 9 U of pyruvate kinase were added. Reaction mixtures were shaken at 30°C for 20 min before deproteinization by the addition of trichloroacetic acid to a final concentration of 4%. Appropriate controls were performed to determine initial inorganic phosphate levels by the addition of trichloroacetic acid before the enzyme source. The possible presence of nonspecific phosphatases was determined, and corrected for when necessary by omitting Mg^{++} from the assay system. After incubation and deproteinization, the mixtures were centrifuged and inorganic phosphate was analyzed in aliquots of the centrifugate by the method of Fiske and SubbaRow (1925).

Citrate synthase (4.1.3.7 citrate oxaloacetate-lyase [CoA-acetylating]) was assayed according to Srere (1969), employing 5,5'-dithiobis-(2-nitrobenzoate) to measure the appearance of released coenzyme A during the reaction. Cytochrome *c* reductase (1.6.99.3 reduced-NAD:[acceptor]oxidoreductase) was estimated by the procedure of Mahler (1955). Protein was determined according to Lowry et al. (1951).

Electron Microscopy

Samples of the mitochondrial and submitochondrial fractions were rapidly fixed, dehydrated, and embedded as previously described by Schnaitman et al. (1967). Small volumes (5–20 μl) were placed on top of 200 μl of 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, contained in Beckman microfuge tubes and immediately centrifuged for 5 min. The supernatant fluid was decanted, and fresh fixative was added. Fixation was continued at 4°C for 2 h. Fixed pellets were stored overnight in 0.1 M phosphate buffer. The micropellets were then postfixed in 1.5% OsO_4, pH 7.2, in 0.067 M collidine buffer (Bennett and Luft, 1959). After dehydration by rapid passage through a cold ethanol series, the pellets were embedded in Spurr's low-viscosity resin as described by Spurr (1969). Thin sections were cut with a diamond knife on a Porter-Blum MT2-B microtome before staining with saturated uranyl acetate for 20 min at room temperature, followed by lead citrate for 10 min at room temperature (Reynolds, 1963).

Membrane fractions that were not fixed were negatively stained with 2% potassium phosphotungstate, pH 6.8, by the drop method (Horne, 1967). A single drop of the fraction was applied to Formvar-carbon-coated grids for 1 min and the excess suspension was removed with filter paper. The remaining thin film was air dried. A similar procedure was utilized for the application and removal of the potassium phosphotungstate.

Samples were examined in an AEI 801 operated at 60 kV; plate magnification ranged from 4,000 to 63,000.

RESULTS

Electron Microscopy

Ultrastructural features of *Ascaris* mitochondria were evaluated after each step in the fractionation procedure (Sottacasa et al., 1967). At each step, the nematode organelles behaved like mammalian mitochondria.

In an environment of hypotonic Tris buffer, swelling occurred, with the inner and outer membranes nearly coming into contact with each other. The addition of ATP and Mg^{++} to this suspension resulted in a contraction of the inner membrane and matrix away from the outer membrane, whereupon further treatment by sonication appeared to dissociate the outer membrane from the inner membrane plus matrix (Fig. 2). The mixture of IMM and OM was placed on a discontinuous sucrose gradient, centrifuged, and after 2 h the resultant pellet (IMM) and two membrane-containing layers were examined. The lower membrane layer contained both single- and double-membrane ghosts which upon negative contrast displayed a mixture of 90 Å particle-free and particle-present membranes. The upper membrane layer was primarily composed of single-membrane vesicles of varying diameter which appeared free of electron-dense contents by section techniques (compare Figs. 2 and 3 *a*). Examination of this layer by negative-contrast techniques displayed essentially particle-free membranes, a typical example of which is shown in Fig. 3 *b*.

