SUBCELLULAR LOCALIZATION OF GLYOXYLATE CYCLE ENZYMES IN *ASCARIS SUUM* LARVAE

HARRIS RUBIN and RICHARD N. TRELEASE

From the Departments of Zoology, and of Botany and Microbiology, Arizona State University, Tempe, Arizona 85281

ABSTRACT

Evidence is presented on the particulate nature of glyoxylate cycle enzymes in metazoa with the use of 15-day old larvae of the nematode *Ascaris suum*. Homogenization procedures were developed to disrupt the resistant nematode cuticle. Malate synthase and isocitrate lyase, key enzymes of the glyoxylate cycle, consistently sedimented with mitochondrial enzymes in differential pellets while catalase, a major peroxisomal enzyme, was always soluble. Isopycnic sucrose gradient centrifugation of the differential pellet yielded two protein peaks: one at 1.18 g/cm^3 (characteristic for mitochondria), and another at 1.23 g/cm^3 (common for glyoxysomes and peroxisomes). Electron microscopy of these fractions revealed that the lighter peak consisted primarily of mitochondria, while the heavier band contained proteinaceous bodies termed "dense granules" morphologically resembling microbodies. Significantly, both malate synthase and isocitrate lyase cosedimented with the mitochondrial marker enzymes in the lighter peak (1.18 g/cm^3) and not with the dense granules. Further purification of mitochondria, accomplished by separating dense granules with a step gradient before isopycnic centrifugation, substantiated the evidence that microbodies (glyoxysomes) do not occur in these nematode larvae. Rough-surfaced membranes were alternatively considered as the subcellular site, but the evidence tends to favor localization of the glyoxylate bypass enzymes in the mitochondria.

The glyoxylate cycle, a metabolic bypass of the tricarboxylic acid cycle, is an anaplerotic pathway providing 4-carbon dicarboxylic acids for biosynthetic intermediates of the cell. It was originally delineated for bacteria (10) and subsequently elucidated in a number of eucaryotic groups (9). Among metazoa, however, only the nematodes have been reported to possess this cycle (3, 18, 19).

In embryos of the nematode parasite, *Ascaris suum*, the glyoxylate bypass serves as the metabolic pathway for the resynthesis of glycogen from storage triglyceride (3). After a marked decline in stored glycogen, this resynthesis occurred between the 12th and 20th day of development when the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, were also reported to be maximal. With electron microscopy, we demonstrated that triglyceride lipid droplets, which serve as the metabolic precursors for the resynthesis, were localized in the posterior region of the worm, and that increases in α-glycogen occurred principally in this area (21). Dense granules, single membrane-bounded organelles, were also specifically localized in the posterior region and have a demonstrable close association with both the

lipid and glycogen stores. These organelles possess morphological characteristics reminiscent of microbodies (glyoxysomes) which are known to contain the enzymes to the glyoxylate cycle in higher plants, fungi, algae, and protozoa (2, 14, 26).

Although Nematoda represents the highest level phylogenetically among metazoa at which the glyoxylate cycle prevails, little data are available on the subcellular localization of its enzymes. Our preliminary work (20) indicated that the enzymes were particulate. On the basis of these findings, and the topographical location of organelles seen in our ultrastructural studies, we postulated that the dense granules were a type of nematode microbody containing the glyoxylate cycle enzymes. To test this hypothesis, homogenization and fractionation procedures were developed for disrupting nematode cuticles and obtaining intact organelles. In the present report, we present evidence that the glyoxylate cycle enzymes are not associated with the isolated dense granules, but cosediment with mitochondria during rate and equilibrium centrifugations.

MATERIALS AND METHODS

Preparation of Eggs

Adult *Ascaris suum* worms were obtained from a local slaughterhouse and maintained in 0.85% saline at 5°C until needed. Extraction and incubation of eggs were done essentially according to the methods of Ward and Fairbairn (27). The anterior third of adult uteri containing undeveloped, fertilized eggs were removed by dissection, homogenized briefly in 0.5 N sodium hydroxide with a Dounce homogenizer (Kontes Glass Co., Vineland, N. J.), and stirred in 3–4 vol of 0.5 N sodium hydroxide for 25 min at room temperature to remove the uterine coat from the eggs. The extracted eggs were washed twice in distilled water by centrifugation (1,000 *g*, 5 min), and incubated in a shaker water bath (Precision Scientific Company, Chicago, Ill.) at 30°C in 125-ml flasks containing 0.1 N sulfuric acid as a development medium. Bacterial contamination in the development medium was periodically tested for by plating aliquots onto a trypticase soy agar medium (Baltimore Biological Laboratories, Baltimore, Md.).

Homogenization Procedure

Before homogenization, two of the three outer eggshell layers were removed (decoated) from developing eggs. For various experiments, this procedure was performed at two different times, i.e., either immediately before homogenization (15-day old worms) or 15 h before homogenization. Treatment at both times gave comparable results. Egg batches were decoated by incubation in 5.25% sodium hypochlorite (8) for 45 min. The decoated eggs floated in the neck of the holding flask and were easily removed by pipette. Approx. 8 g (fresh weight) of decoated eggs were recovered from 21–24 g of shelled eggs extracted from the uterine starting material. Those eggs decoated 15 h before homogenization were washed three times in distilled water and maintained overnight at 30°C in 0.85% saline. Bacterial growth during this period was insignificant.

