Comp. Biochem. Physiol., Vol. 64B, pp. 57 to 63
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0305-0491/79/0801-0057$02.00/0

INTESTINAL PEROXISOMES OF GOLDFISH (*CARASSIUS AURATUS*)—EXAMINATION FOR HYDROLASE, DEHYDROGENASE AND CARNITINE ACETYLTRANSFERASE ACTIVITIES

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(*Received* 22 *January* 1979)

Abstract—1. Rate sedimentation and isopycnic centrifugation were used to analyse the subcellular sites of enzymes in homogenates of goldfish intestinal mucosa.

2. The results allowed the following allocations to be made: carnitine acetyl transferase-mitochondrial and peroxisomal, xanthine dehydrogenase and NAD: α-glycerophosphate dehydrogenase soluble phase, NADP: isocitrate dehydrogenase soluble phase and mitochondrial, and 2-naphthyl laurate hydrolase microsomal and/or brush border.

3. Histochemistry confirmed the use of alkaline phosphatase and 1-naphthyl acetate esterase as brush border and microsome markers respectively.

4. Urate oxidase, allantoinase, allantoicase, xanthine oxidase and glycollate/lactate oxidase, activities were undetectable, and 1-naphthyl palmitate hydrolase was present only as a contaminant from pancreas.

INTRODUCTION

Many, possibly all, mammalian tissues contain core-free catalase particles which are smaller than the core containing peroxisomes of liver and kidney (Hruban *et al.*, 1972; Novikoff *et al.*, 1973). Novikoff & Novikoff (1972) have termed these organelles "microperoxisomes" because by electron microscopy they are structurally distinguishable from the originally observed hepatic peroxisomes. Whether all these particles fulfill De Duve's definition of peroxisomes (De Duve & Baudhuin, 1966) is not certain. To do so they would have to be shown to contain, in addition to catalase, at least one oxidase. In only a few tissues have microperoxisomes been studied by biochemical techniques, and in some of these tissues they have been found to contain oxidase activity (Connock *et al.*, 1974; Herzog & Fahimi, 1975; Goldenberg *et al.*, 1976; Peters, 1976) but in others to possibly lack oxidases (Bock *et al.*, 1975; Goldenberg *et al.*, 1978). To discover the function(s) of microperoxisomes it will be necessary to further elucidate their enzyme content.

Novikoff & Novikoff (1972) found that the guinea-pig enterocyte is endowed with "huge numbers" of microperoxisomes. Using guinea-pig small intestinal mucosa we examined the subcellular site(s) of enzymes we suspected might have a microperoxisomal localization (Temple *et al.*, 1976; Martin *et al.*, 1979; and unpublished observations). None of the enzymes we examined were detected in microperoxisomes. Microperoxisomes of small size containing catalase and D-amino acid oxidase are also present in homogenates made from mucosa of the goldfish intestine (Connock, 1973). We now report our examination of these particles for some of the enzymes we previously examined in the guinea-pig. In contrast to guinea-pig, goldfish intestinal microperoxisomes consistently exhibited carnitine acetyltransferase activity. As far as we know the only other biochemical studies of peroxisomes in lower vertebrates are those of Scott *et al.* (1969) on frog (*Rana pipiens*) liver and kidney, and of Kramar *et al.* (1974), Goldenberg (1977) and Goldenberg *et al.* (1978) on liver of the carp (*Cyprinus carpio*).

MATERIALS AND METHODS

Homogenate

Small goldfish were used (less than 7 g weight) and homogenates were prepared as described previously (Connock, 1973). Homogenate from several animals was usually pooled. (With larger fish, at least during the breeding season when the majority of our experiments were done, high proportions of catalase and D-amino acid oxidase in homogenates were soluble rather than particulate.)

Centrifugation

Homogenate was loaded into an aluminium B XIV zonal rotor and subcellular fractions separated as before (Connock, 1973). In some cases samples were centrifuged in tubes in a 6 × 38 ml M.S.E. swinging bucket rotor. The tubes were unloaded by displacement with 60% w/w sucrose. Fractions were equilibrated to the same sucrose concentration before assays were done.