Inner membranes were recovered from the IMM fraction after incubation with Lubrol-WX followed by centrifugation at 269,000 *g* for 60 min. The pellet was subjected to incubation with phosphorylase b to remove most of the glycogen before sectioning and electron microscopy (Fig. 4 *a*). Single-membrane vesicles 60–300 nm in diameter with electron-translucent matrices were noted (compare Figs. 2 and 4 *a*). In some instances glycogen rosettes appeared inside the vesicles, indicating that the membranes had reformed, trapping the glycogen on the inside. Small samples of the pellet were resuspended and examined by means of negative-contrast techniques. Essentially all of the membranes observed displayed 90-Å

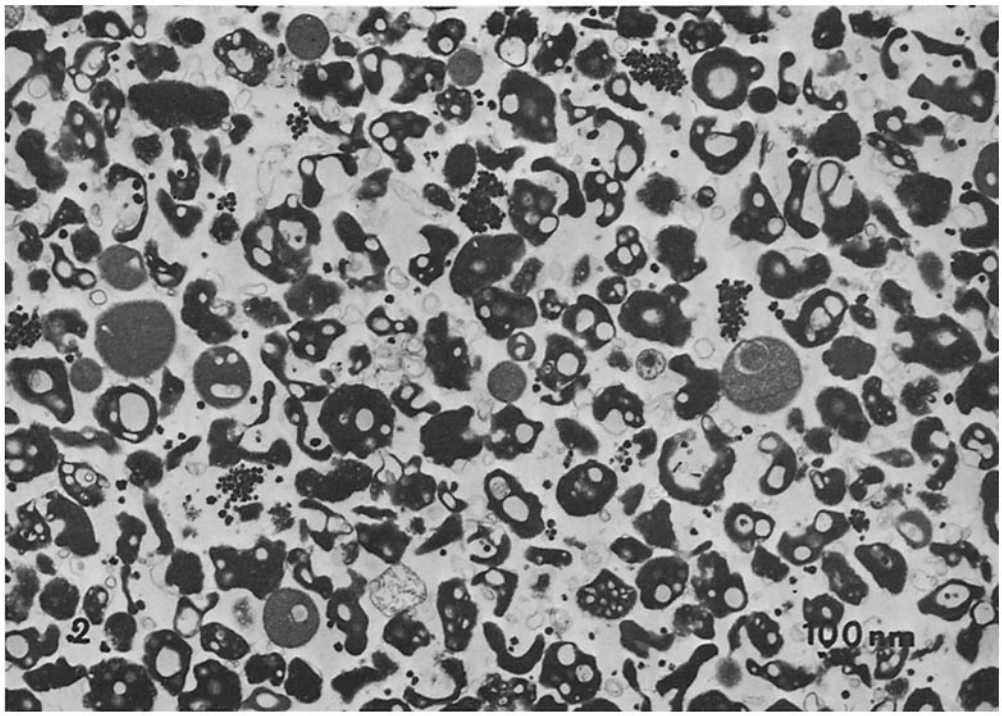


FIGURE 2 The IMM fraction. This fraction consists of an electron-dense matrix surrounded by the inner membrane. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 10,700.