Immediately before homogenization, eggs decoated by either method were collected by centrifugation at 1,000 *g* for 5 min, washed twice with distilled water, and resuspended in 15 ml of grinding medium containing 0.25 M sucrose and 0.2% bovine serum albumin (BSA) in 0.005 M Tris-HCl, pH 8.0. The larvae were immediately freed from their eggshells into the grinding medium by vigorous shaking with sea sand (Fisher Scientific Co., Fair Lawn, N. J.) for 2–5 min. Larval release was monitored by bright-field microscopy. The grinding medium, containing the released worms, was pipetted from the sand, and the worms were separated from the disrupted eggshells by centrifugation at 3,000 *g* for 5 min. 5–6 g (fresh weight) of packed larvae were routinely obtained from 40 g of uterine starting material.

The packed larvae were resuspended in approx. 10 ml of grinding medium and poured into a cold (4°C) mortar. A heavy slurry of worms was prepared by mixing the resuspension with 0.25-mm glass beads (B. Braun Instruments, San Mateo, Calif.) in a vol ratio of 1:3, respectively. Homogenization was initiated by hand grinding the slurry of worms for 3–5 min. The degree of breakage was monitored by bright-field microscopy. This homogenate was filtered through three layers of Miracloth (Johnson & Johnson Filter Products Div., Chicago, Ill.) and washed with an additional 10 ml of grinding medium. The tissue residue, still containing glass beads, was gently scraped from the Miracloth into the mortar and resuspended in a final vol of approx. 10 ml more of grinding medium which was sufficient to maintain the heavy slurry consistency. This sequence of hand grinding, filtration, and resuspension was repeated three times with the tissue residues. This process of several brief homogenizations followed by filtration of the slurry gave the highest yield of intact organelles. Routinely, 70–75 ml of filtrate were recovered.

Differential and Sucrose Density Gradient Centrifugation

The pooled homogenate was differentially centrifuged (Beckman JS-13 swing-out rotor, Beckman Instruments, Spinco Div., Palo Alto, Calif.) two times, first at 522 *g* max for 10 min, to remove worm fragments, nuclei, and cell debris, and then at 17,770 *g* max for 10 min to sediment the glyoxylate cycle enzymes. This pellet was resuspended in 0.005 M Tris-HCl (pH 7.5) containing 0.25 M sucrose, and applied on top of a 30-ml sucrose solution (43% wt/wt, 1.20 g/cm^3) buffered with 0.005 M

Tris-HCl, pH 7.5. This step gradient was centrifuged for 30 min in a SW-25.1 rotor at 40,000 *g* max. The supernatant layer, approx. 8 ml cleared of dense granules, was then collected by pipette and layered onto a continuous, 50-ml sucrose gradient (25–57% wt/wt) buffered with 0.005 M Tris-HCl, pH 7.5. Particles were centrifuged to equilibrium in a SW-25.2 rotor for 4 h at 38,500 *g* max. 2-ml fractions were drop-collected with a Gilson microfractionator (Gilson Medical Electronics Inc., Middleton, Wis.) from the bottom of cellulose nitrate tubes.

Enzyme Assays

The supernates and resuspended pellets were disrupted in a French pressure cell (10,000 lb/inch^2) before conducting each assay. Fumarase was assayed spectrophotometrically at 240 nm (15). Citrate synthase activity was determined by measuring the appearance of released CoA at 412 nm (23). Malate dehydrogenase was assayed by measuring the rate of nucleotide oxidation at 340 nm (28). Malate synthase (EC 4.1.3.2) was assayed by following the cleavage of the thioester bond in acetyl-CoA at 232 nm (5). Catalase was assayed spectrophotometrically at 240 nm by the method described by Lück (13). Glucose-6-phosphatase (G-6-Pase) activity was determined by a discontinuous assay after the procedure of Leskes et al. (11). Isocitrate lyase (EC 4.1.3.1) activity was determined by measuring the formation of glyoxylate from isocitrate in a discontinuous assay (17). The reaction mixture contained 92 mM morpholinopropane sulfonic acid (MOPS) pH 7.7, 5 mM magnesium chloride, 1 mM sodium ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.1 ml of enzyme, and 4.4 mM isocitrate in a final vol of 3.6 ml. Protein was measured by the method of Lowry et al. (12).

Electron Microscopy

After appropriate gradient fractions were pooled, a final concentration of 3% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) was added, and the mixtures were allowed to fix for 10 min. To dilute the sucrose concentration for centrifugation, 3% glutaraldehyde, buffered with 0.05 M potassium phosphate, pH 7.4, was added in a 1:1 ratio to the fixed fractions. The fixed material was collected by centrifugation (17,700 *g*, 10 min) and washed three times in 0.05 M potassium phosphate, pH 7.4. It was finally resuspended in 1.0 ml of 0.05 M potassium phosphate (pH 7.4), dispersed onto a Millipore filter (0.45 μm) (Millipore Corp., Bedford, Mass.) by means of a Swinney adaptor, and encased in warm 2% agar. The agar-encased filter was washed once more in phosphate buffer (pH 7.4), postfixed in phosphate-buffered 2% osmium tetroxide for 1 h, dehydrated in a graded series of acetones, and embedded in a mixture of low viscosity resins (22). Whole organisms were fixed and embedded as previously described (21). Silver-grey sections were obtained with a Sorvall MT-2 ultramicrotome (DuPont Instruments, Sorvall Operations, Wilmington, Del.) and examined on a Philips EM 300 electron microscope after poststaining with aqueous 2% uranyl acetate and lead citrate.