Microperoxisome enriched samples were prepared by two methods:

(1) Homogenate from 18 goldfish intestines was centrifuged in a zonal rotor as described in Fig. 1. Ninety per cent of the volume of the microperoxisome-rich fractions (checked by marker analyses and equivalent to region b in Fig. 1) was pooled, centrifuged ($1.6 \times 10^6\ g_{av}$ min) and the pellet resuspended in 6.5 ml of 25% (w/w) sucrose.

(2) Five ml of homogenate from a single goldfish was loaded on to a 28-ml linear sucrose gradient (20–35% w/w) in a tube and centrifuged ($7 \times 10^5\ g_{av}$ min) in a 6 × 38 ml swing-out rotor. Equal aliquots of microperoxisome-rich

fractions (indicated by assay for catalase) from the middle of the gradient were pooled.

Estimations

Succinic dehydrogenase, D-amino acid oxidase, catalase, alkaline and acid phosphatases and protein were assayed as previously (Connock, 1973). Other assays were as follows: 1-naphthyl acetate esterase, 2-naphthyl laurate hydrolase (at pH 6.5 without triton X-100 but with 3 mg/ml of BSA), 1-naphthyl palmitate hydrolase (at pH 5.8 with no triton X-100), and FAD and NAD-dependent α-glycerophosphate dehydrogenases (Temple *et al.*, 1976); carnitine acetyltransferase (Markwell *et al.*, 1973); NADH cytochrome *c* reductase (Donaldson *et al.*, 1972); DNA (Burton, 1956); xanthine oxidase, L-α hydroxyacid oxidase (lactate and glycollate as substrates) and urate oxidase were assayed by methods dependent on measuring the production of hydrogen peroxide (Guilbault *et al.*, 1966; Gaunt & De Duve, 1976) but all three oxidases were undetectable as also were allantoinase and allantoicase assayed by methods dependent on the conversion of reaction product to glyoxyllate (Vogels & Van der Drift, 1970). Xanthine dehydrogenase and NADP: isocitrate dehydrogenase were assayed by similar methods to that for NAD:α-glycerophosphate dehydrogenase, the media contained: (a) for isocitrate dehydrogenase (Leighton *et al.*, 1968) 6.6 mM isocitrate, 68 mM K phosphate buffer (pH 7.4), 2.9 mM INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-24-tetrazolium chloride), 0.59 mM PMS (phenazine methosulphate), 0.16% triton X-100, 0.11 mM NADP and 3.8 mM $MgCl_2$; (b) for xanthine dehydrogenase (Cleer *et al.*, 1976) 0.26 mM xanthine, 46 mM K phosphate buffer (pH 7.7); 2.9 mM INT, 0.58 mM PMS, 0.1% triton X-100, and 0.57 mM NAD.

Histochemistry

Unfixed cryostat sections were processed for the following enzymes: (a) alkaline phosphatase according to Stutte (1967), and also by the calcium salt method of Chayen *et al.* (1969) except that 1-naphthyl phosphate was used instead of β-glycerophosphate as substrate, (b) 1-naphthyl phosphate method for acid phosphatase (Barka & Anderson, 1962), (c) 1-naphthyl acetate esterase (Davis & Ovastein, 1959), (d) An attempt was made to localize 2-naphthyl laurate hydrolase using esterase methods with the substrate dissolved in acetone or *N*,*N*-dimethyl formamide and the coupler being hexazitized pararosaniline or new fuchsin. No activity could be detected. Similar methods were used for 1-naphthyl palmitate hydrolase activity. The successful medium was: 3.2 ml of hexazotised pararosaniline (i.e. equal mixture of 4% pararosaniline in 2 N NCl and 0.68 M $NaNO_2$), plus 40 ml of 0.1 M acetate buffer pH 6, the pH readjusted to 6 (with 2 N NaOH), volume made to 70 ml with distilled water, and finally 7 ml of acetone containing 10 mg 1-naphthyl palmitate was added. This medium was replaced every 30 min with fresh medium over an incubation period of 4 hr.

For all histochemical methods control sections were incubated in complete media except that substrates were omitted. Such sections failed to accumulate specific dye product.

Presentation of results

Data for distribution of enzymes in subcellular fractions are presented in the form of histograms as described by De Duve (1967). The ordinates (Figs 1, 2 and 4) represent the relative concentration of the enzyme in the fraction; this is defined as: the concentration of the biochemical in the fraction divided by the concentration of the biochemical if it were distributed uniformly throughout the total analysed volume unloaded from the rotor (or tube). The abscissae are divided according to the percentage volume of the fractions.