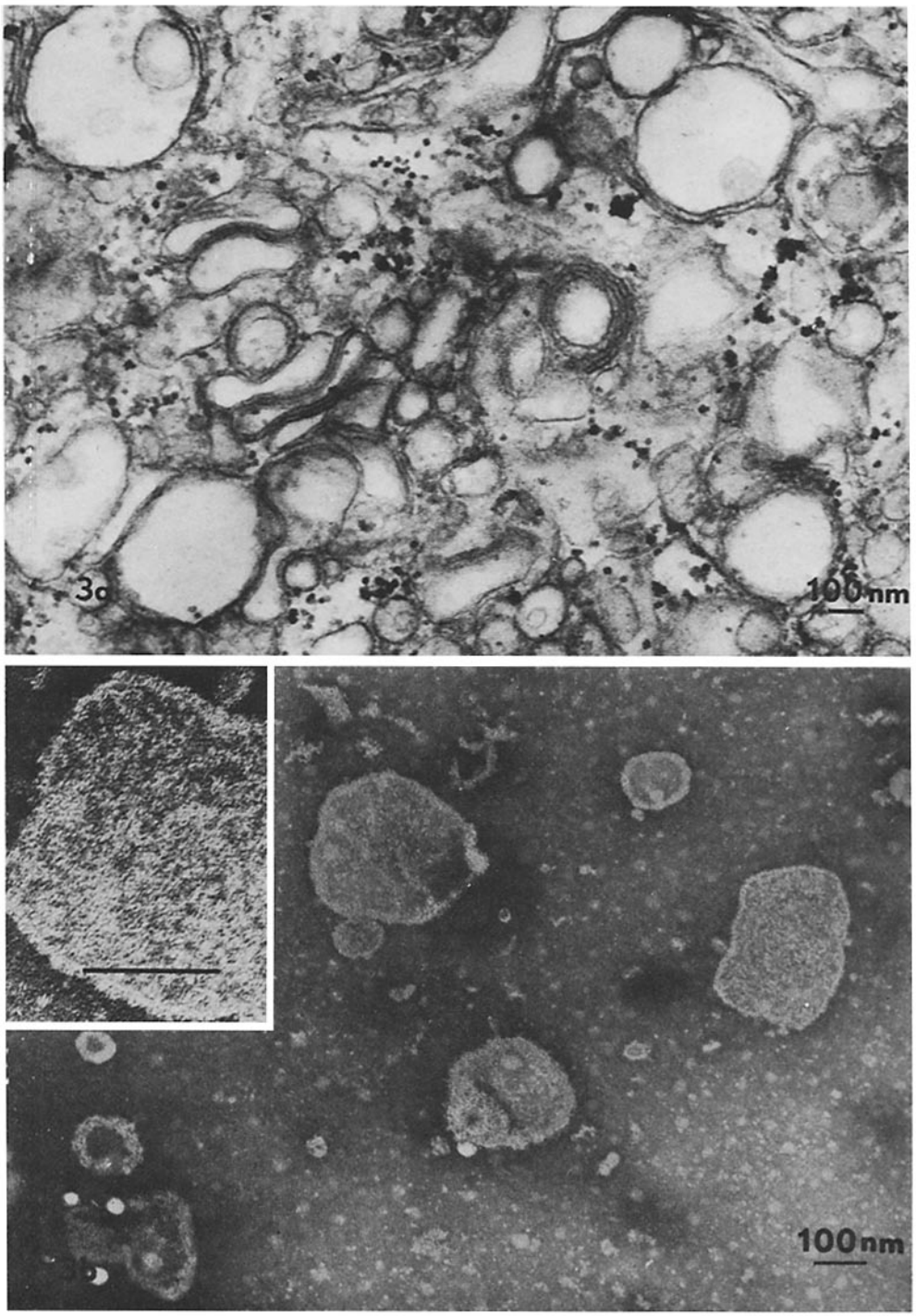
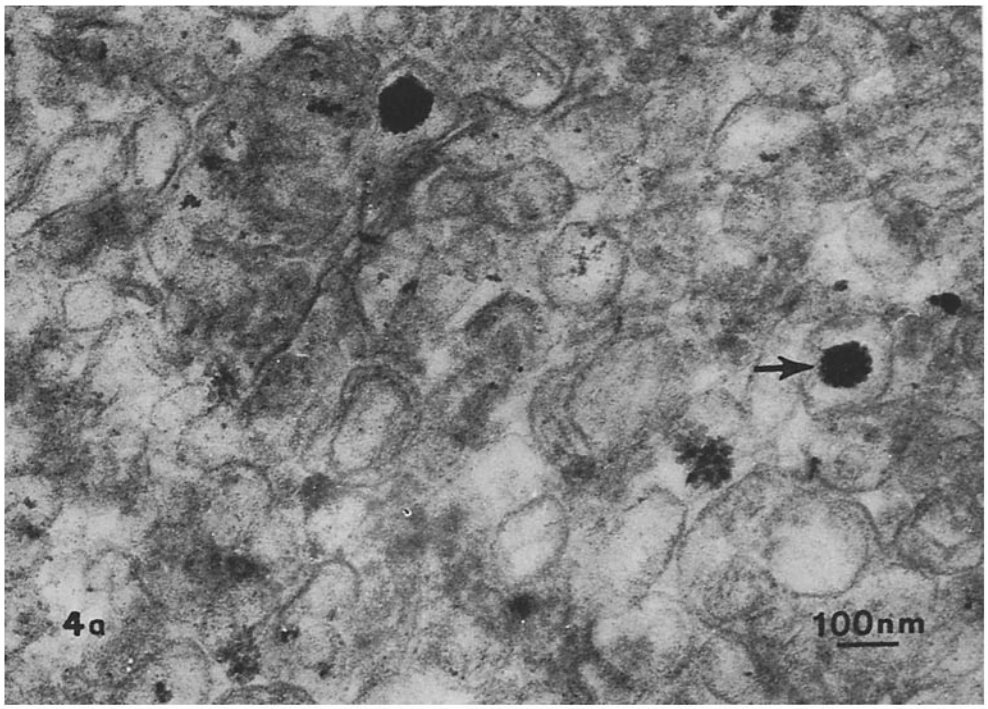


FIGURE 3 *a* The OM fraction. This preparation consists of vesicles with electron-translucent contents. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 48,000.