RESULTS

All experiments on fractionation and isolation of organelles were done with 15-day old *Ascaris* larvae because at this stage malate synthase and isocitrate lyase activities were near maximal levels (3) and glycogen was being actively resynthesized (21). The site of the lipid-to-glycogen interconversion was found to be the posterior region of the larvae (21). This area was occupied by deposits of lipid bodies closely associated with α-glycogen (Fig. 1). Mitochondria and especially dense granules were specifically localized among the lipid bodies, whereas few other organelles or membrane systems were observed in this region. The majority of other common cell organelles were found in the peripheral somatic muscle cells and the cell types of the anterior region (21).

Because there were no known techniques in the literature for isolation of intact organelles from *Ascaris* larvae, development of a satisfactory homogenization technique had to be empirical (Table I). Citrate synthase, common to both glyoxylate and tricarboxylic acid cycles, was used as an indicator enzyme. Pressing the larvae at 10,000 lb/in^2 (no. 1 and 2, Table I) was initially tested since it had been successful for demonstrating glyoxylate cycle and β-oxidation activities in larval extracts (3, 27). High pressure pressing (no. 1, Table I) left an unusually high amount of intact cells or larvae (755 *g* pellet); those cells that were homogenized exhibited consistently low activities in the 10,500 *g* pellet. Shearing plus pressing (no. 2, Table I) was attempted to increase the yield, but it was too harsh for obtaining intact particles. In fact, whenever two homogenizing techniques were used sequentially (see nos. 2, 3, and 6, Table I), the 10,500 *g* pelletable activity was always low. Substantial improvement was evident when worms were first released from their eggshells (see Materials and Methods) and then sheared in a motor-driven Teflon pestle (no. 4, Table I). Bloom and Entner (4) produced a mitochondrial pellet with this homogenizer, but no data were given for recoveries in their pellets. We found this technique too mild for breaking larval cuticles because a reasonable yield of particles was not reliably obtained even after lengthy time periods. In other experiments, lower pressures in the French press

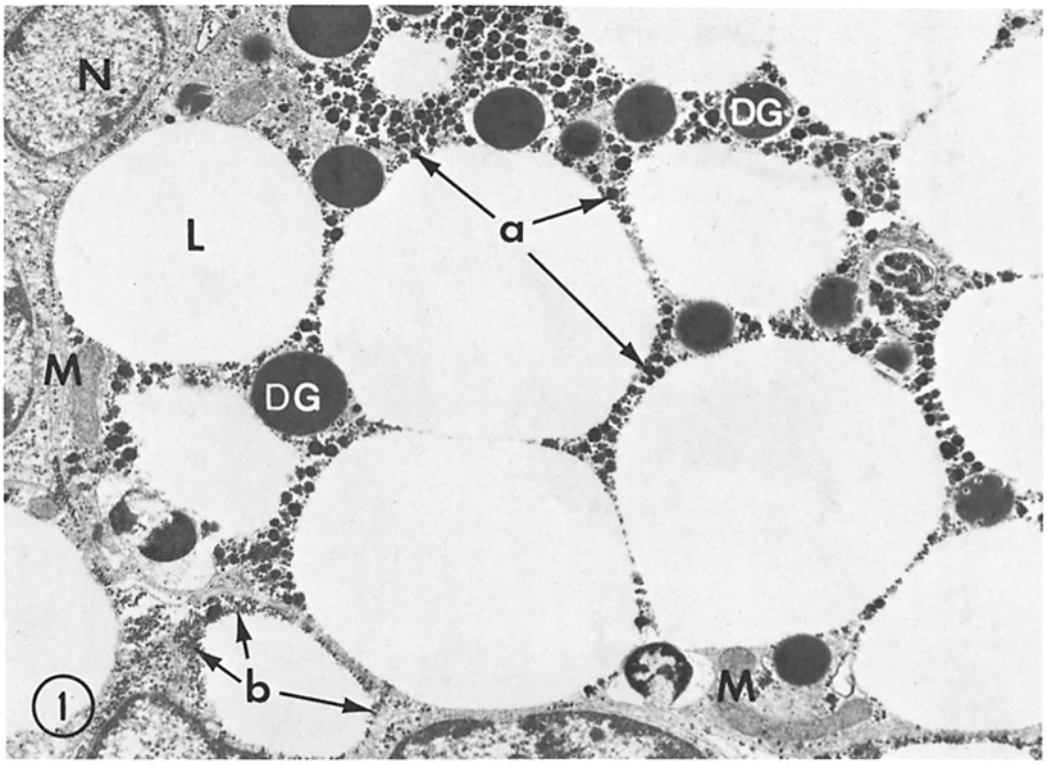


FIGURE 1 Electron micrograph of a longitudinal section view through the posterior region of a 15-day old *Ascaris* larva. Lipid bodies (*L*), which are abundant and closely appressed at this stage of development, are restricted to this posterior region where they occupy the central noncellular area. Osmiophilic, dense granules (*DG*) are also confined to the posterior region and are localized among the numerous lipid bodies. Accumulations of α-glycogen particles (*a*) are distributed among the lipid droplets as are mitochondria (*M*) which are rather pleiomorphic and of the same approximate size as the spherical dense granules. Profiles of rough ER are noticeably absent in this area. Bordering the central region are nucleated (*N*) somatic muscle cells characterized by β-glycogen (*b*) rather than α-glycogen particles. × 22,000.