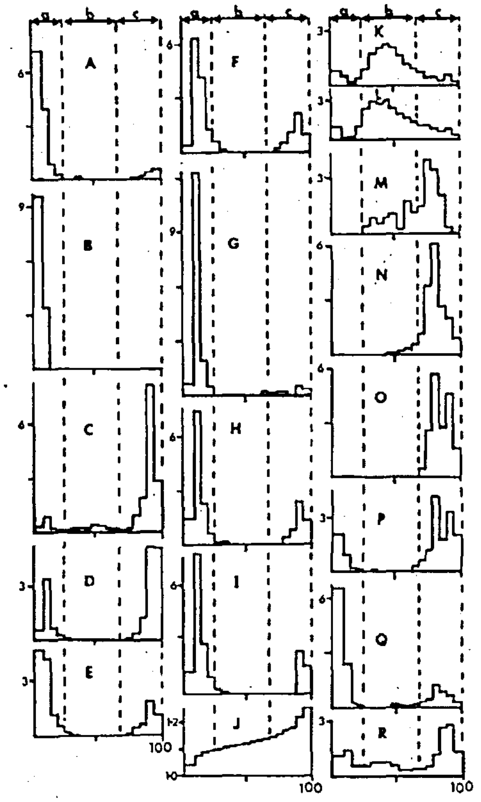

Fig. 1. Distribution of markers and enzymes after rate sedimentation. 10.5 ml of homogenate from 19 goldfish intestines was loaded onto 350 ml of 20–35% (w/w) linear sucrose gradient resting on 100 ml of 40–60% (w/w) linear sucrose gradient. Centrifugation was for 45 min at 18,500 rpm in a BXIV zonal rotor. Discontinuous vertical lines divide gradient into zones (a, b and c) which are discussed in text. (A) Acetyl-CoA hydrolase; (B) Xanthine dehydrogenase; (C) 1-naphthyl palmitate hydrolase; (D) DNA; (E) protein; (F) alkaline phosphatase; (G) NADH cytochrome *c* reductase; (H) 2-naphthyl laurate hydrolase; (I) 1-naphthyl acetate esterase (J) density, (ordinate—g cm^{-3}); (K) catalase; (L) D-amino acid oxidase; (M) Carnitine acetyltransferase; (N) succinic dehydrogenase; (O) α-glycerophosphate dehydrogenase (FAD); (P) α-glycerophosphate dehydrogenase (NAD + FAD); (Q) isocitrate dehydrogenase; (R) acid phosphatase.

RESULTS

Brief centrifugation of homogenate into a shallow sucrose gradient gave a rate-dependent separation of organelles (Fig. 1). Markers used were: succinic dehydrogenase and FAD linked α-glycerophosphate dehydrogenase for mitochondria, alkaline phosphatase for brush borders, acid phosphatase for lysosomes, 1-naphthyl acetate esterase and NADH cytochrome *c* reductase for microsomes (ER component), DNA for nuclei and catalase and D-amino acid oxidase for microperoxisomes. Microperoxisomes are found in the middle of the gradient (region b, Fig. 1) well separated from microsomes and brush border fragments (region a), and from the mitochondria and most of the lysosomes (region c). Xanthine dehydrogenase, acetyl-CoA hydrolase (the "control" in the carnitine acetyltransferase assay) and some of the NAD dependent α-glycerophosphate dehydrogenase and isocitrate dehydrogenase activities have remained in the sample layer indicating the presence of soluble phase enzymes. The latter two enzymes are also found in the mitochondrion rich region of the gradient (region c). This α-glycerophosphate dehydrogenase activity however can be wholly explained by the influence of the FAD-dependent enzyme on the assay system employed. Carnitine acetyltransferase has a peak corresponding to mitochondrial enzyme but also shows considerable activity in the microperoxisome-rich region (b) of the gradient. 2-Naphthyl laurate hydrolase distribution is similar to that of microsomal and brush border markers, but that of 1-naphthyl palmitate hydrolase bears only a partial resemblance to DNA and acid phosphatase. In a repeat of this experiment the distribution of markers was the same but 1-naphthyl palmitate hydrolase was mainly found in fraction 1 corresponding to the sample layer.