FIGURE 3 *b* An unfixed preparation of the OM fraction negatively stained with phosphotungstate. This fraction consists of somewhat ragged vesicles of variable size. × 68,000. *Inset* displays outer membrane. × 190,000.



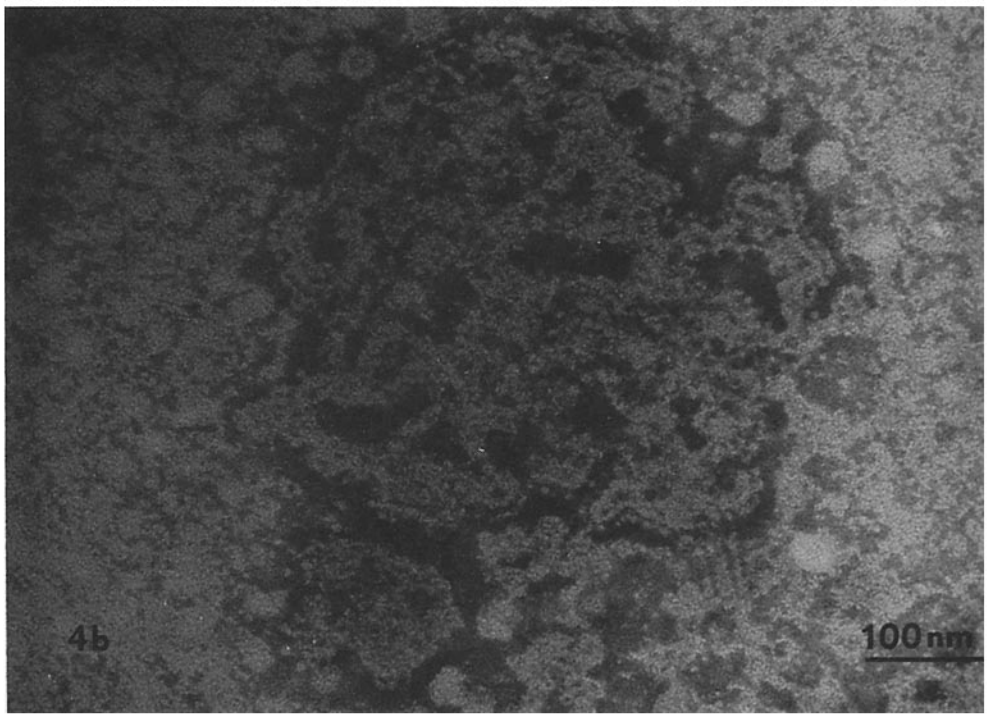


FIGURE 4 *a* The inner membrane preparation treated with phosphorylase b. Note the presence of glycogen rosettes within certain vesicles (arrow). Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 88,000.

FIGURE 4 *b* An unfixed preparation of the inner membrane negatively stained with phosphotungstate. Note the 90-Å stalked particles on the exterior of the membranes. The disrupted membrane often appears in the form of smaller vesicles and strands. × 170,000.

stalked particles which presumably contain the mitochondrial ATPase (Racker and Horstman, 1967). A typical field is shown in Fig. 4 *b*. It would appear, therefore, that the OM and IM fractions obtained are morphologically indistinguishable from the corresponding fractions observed in mammalian mitochondria.

Distribution of Mitochondrial Enzymes

Ascaris mitochondria were fractionated and the distributions of a number of enzymes were examined (Table I). Activities recovered in each fraction were compared with whole mitochondrial suspensions which had been incubated and assayed in hypotonic Tris buffer in order to minimize permeability effects. Each enzymatic activity of such preparations of "whole mitochondria" was arbitrarily assigned a value of 100% and the percent recoveries of activities in the other fractions were calculated on this basis.