were used (no. 5, Table I) in conjunction with the Teflon pestle (no. 6, Table I); these procedures resulted in apparent enzyme inactivation (low recoveries) and low proportions of pelletable activities. Hand grinding free larvae in a mortar with a slurry of glass beads (no. 7, Table I) consistently gave high recoveries but only 48–65% pelletable citrate synthase activity. This procedure provided reasonable amounts of intact mitochondria and/or presumptive glyoxysomes which could be reliably sedimented from larval homogenates.

Having developed a satisfactory homogenizing procedure, we determined an optimal rate-sedimentation for selected glyoxylate and tricarboxylic acid cycle enzymes (malate synthase, citrate synthase, and fumarase). The highest proportion of particulateness and specific activities were recovered in the 17,770 *g* (10 min) pellets. Higher-rate centrifugations gave significant specific activities for each enzyme, but only 4–7% of the activities were pelletable. Omitting the 17,770 *g* spin after the first centrifugation did not improve the percent particulateness or specific activities in a 10,200 *g* (25 min) pellet, but only succeeded in tightly packing the particles, making resuspension difficult.

Table II shows that both mitochondria and glyoxylate cycle enzymes cosediment at 17,770 *g* (10 min). 49% of fumarase activity was sedimentable, with 30% and 47% of malate synthase and isocitrate lyase, respectively, in the 17,770 *g* pellet. Citrate synthase, common to both cycles, sedimented with these three enzymes while the majority of the activity for malate dehydrogenase, also a shared enzyme, was apparently soluble. This was not unexpected, since Zee and Zinkham (28) re-

TABLE I

Comparison of Citrate Synthase Pelletability from Homogenates of 15-Day Old Ascaris Larvae

Procedure no.	Homogenization technique	Percent of activity in the 755 *g* supernate				Percent recovery from 755 *g* supernate
		755 *g* supernate	755 *g* pellet	10,500 *g* supernate	10,500 *g* pellet	
1	French press (10,000 lb/in²)	100	30	84	13	97
2	Blender + French press (10,000 lb/in²)	100	ND*	113	3	116
3	Potter-Elvehjem + sonication	100	3	77	15	92
4	Potter-Elvehjem	100	ND*	58	31	89
5	French press (3,000 lb/in²)	100	16	55	21	76
6	French press (500 lb/in²) + Potter-Elvehjem	100	16	41	8	49
7	Mortar and pestle + glass beads	100	5	45	48	93

Larvae contained within eggshells were used as the starting material for procedures no. 1, 2, and 3 whereas larvae released from their eggshells with sea sand after hypochlorite treatment were used in procedures no. 4–7. The homogenized material was centrifuged at 755 *g* for 15 min; this supernate was centrifuged again at 10,500 *g* for 30 min.

* ND = not determined.

TABLE II

Percent Distribution of Enzyme Activity in 15-day Ascaris Larvae Recovered in Differential Sedimented Fractions

Enzyme	Homogenates	522 *g* (10 min)		17,700 *g* (10 min)		110,000 *g* (60 min)	
		Supernate	Pellet	Supernate	Pellet	Supernate	Pellet
Fumarase	100	96	2	45	49	28	11
Citrate synthase	100	98	5	44	48	34	10
Isocitrate lyase	100	81	1	38	47	16	4
Malate synthase	100	83	3	54	30	43	13
Malate dehydrogenase	100	98	2	90	2	ND*	ND*
G-6-Pase	100	82	7	84	12	72	10
Catalase	100	99	3	77	5	70	5

The supernate from each step was sequentially centrifuged at a higher *g* force. All percent values are calculated from activities in the filtered homogenates.

* ND = not determined.

TABLE III

Comparison of Specific Activities for Glyoxylate and Krebs Cycle Enzymes in the Differential Pellet and in Bands from Continuous Gradients

Enzyme	Sp act (nmol/min/mg protein)		
	Differential pellet 17,700 *g* (10 min)	Gradient peak (1.189 g/cm³) without step gradient	Gradient peak (1.182 g/cm³) with step gradient
Fumarase	173	562	1,269
Citrate synthase	137	467	931
Malate synthase	23	52	105
Isocitrate lyase	28	57	129

Values are averages from four separate experiments.

ported the occurrence of one mitochondrial and three cytoplasmic isozymes for malate dehydrogenase.

In all of our experiments, the greatest proportion of the microbody enzyme catalase was always recovered in supernatant fractions. We often experienced a loss in activity for catalase during fractionation; nevertheless, when the enzyme was subjected to 110,000 *g* microsomal centrifugation, nearly 90–99% of the activity was consistently found soluble (Table II). G-6-Pase, an endoplasmic reticulum (ER) marker, typically followed the distribution of catalase, suggesting that these two enzymes were not compartmentalized with the glyoxylate bypass enzymes.