Figure 2 depicts the distribution of enzymes after isopycnic fractionation of homogenate in a sucrose gradient. The non-marker enzymes, other than 1-naphthyl palmitate hydrolase, have distributions consistent with those observed in rate sedimentation experiments. 2-Naphthyl laurate hydrolase distribution again resembles microsomal and brush border markers, isocitrate dehydrogenase mostly remained in the sample layer (soluble activity) but has a distinct peak of activity corresponding to the mitochondria. "Apparent" NAD-linked α-glycerophosphate dehydrogenase activity in this gradient was almost all contributed by the influence of the FAD enzyme on the assay system but a small amount of genuine NAD-dependent enzyme remained in the sample layer. Xanthine dehydrogenase and most of the acetyl-CoA hydrolase remained in the sample layer (soluble enzyme) but some of the latter enzyme must also reside in the microsomes or brush border fragments. Carnitine acetyltransferase distribution is again mainly mitochondrial but there is distinct activity in the microperoxisome-rich region of the gradient. 1-Naphthyl palmitate hydrolase was found in the densest region of the gradient with a distribution unlike any of the markers. The distribution of DNA in several zonal rotor experiments indicated that a variable proportion (range 30–70%) of the nuclei were damaged. The released DNA from these nuclei was contained in material with a lower density and slower sedimentation rate than intact nuclei. There was no other evidence of damaged organelles and it is assumed that the nuclei may be especially susceptible to damage.

The results of our zonal rotor experiments raise several questions:

(a) Is it justified to use alkaline phosphatase and 1-naphthyl acetate esterase as markers for brush borders and microsomes (ER component) respectively when these enzymes give almost identical distributions in both rate and isopycnic separations?

(b) What is the origin of 1-naphthyl palmitate hydrolase in the homogenates? Its distribution in fractionation experiments is inconsistent and does not correspond with any of the markers employed.

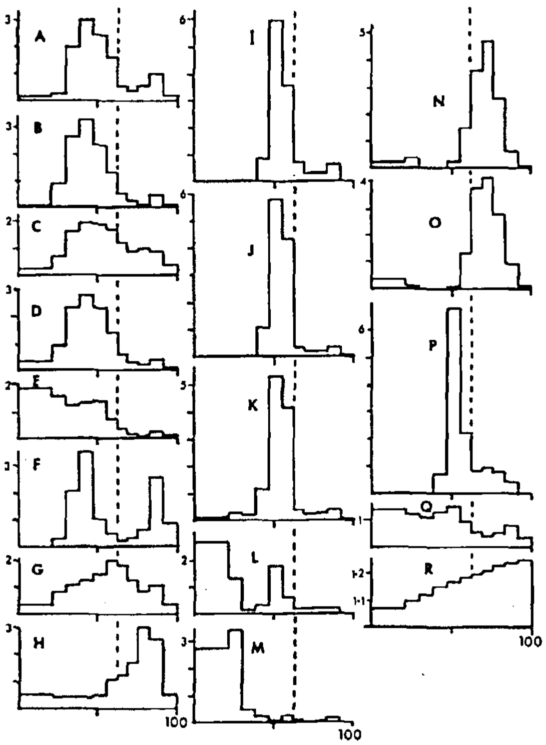

Fig. 2. Distribution of markers and enzymes after isopycnic centrifugation. 13.5 ml of homogenate from 20 goldfish intestines was loaded onto 100 ml of 30–52% (w/w) linear sucrose gradient. Centrifugation was at 29,000 rpm for 3 hr in a B XIV zonal rotor. The discontinuous vertical line marks a density of 1.19 g cm^{-3} which separates the mitochondrial and peroxisomal peaks. (A) Alkaline phosphatase; (B) NADH cytochrome *c* reductase; (C) 2-naphthyl laurate hydrolase; (D) 1-naphthyl acetate hydrolase; (E) acetyl-CoA hydrolase; (F) DNA; (G) acid phosphatase; (H) 1-naphthyl palmitate hydrolase; (I) succinic dehydrogenase; (J) α-glycerophosphate dehydrogenase (FAD); (K) α-glycerophosphate dehydrogenase (NAD + FAD); (L) isocitrate dehydrogenase; (M) xanthine dehydrogenase; (N) catalase; (O) D-amino acid oxidase; (P) carnitine acetyltransferase; (Q) protein; (R) density (ordinate—g cm^{-3}).