Mitochondrial activities of pyruvate dehydrogenase, succinate dehydrogenase, NADH oxidase, and the magnesium-dependent ATPase were found predominantly in the pellet after centrifugation of the Lubrol-treated IMM fraction. Whether pyruvate dehydrogenase is directly associated with the *Ascaris* inner membrane or whether it is a particulate matrix component which pellets with the IM fraction can not be definitively differentiated from these studies. The findings of Schnaitman and Greenawalt (1968) indicate the possibility of a soluble matrix localization of this complex in rat liver after centrifuging at a lower speed than employed in these studies (144,000 *g* vs. 269,000 *g*). The remaining enzymes of this fraction, however, are presumed to be localized in the inner mitochondrial membrane as is also the case for the corresponding enzymes in mammalian mitochondria (Levy et al., 1967). The total recovery of NADH oxidase was low in most experiments,

TABLE I

*Localization of Enzymes in Mitochondria from Ascaris Muscle and Rat Liver**

Enzyme (source)	Whole mitochondria‡		Matrix		Inner membrane		Intermembrane space		Outer membrane	
	Activity	Total	Activity	Total	Activity	Total	Activity	Total	Activity	Total
		%		%		%		%		%
Succinate dehydrogenase (*Ascaris*)	0.78	100	0.09	11	0.52	*67*	0.04	5	0.01	1
Pyruvate dehydrogenase (*Ascaris*)	1.92	100	0	0	1.52	*70*	0	0	0.1	6
NADH oxidase (*Ascaris*)	1.35	100	0.09	0	0.56	*41*	0.17	12	0.01	1
ATPase (Mg^{++}) (*Ascaris*)	0.40	100	0	0	0.21	*53*	0	0	0	0
Cytochrome *c* reductase rotenone insensitive (*Ascaris*)	—	—	0	0	—	—	0	0	0.12§	—
Cytochrome *c* reductase rotenone sensitive (*Ascaris*)	—	—	0	0	0.85‖	—	0	0	0	0
Adenylate kinase (*Ascaris*)	1.8	100	0.15	8	0.3	17	1.3	*72*	0.1	6
Citrate synthase (*Ascaris*)	0.38	100	0.27	*71*	0.03	8	0.02	5	0	0
Fumarase (*Ascaris*)	6.39	100	0.28	4	0.01	1	5.3	*83*	0.1	2
Malic Enzyme (*Ascaris*)	8.63	100	2.76	*32*	0.25	2	5.22	*60*	0.56	6
Adenylate kinase (rat liver)	3.24	100	0.12	4	0	0	3.15	*97*	0.03	1
Citrate synthase (rat Liver)	2.40	100	1.23	*61*	0.36	15	0.42	18	0.01	0
Fumarase (rat Liver)	0.44	100	0.32	*72*	0.02	5	0.08	18	0	0

* Activities represent total activities in mitochondrial fractions obtained from 10 g of tissue expressed as micromoles of substrate utilized per minute.

‡ Mitochondria in hypotonic Tris-phosphate buffer (10 mM; pH 7.5).

§ Not inhibited by 1×10^{-4} M rotenone.

‖ Inhibited 72% by 1×10^{-4} M rotenone.

although essentially all of that activity which was recovered resided with the IM fraction. It is of interest that an almost complete recovery was obtained in the IMM fraction. A loss in activity resulted upon Lubrol treatment, indicating a possible inactivation of the enzyme by this detergent. Although the succinate, NADH oxidase, and possibly pyruvate dehydrogenase appeared to be rather firmly attached to the membranes, in some experiments a variable amount of the magnesium-dependent ATPase activity was found to be associated with the soluble matrix fraction, indicating some dissociation from the membrane under these conditions.

Both the IM and OM fractions from *Ascaris* mitochondria possessed NADH-cytochrome *c* reductase activities (Table I). The activity associated with the inner membrane, however, was inhibited 72% by 1×10^{-4} M rotenone, whereas the corresponding activity of the outer membrane was completely insensitive to this concentration of rotenone. These findings are again similar to the distributions found in mammalian mitochondria where the rotenone-insensitive enzyme serves as a marker for the outer membrane (Sottocasa et al., 1967). The fact that the cytochrome *c* reductase activity of the outer membrane was not at all inhibited by rotenone agrees with the electron microscope findings that this fraction was composed almost exclusively of outer membranes.