In attempts to define the particulate nature of

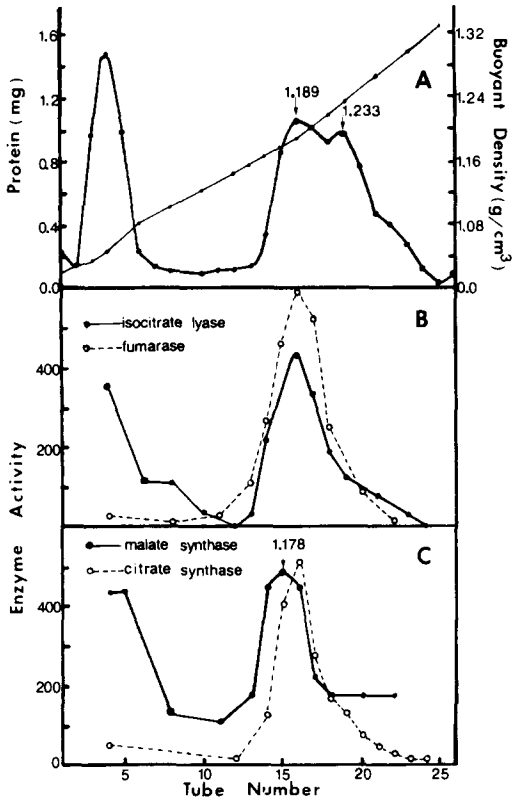


FIGURE 2 Protein and enzyme distribution after equilibrium centrifugation. A resuspended 17,770 *g* (10 min) pellet was layered on top of a continuous gradient ranging from 16 to 60% (wt/wt) sucrose. (A) Two protein peaks are resolved in the gradient at 1.189 and 1.233 g/cm³. (B and C) Both the glyoxylate and Krebs cycle enzyme activities coband in the lighter protein region, showing only minimal activities in the heavier 1.233 g/cm³ region. Only malate synthase does not precisely coband; however, the activity is only shifted to the lighter side by one tube. Some solubilized activities are apparent for malate synthase and isocitrate lyase. Enzyme activities: fumarase and citrate synthase, nmoles/minute/2 ml fraction; malate synthase and isocitrate lyase, × 10. Percent recovery values obtained from the sum of gradient activities × 100 per activity in the layer is approx. 54, 60, 38, and 42% for the above enzymes, respectively, and 66% for protein.

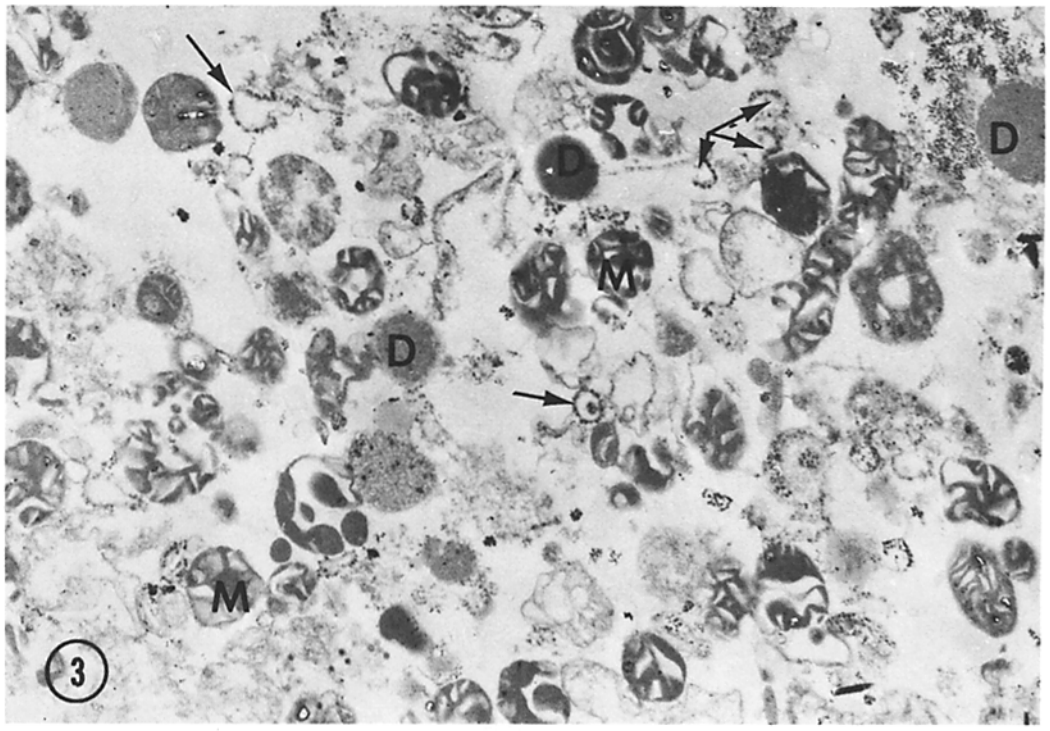
glyoxylate and tricarboxylic acid cycle enzymes, the 17,700-*g* particulate fraction was subjected to isopycnic density-gradient centrifugation. The profiles of the protein and enzyme distributions after centrifugation are shown in Fig. 2. Two protein peaks were apparent: the lighter occurred at a density of 1.189 g/cm³, and the denser peak banded at 1.233 g/cm³. The profiles for fumarase, isocitrate lyase, and citrate synthase cobanded with the lighter protein peak. Malate synthase was not precisely coincident with the other enzyme profiles, but the majority of its activity was in the lighter protein band. Comparisons of specific activities in the lighter protein band with those in the 17,770 *g* differential pellet showed nearly a threefold purification for fumarase and citrate synthase, and a twofold purification for malate synthase and isocitrate lyase (Table III). It was apparent that both mitochondrial and glyoxylate cycle enzyme activities were associated in the same protein fraction. Electron microscopy revealed that the lighter protein peak was primarily a mitochondrial fraction, contaminated with dense granules and membrane fragments (Fig. 3). The 1.233 g/cm³ band essentially lacked any of these enzyme marker activities and was principally composed of dense granules, with only relatively few mitochondria and membrane fragments (Fig. 4).