(c) Is 2-naphthyl laurate hydrolase localized in microsomes and/or brush borders?

(d) Can the localization of carnitine acetyltransferase in microperoxisomes be confirmed by fractionation experiments using samples with a greater ratio of microperoxisomes to mitochondria than is found in whole homogenate?

In an attempt to answer the first three of these questions we employed histochemistry. Staining cryostat sections for alkaline phosphatase using two methods clearly demonstrated the genuine brush border localization of this enzyme (Fig. 3b, c, d). In contrast 1-naphthyl acetate esterase staining is found in the enterocyte cytoplasm between the brush border and nucleus (Fig. 3e,f). The dye deposit is diffuse and consistent with an ER localization. In addition a strong reaction for esterase is found in tissue (presumed to be pancreas) closely applied to the outer wall of the intestine. Unfortunately no positive reaction could be obtained in histochemical tests for

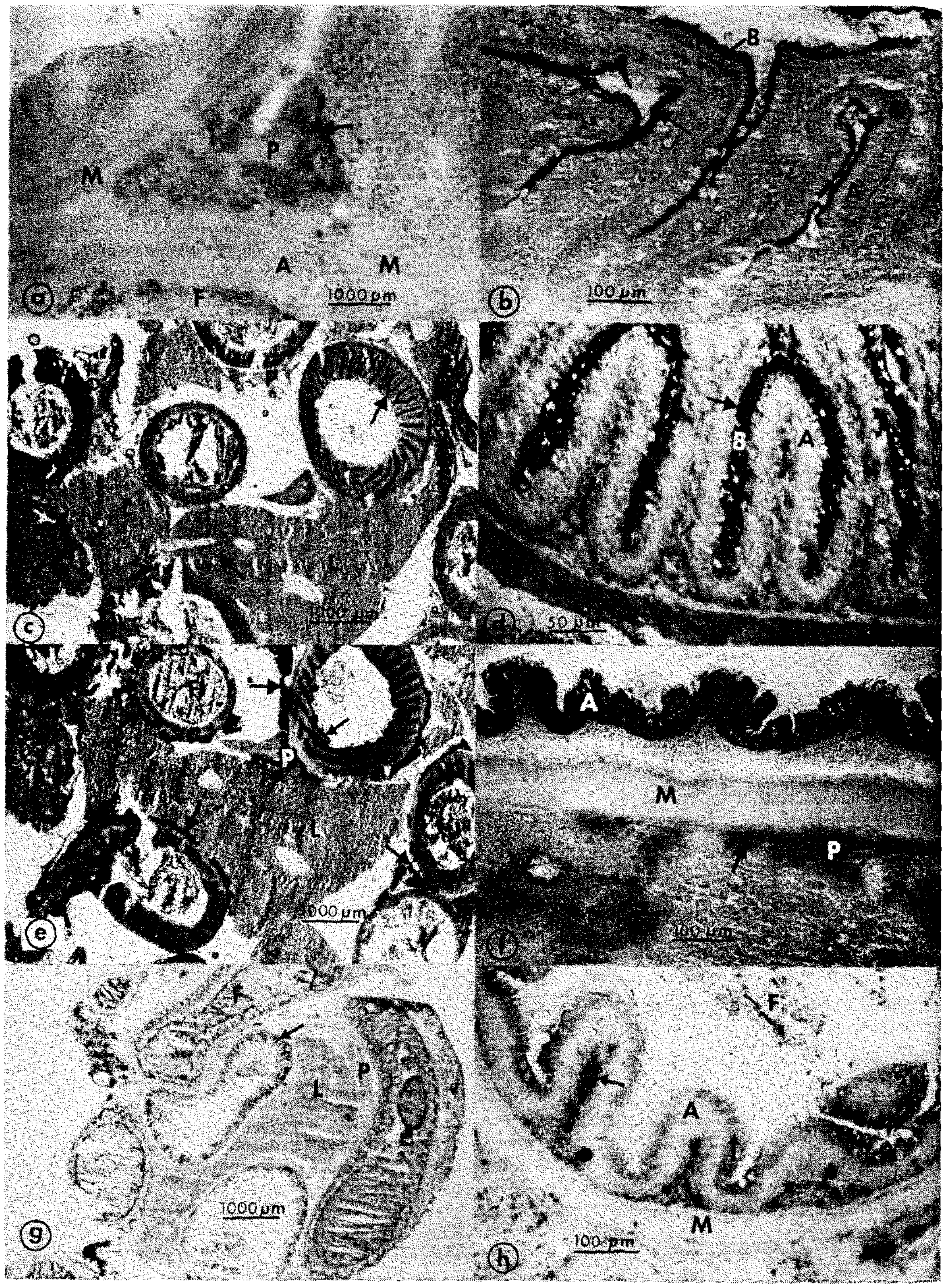

Fig. 3. Histochemical localization of enzyme activities in tissue sections. Arrows point to sites of activity. A = absorptive cell layer; B = brush border. F = food in intestinal lumen; L = liver; M = muscular wall of intestine; P = presumed pancreas; (a) 1-naphthyl pamitate hydrolase—note reaction in pancreas; (b) alkaline phosphatase (method of Chayen, 1969); (c) and (d) alkaline phosphatase (method of Stutte, 1967)—note reaction in liver and brush border of absorptive cells; (e) and (f) 1-Naphthyl acetate hydrolase—note strong reaction in pancreas and absorptive cells of intestine; (g) and (h) acid 1-naphthyl phosphatase—note reaction in cells in core of mucosal folds and diffuse reaction in absorptive cells.

2-naphthyl laurate hydrolase. After prolonged incubation for 1-naphthyl palmitate hydrolase the only diazo dye deposited was in tissue (presumed to be pancreas) closely applied to the outer wall of the intestine. We suspect therefore that traces of this tissue may contaminate our homogenates. The mucosal tissue for homogenization was obtained by scraping the length of intestine along the outside with a microscope slide and collecting the material expelled from the lumen. To test the possibility of contamination we compared 1-naphthyl palmitate hydrolase activity in a sample obtained by scraping the outer wall of the intestine with that of a sample obtained by lightly scraping the inside of the slit open intestine. Ninety-six per cent of the activity was detected in the former sample. We conclude that the source of 1-naphthyl palmitate hydrolase activity is not the intestinal mucosa but probably pancreas, or some other tissue, closely applied to the outer wall of the intestine.