The distribution and activities of two soluble *Ascaris* mitochondrial enzymes, adenylate kinase and citrate synthase, were examined (Table I). 72% of the former was recovered with the IMS fraction, and 71% of the latter activity was found to be associated with the Lubrol-soluble or matrix portion. These enzymes, therefore, could be utilized as markers for their respective compartments in the *Ascaris* mitochondrion. The corresponding enzymatic activities serve as markers for the identical compartments in mammalian mitochondria (Schnaitman and Greenawalt, 1968). It should be noted that only 5% of the predominantly matrix enzyme, citrate synthase, was recovered in the IMS fraction. This indicated a relatively small leakage of the matrix component into the IMS fraction which presumably would result from damage to the inner membrane during fractionation.

Most interesting was the finding that 83% of the *Ascaris* fumarase activity was recovered from the IMS fraction (Table I). This is in direct contrast to the findings with mammalian mitochondria obtained by Brdiczka et al. (1968) and Parsons et al. (1967) who reported that fumarase was localized primarily in the matrix. In conjunction with the *Ascaris* localization of fumarase, it was also of interest that the nematode mitochondrial "malic" enzyme activity was recovered from both the IMS and matrix fractions, with the largest recovery (60%) in the former.

In view of the apparent differences in fumarase localization between the nematode and mammalian mitochondria, efforts were made to test the isolation procedures employed. Mitochondria and submitochondrial fractions were prepared from 10 g of rat liver by procedures identical to those used with *Ascaris*. In accord with previous findings, the rat liver mitochondrial adenylate kinase and citrate sythase were found primarily in the intermembrane space (97%) and matrix (61%), respectively. However, in direct contrast with the *Ascaris* system, fumarase was confined primarily to the matrix fraction (72%) in the liver mitochondria. These findings would support the supposition that *Ascaris* fumarase occupies a site different from that of the corresponding enzyme in the mammalian system. No attempt was made to localize the liver malic enzyme, since this differs from the *Ascaris* system in that the former is NADP linked.

To further elucidate the validity of the apparent intermembrane space localization of fumarase and malic enzyme in the helminth mitochondria, the technique of Schnaitman and Greenawalt (1968) was employed. Aliquots of intact *Ascaris* mitochondria were incubated with increasing concentrations of digitonin per milligram of mitochondrial protein. After a 10-min incubation period, the digitonin was diluted by the addition of mitochondrial medium, centrifuged at 27,000 *g*, and the enzymatic activities in the pellet were assayed. It would be expected that enzymes localized in the space between the inner and outer membranes would be released by lower concentrations of the detergent than would be required for release of the inner membrane or matrix enzymes, since the outer membrane would be ruptured before the inner membrane. It is of interest that considerably higher concentrations of digitonin were necessary to disrupt the nematode mitochondria (Fig. 5) than were employed for the mammalian organelles (Schnaitman and Greenawalt, 1968).

Fumarase, adenylate kinase, and malic enzyme

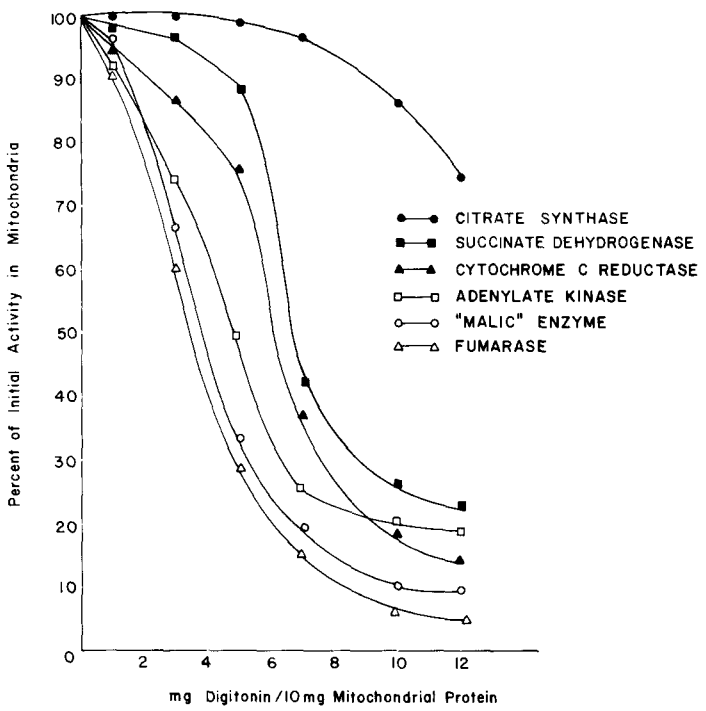


FIGURE 5 Effect of increasing digitonin concentrations on the release of enzymes from *Ascaris lumbricoides* var. *suum* mitochondria. Suspensions of *Ascaris* mitochondria were incubated with the indicated concentrations of digitonin for 10 min. Mixtures were then diluted with mitochondrial media, the particulate fraction was recovered by centrifugation, and the percentage of the indicated enzymatic activities remaining were determined.