In an attempt to purify the lighter protein band, the resuspended differential pellets were layered first onto a 43% wt/wt sucrose solution (1.20 g/cm³) and centrifuged to separate dense granules from the mitochondria. Electron microscopy showed that the pellet (material heavier than 1.20 g/cm³) was composed principally of dense granules contaminated with some nuclei, damaged mitochondria, and membrane fragments. Only 5–12% of the activities (nmoles/minute) for mitochondrial and glyoxylate cycle enzymes was found in this pellet; the remainder was primarily associated with the supernatant fraction remaining on top of the 43% sucrose.

Isopycnic centrifugation of this supernate resulted in a single protein peak at a density of 1.182 g/cm³ (Fig. 5). Mitochondrial and glyoxylate cycle enzymes again cobanded with this protein peak. Electron microscopy indicated that the 1.182 g/cm³ protein band was a highly enriched mitochondrial fraction with some rough-surfaced membrane fragments as the principal source of contamination (Fig. 6). Significantly, the elimination of dense granules from the 1.182 g/cm³ mitochondrial peak was reflected by a twofold increase in specific activity for both tricarboxylic acid and glyoxylate cycle enzymes (Table III).

DISCUSSION

In a preliminary report (20), we presented the first evidence for the particulate nature of glyoxylate cycle enzymes in metazoa, using larvae of *Ascaris suum*. However, the specific subcellular localiza-



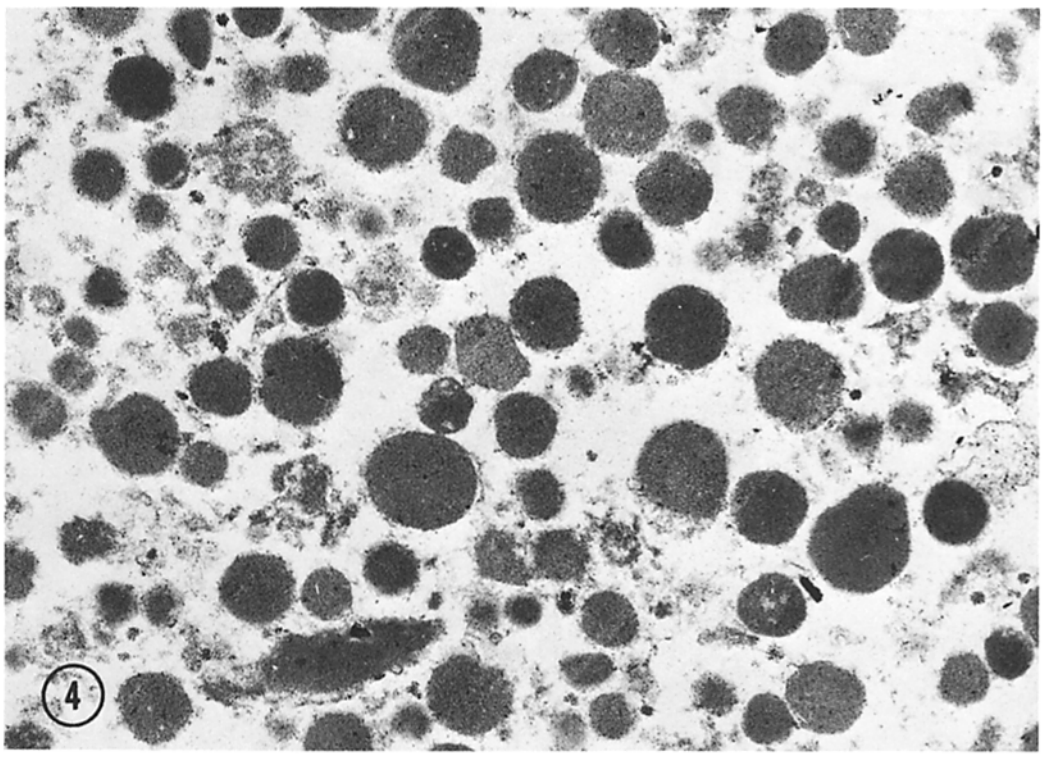


FIGURE 3 Representative electron micrograph of particles banding at 1.189 g/cm³ in gradients similar to that shown in Fig. 2. Mitochondria (*M*) which are always seen in a condensed configuration constitute the largest proportion of the particles present. Osmiophilic dense granules (*D*), which are distinguishable by their homogeneous matrix, are always prevalent but in relatively fewer numbers than the mitochondria. Fragments of rough-surfaced membranes (arrows) are also commonly observed in this fraction. × 25,500.