We also stained tissue sections for acid 1-naphthyl phosphatase activity (Fig. 3g,h). Most diazo dye was deposited at particulate sites, below the enterocytes, in cells in the core of the mucosal folds. Some diffuse stain was observed in the enterocytes. Thus most of the lysosomal activity with this substrate was in non-absorptive cells. The staining in absorptive cells is consistent with an ER localization or may possibly originate from damaged lysosomes. Gauthier & Landis (1972), using β-glycerophosphate as substrate, observed lysosomal particulate localization in both enterocytes and non-absorptive cells. Thus acid β-glycerophosphatase would appear to be the better marker for lysosomes, a conclusion in agreement with our previous work on this tissue (Connock, 1973).

To examine the microperoxisomal localization of carnitine acetyltransferase we submitted microperoxisome enriched samples to isopycnic centrifugation in sucrose gradients. In the first experiment a rate separation of whole homogenate in a zonal rotor was carried out and the microperoxisome rich fractions from the middle of the gradient were pooled (equivalent to region b of Fig. 1). The particles in these pooled fractions were concentrated by centrifugation and resuspension, and were then submitted to isopycnic centrifugation. From the similar distributions of protein and succinic dehydrogenase in this gradient it can be seen (Fig. 4a) that mitochondria are the most abundant particles in the sample. Despite this the carnitine acetyl-transferase activity has a distribution far more similar to catalase than to succinic dehydrogenase. These results clearly demonstrate microperoxisomal carnitine acetyltransferase activity. However, this isopycnic separation of microperoxisomes from mitochondria was not as great as expected. This was probably due to agglutination of particles resulting from the concentration step in the experimental procedure. A second experiment was done in which a rate separation in a swing-out rotor was used to yield a microperoxisome enriched sample. This sample (which was not as enriched as that in the previous experiment) was submitted to isopycnic centrifugation without a preliminary concentration step. The agglutination of particles was avoided and as expected a better separation of mitochondria and microperoxisomes was achieved. Protein distribution in this gradient shows that microperoxisomes were a minor constituent of the sample. Carnitine acetyl-transferase distribution is again consistent with its localization in microperoxisomes as well as mitochondria (Fig. 4b,c).