were all released to the medium at a lower digitonin concentration than any of the other enzymes assayed (Fig. 5). These findings are in agreement with the supposition that all three enzymes are present primarily in the intermembrane space. That they are not present in the matrix was indicated by the finding that considerably higher concentrations of digitonin were required to release the matrix marker citrate synthase from the submitochondrial particles. Similarly, the inner and outer membrane markers, succinate dehydrogenase and rotenone-insensitive cytochrome *c* reductase, respectively, both require higher concentrations of digitonin for release into the soluble fraction. As might be expected, the release of the rotenone-insensitive cytochrome *c* reductase from the outer membrane required higher concentrations of digitonin, since low concentrations of the surfactant rupture the outer membrane without removing it from the inner membrane plus matrix.

DISCUSSION

If, as found in these studies, fumarase and malic enzyme are distributed in the intermembrane space of the *Ascaris* mitochondrion, then the question of the physiological significance of this altered distribution arises. Malate serves as the mitochondrial substrate in this and in other invertebrate systems, where it undergoes a dismutation reaction (Fig. 1). The malic enzyme oxidizes malate to pyruvate, resulting in the formation of intramitochondrial NADH. Concurrently, fumarase dehydrates malate to fumarate which, in turn, is reduced by the NADH to form succinate and ATP. If both malic enzyme and fumarase are present in the intermembrane space, then in contrast with the mammalian organelles, fumarate and NADH would be generated in the intermembrane compartment of the nematode mitochondrion. The nematode Mg^{++}-dependent ATPase and the succinate dehydrogenase with its associated electron transport system

appear to have a distribution similar to that reported in mammalian mitochondria. In addition, the inner membranes of both species of mitochondria are presumed to be impermeable to the nucleotide NADH (Lehninger, 1951). If this nucleotide is required by the worm for the reduction of fumarate to succinate and the electron transport-associated generation of ATP, then where and how does this energy-yielding reduction of intermembrane space fumarate by intermembrane space NADH take place?

Seligman et al. (1968) reported cytochemical studies which indicated that succinate dehydrogenase was associated with the intermembrane space side of the inner membrane in mouse kidney and rat heart mitochondria. If this were true in *Ascaris*, NADH could reduce fumarate directly on this side of the membrane, although phosphorylation would have to occur on the opposite side of the membrane (ATPase). The findings of Seligman et al. (1968), however, are not in agreement with those of Harris et al. (1967) and of Quagliariello and Palmieri (1968) that succinate can reach the respiratory chain preferentially from the matrix side of the inner membrane in mammalian mitochondria.

It appears more likely that fumarate formed in the intermembrane space compartment would traverse the inner membrane where it would then be reduced to succinate with the concomitant generation of ATP. Such a mechanism, however, would require a means of transferring reducing power from the NADH formed in the intermembrane space across the inner membrane. Fioravanti and Saz (1973) reported the presence of an active NADH/NAD transhydrogenase associated with the mitochondrial inner membrane of *Ascaris*. Interestingly, NADPH-NAD transhydrogenase activity was not demonstrable. Since transhydrogenases recently have been incriminated in the process of proton translocation across the mitochondrial inner membrane (Moyle and Mitchell, 1973), the possibility has been suggested that the *Ascaris* NADH-NAD transhydrogenase activity may serve to transport protons from NADH in the intermembrane space, across the inner membrane, resulting in NADH accumulation within the matrix.

This postulation is supported to some extent by the earlier findings of Papa et al. (1970). These authors reported a requirement of inorganic phosphate for the production of succinate from malate in *Ascaris* mitochondria. They suggested that inorganic phosphate was necessary for malate transport. The addition of malate to *Ascaris* mitochondria in the absence of phosphate resulted in an accumulation of fumarate and pyruvate, but little or no succinate was formed. The addition of phosphate increased succinate accumulation. These findings would be explained if fumarase and the malic enzyme were located in the intermembrane space as suggested above.

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