FIGURE 4 Representative electron micrograph of particles banding at 1.233 g/cm³ in gradients similar to that shown in Fig. 2. These particles are identified as dense granules by their homogeneous, osmiophilic matrices, and their general spherical appearance. × 19,000.

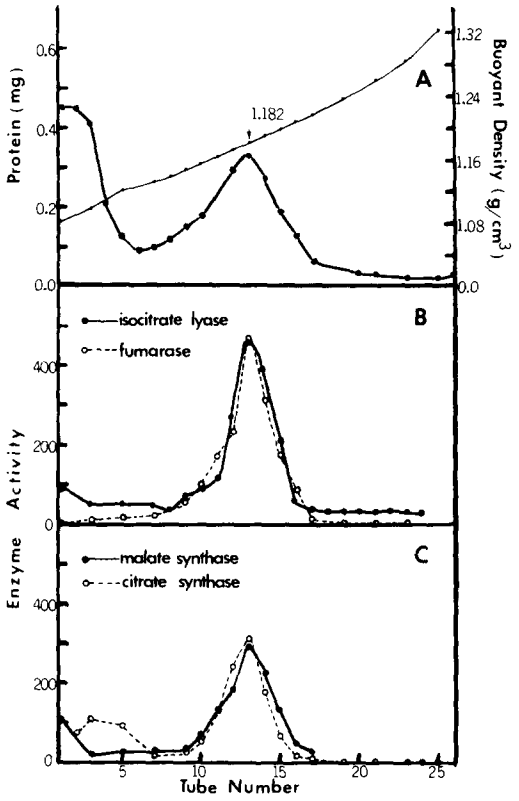


FIGURE 5 Protein and enzyme distribution after equilibrium centrifugation. (A) The step-gradient supernate was layered onto a continuous gradient ranging from 25 to 57% (wt/wt) sucrose. A single protein peak is apparent in the gradient at 1.182 g/cm³. (B and C) Both the glyoxylate and Krebs cycle enzymes coband in this one region of the gradient. Little solubilized activities are apparent at the top of the gradient. Enzyme activities: fumarase and citrate synthase, nmoles/minute/2 ml fraction; malate synthase and isocitrate lyase, × 10. The percent recoveries (sum of enzyme activity × 100/activity in the layer) for these enzymes, respectively, are approx. 62, 57, 38 and 69% and 74% for protein.

tion of these enzymes had not been demonstrated. This was primarily due to the difficulty in isolating intact organelles from nematode larvae that had resistant cuticles and were contained within multilayered egg shells. This problem is shown in Table I, where the percent pelletability of enzymes is clearly a reflection of the homogenization process. Our best homogenization procedure yielded only 30–47% pelletability for malate synthase and isocitrate lyase, respectively (Table II). These values did not directly demonstrate that the glyoxylate cycle was totally operative within organelles, but we could also only demonstrate approx. 50% pelletable activity for the cosedimenting mitochondrial enzymes. The soluble activities probably resulted from disruption of the organelles rather than from differential enzyme leakage because of the relatively uniform solubilities of all the particulate enzymes studied (see Table II). Under these conditions, where homogenization is problematic, we consider 30–47% reasonably representative of particulate enzyme activity, and conclude that this pathway is probably housed within organelles.

The dense granules found in the posterior region of *Ascaris* larvae were considered as the possible subcellular site for the glyoxylate-cycle activity. This notion was based on their being morphologically similar to microbodies (glyoxysomes), their *in situ* localization (21), and their buoyant density at 1.23 g/cm³ (present work). The biotase activity was undetectable in the gradient fractions, and G-6-Pase activity was mostly soluble (Table III). However, the findings that catalase was not particulate, that malate synthase cytochemical reactivity was lacking (25), and that membrane profiles were absent in the posterior region of *Ascaris* (21) and another nematode, *Meloidogyne incognita* (6), all tend to cast doubt on the proposal that these membranes are functioning as "microglyoxysomes." It seems more likely that the mitochondria in these fractions are responsible for the glyoxylate cycle activities.
chemical results showed, however, that the glyoxylate cycle enzymes did not band at 1.23 g/cm³, or in pellets obtained from the step gradients, but followed the distribution of Krebs cycle enzymes at 1.18 g/cm³. The consistently soluble activities for catalase also pointed to the conclusion that dense granules were not microbody-type organelles. It seems likely that the original postulate for dense granule function is still tenable, i.e., that they are utilized as structural components during development of the intestine (7).