DISCUSSION

The enzymes we examined have previously been reported to reside in peroxisomes of one tissue or another. Peroxisomal carnitine acetyltransferase has been found in mammalian liver (Markwell *et al.*, 1973) and cold-adapted brown adipose tissue (Pavelka *et al.*, 1976), and in carp liver (Goldenberg *et al.*, 1978). Goldfish intestine is thus, as far as we know, only the second extra hepatic microperoxisomal site reported for this enzyme. Xanthine dehydrogenase has been found in liver and kidney peroxisomes of chicken (Scott *et al.*, 1969), but in goldfish intestine we found that it was totally soluble phase as is xanthine oxidase in carp liver (Goldenberg, 1977). Our results agree with those of Krenitsky *et al.* (1974) in that NAD was found to be a more efficient electron acceptor than O_2 for xanthine oxidation. The uricolytic enzymes urate oxidase and allantoinase were found in frog liver peroxisomes by Scott *et al.* (1969) and also in carp liver peroxisomes (Goldenberg, 1977) but in the latter tissue most of the allantoinase was soluble as also was allantoicase. These enzymes seem to be absent from goldfish intestine as also is glycolate/lactate oxidase activity which is widely found in reno-hepatic peroxisomes (Masters & Holmes, 1977). NADP-dependent isocitrate dehydrogenase has been found in peroxisomes of rat and carp liver (Leighton *et al.*, 1968; Goldenberg *et al.*, 1978) together with mitochondrial and soluble phase activities, only the latter two localizations could be identified in goldfish intestine. NAD-dependent α-glycerophosphate dehydrogenase has been found in avian and mammalian liver and kidney peroxisomes (Gee *et al.*, 1974) but in carp liver (Goldenberg *et al.*, 1978) as in goldfish intestine only soluble enzyme was detected.

Although hydrolysis of 1-naphthyl palmitate and 2-naphthyl laurate has not previously been reported in peroxisomes, these substrates might be expected to be attacked by lipases such as monoglyceride acyl hydrolase. The latter enzyme is found in peroxisomes of fatty seeds where it provides fatty acids for oxidation. Recently rat liver peroxisomes were found to be capable of β-oxidation of fatty acids (Lazarow, 1978) and to contain fatty acid activating enzyme (Krisans & Lazarow, 1978) and thus might be expected to have monoglyceride acyl hydrolase activity. Guinea-pig enterocyte microperoxisomes lack palmitoyl-CoA ligase activity (Martin *et al.*, 1979) while 2-naphthyl laurate and 1-naphthyl palmitate hydrolases are localized in microsomes and lysosomes respectively (Temple *et al.*, 1976). In the goldfish intestine the latter enzyme appears to be absent while the former is a microsomal and/or a brush border enzyme.

The physiological function of peroxisomess especially of microperoxisomes in extra reno-hepatic tissues of vertebrates, awaits further investigation. While agreeing with Masters & Holmes (1977) who, in a recent review, point out the virtue of varied approaches in these investigations we would stress the