What, then, is the particulate site of the glyoxylate cycle? The cosedimentation of both Krebs and glyoxylate cycle enzymes during rate centrifugations does not by itself indicate that these enzymes act within mitochondria, because peroxisomes, glyoxysomes, and mitochondria typically sediment at the same rate (9, 24). The lack of evidence for any conventional appearing microbodies and the preponderance of mitochondria in these fractions do, however, make the mitochondria likely candidates. Moreover, removal of dense granules from these fractions showed that the specific activities for both Krebs and glyoxylate cycle enzymes be-

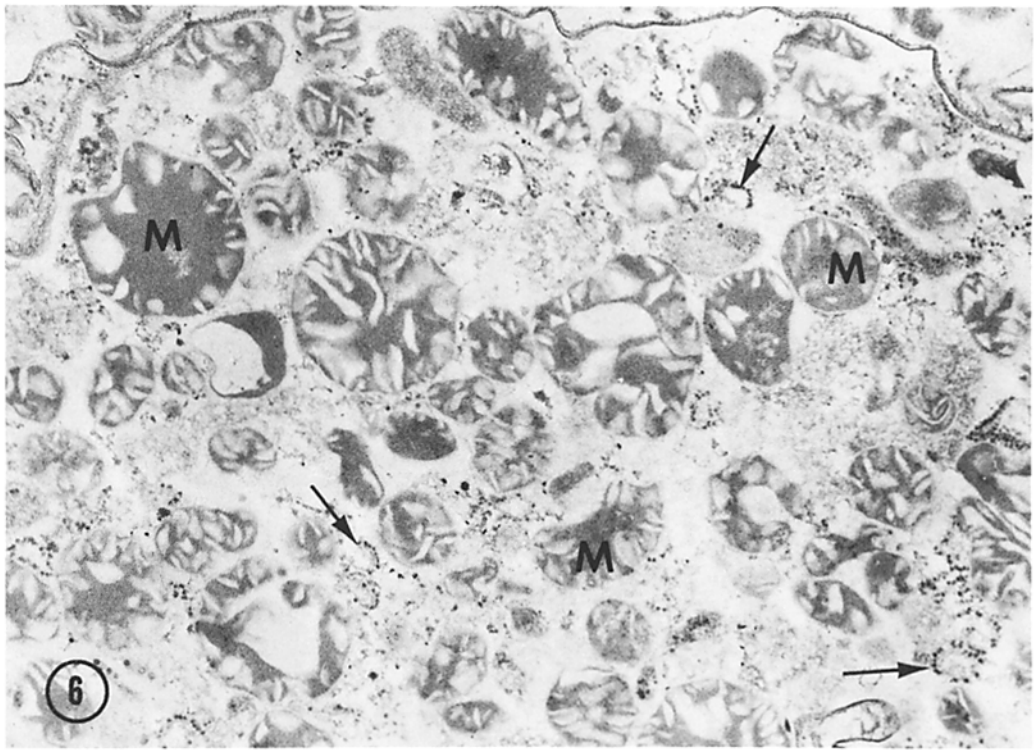


FIGURE 6 Representative electron micrograph of particles banding at 1.182 g/cm³ in gradients similar to that shown in Fig. 5. Mitochondria (*M*) in the condensed form comprise the major population of particles that are present in the band. Rough-surfaced membranes (arrows) persistently occur in this fraction. × 30,000.

haved the same, i.e., they both increased twofold (Table III). This would be expected if both metabolic pathways were localized within the same organelle.

The persistent occurrence of rough-surfaced membranes in the 1.18 g/cm³ bands makes them possible sites of the glyoxylate cycle activities. That they should be seriously considered stems from the well-known ontogenetic relationship between membrane precursors and microbodies (glyoxysomes and peroxisomes) (9, 16). From our data, we cannot be certain whether these are rough ER fragments or broken membranes with absorbed glycogen. NADPH cytochrome *c* reductase activity was undetectable in the gradient fractions, and G-6-Pase activity was mostly soluble (Table III). However, the findings that catalase was not particulate, that malate synthase cytochemical reactivity was lacking (25), and that membrane profiles were absent in the posterior region of *Ascaris* (21) and another nematode, *Meloidogyne incognita* (6), all tend to cast doubt on the proposal that these membranes are functioning as "microglyoxysomes." It seems more likely that the mitochondria in these fractions are responsible for the glyoxylate cycle activities.

The idea that glyoxylate cycle enzymes may be housed within mitochondria is not without precedent. Avers (2) believes that, in yeast, isocitrate lyase is contained within both peroxisomes and mitochondria. In *Tetrahymena*, the glyoxylate cycle enzymes are not solely localized in the glyoxysomes, but are shared with the mitochondria (14). Working with the free-living nematode *Turbatrix aceti*, Aueron and Rothstein (1) reported the equilibration of isocitrate lyase, catalase, and two other peroxisomal enzymes in a mitochondria-rich fraction. On the basis of differential enzyme release from mitochondrial pellets, they suggest that peroxisomes actually contain the isocitrate lyase and that these organelles simply band with the mitochondria. However, they clearly admit that their evidence for the existence of two types of organelles is not conclusive. Thus, it appears

reasonable from our data and the information in the literature that nematode mitochondria could house the glyoxylate cycle enzymes. This proposal is particularly significant because in all eucaryotes known to compartmentalize the enzymes of the glyoxylate cycle, the enzymes are housed within a glyoxysome-type organelle. Since our data do not support this spatial separation of particles, the occurrence of glyoxysomes in metazoa has yet to be demonstrated.

The authors wish to express their appreciation to the Cudahy Co. (Phoenix, Ariz.) for the time and consideration given in providing the adult ascarid worms used in this investigation. They are also grateful for the technical assistance provided by Ms. Mary Kimble and Ms. Anna Hill.

This work was supported by National Science Foundation grant GB-43636.

Received for publication 30 December 1975, and in revised form 5 April 1976.

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