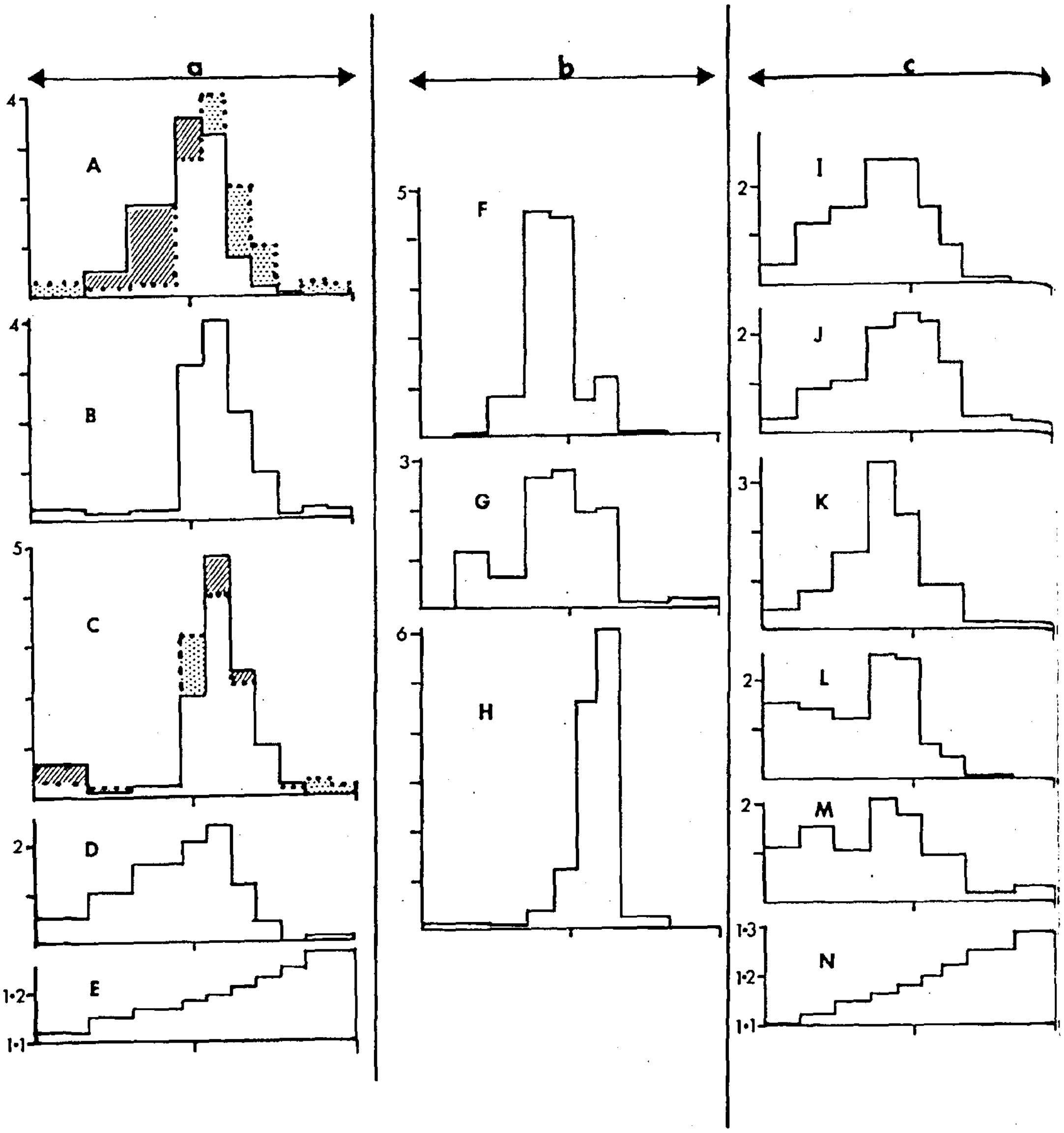

Fig. 4. Isopycnic centrifugation of microperoxisome enriched samples. Column a—Sample prepared by method 1. Centrifugation (1.6 × $10^6 g_{av}$ min) was in a tube containing 25 ml of linear sucrose gradient (30–52%); distributions shown are (A) Succinic dehydrogenase (solid) and carnitine acetyl-transferase (dotted); (B) carnitine acetyltransferase; (C) catalase (solid) and carnitine acetyltransferase (dotted); (D) protein; (E) density (ordinate—g cm^{-3}). Columns b and c—Sample prepared by method 2. Centrifugation (21 × $10^6 g_{av}$ min) into an 18 ml gradient (30–52%). Distributions shown are (F) succinic dehydrogenase; (G) carnitine acetyltransferase; (H) catalase; (I) 1-naphthyl acetate esterase; (J) 2-naphthyl laurate hydrolase; (K) alkaline phosphatase; (L) acetyl CoA hydrolase; (M) protein; (N) density (ordinate—g cm^{-3}).

central importance of fractionation experiments which can establish the enzyme complement of these organelles. This appears especially important in view of the differential partition of many peroxisomal enzymes between multiple subcellular compartments.

SUMMARY

Histochemistry and subcellular fractionation have been used to allocate, in the goldfish intestine, the subcellular sites of enzymes reported to be present in peroxisomes of various tissues. Xanthine oxidase, L-α-hydroxyl acid oxidase and uricolytic enzymes were not detectable. Xanthine dehydrogenase and NAD α-glycerophosphate dehydrogenase were localized exclusively in the soluble phase, while NADP isocitrate dehydrogenase was predominantly soluble but also localized in mitochondria. Carnitine acetyltransferase was mainly mitochondrial but 10–20% was microperoxisomal. 2-Naphthyl laurate hydrolase