

## Zonal rotor study of the subcellular distribution of acyl-CoA synthetases, carnitine acyl transferases and phosphatidate phosphatase in the guinea pig small intestine

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### *Intestine—subcellular fractionation—acyl-CoA synthetases—carnitine acyl transferases—phosphatidate phosphohydrolase*

Homogenates made from the mucosa of the guinea pig small intestine were fractionated in a zonal rotor by rate and isopycnic centrifugation in sucrose gradients. Density perturbation of endoplasmic reticulum vesicles was done by treating homogenate with pyrophosphate and was analysed by isopycnic centrifugation.

Subcellular fractions were analysed for the distribution of markers for brush borders, basolateral plasma membrane, lysosomes, peroxisomes, mitochondria, nuclei and endoplasmic reticulum. Fractions were also analysed for the distribution of propionyl-, butyryl-, and palmityl-CoA synthetases, for carnitine acetyl and palmityl transferases, and for phosphatidate phosphatase.

Comparison of marker and unknown distributions shows that palmityl-CoA synthetase is located on the endoplasmic reticulum, while propionyl- and butyryl-CoA synthetases and carnitine acetyl and palmityl transferases are exclusively mitochondrial.

Phosphatidate phosphatase has complex subcellular localisation with activity in brush borders, microsomes (possibly not the endoplasmic reticulum component) and possibly lysosomes.

### Introduction

Peroxisomes of mammalian liver are now known to play a role in lipid metabolism. There is a marked proliferation of hepatocyte peroxisomes after treatment of the animal with a variety of hypolipidaemic drugs (e.g. ref [15]). Lazarow and de Duve [20] have shown that rat liver peroxisomes oxidise fatty acid (palmityl-CoA) and Jones and Hajra [18] have localised dihydroxyacetone phosphate acyltransferase in peroxisomes of guinea pig liver. Investigations of extra-hepatic peroxisomes have mostly employed electron microscopy which has revealed details of their distribution, abundance and structure, but little about their enzyme content. Some progress has been made with enterocyte peroxisomes where zonal rotor fractionation studies have shown that in guinea pig [9], rat [27] and man [26] these particles contain catalase and D-amino acid oxidase. Mouse enterocyte peroxisomes proliferate in response to the hypolipidaemic drug nafenopin [28], and Svoboda [36] ob-

served a similar response to clofibrate by rat enterocyte peroxisomes. Lipid metabolism is known to be active in the small intestine [5] but studies of its subcellular compartmentation have previously never taken peroxisomes into account. These considerations have prompted us to investigate the subcellular localisation in guinea pig small intestine of a number of enzymes related to lipid metabolism. We have studied acyl-CoA synthetases, carnitine acyl transferases, and phosphatidate phosphatase. In conjunction with rate sedimentation and isopycnic separations in a zonal rotor we have employed extensive subcellular markers and density perturbation in order to localise the enzymes of interest. Although none of the enzymes was localised in peroxisomes, our results extend and refine knowledge of their subcellular sites in intestinal tissue.

### Materials and methods

#### *Chemicals and enzymes*

Acetyl-CoA, ATP, bovine serum albumin (fraction V), CoA, 5,5'-dithiobis-(2-nitrobenzoic acid), dithiothreitol, Dowex ion exchange resin, NAD and phosphatidic acid were purchased from Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Phenazine methosulphate and trifluoroacetic acid were from BDH Chemicals Ltd., Poole, Dorset, U.K., (-)-carnitine chloride, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride and trifluoroacetic anhydride were from Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K. All radiochemicals were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. Palmityl(-)-carnitine was prepared by a method based on that of Chase and Tubbs [7] as modified by Sanchez et al. [31]. (±)-[Me<sup>3</sup> H] carnitine was prepared by the method of Carter and Bhattacharyya [6]. Before use the (±)-[Me<sup>3</sup> H] carnitine was diluted with nonradioactive (-) carnitine. Carnitine palmityl transferase E.C.2.3.1.23 was prepared from bovine liver by the method of Norum [2] as modified by Sanchez et al. [31]. The final solution had a protein concentration of 16 mg/ml. This was stored as 1 ml aliquots at -20°C until used. Aqueous dispersions of phosphatidate were prepared according to Sedgwick and Hubscher [34].

#### *Preparation of homogenate and subcellular fractionation*

The procedures employed were as described previously [37].

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### Pyrophosphate treatment of homogenate [2]

In some experiments freshly prepared homogenate was treated with pyrophosphate before being loaded into the zonal rotor. 25 ml of homogenate was mixed with 5 ml of ice cold 100 mM pyrophosphate (pH 8.2) in 10% (w/w) sucrose. The density gradient in these experiments was extended at the light end so as to separate pyrophosphate in the sample layer from the density equilibrated particles in the gradient. Pyrophosphate treatment caused some of the DNA in the homogenate to shift to a lower density. This translocated DNA interfered with refractive index measurements and thus density estimations for these fractions were made by interpolation.

**Biochemical analyses.** The following were assayed as described previously [37]: DNA, protein, acid phosphatase, alkaline phosphatase, glucose-6-phosphatase,  $\alpha$ -naphthyl acetate hydrolase,  $\beta$ -naphthyl laurate hydrolase, succinic dehydrogenase,  $\alpha$ -glycerophosphate dehydrogenase (FAD) and catalase.  $\text{Na}^+/\text{K}^+$ -ATPase was assayed according to Lewis et al. [21] and carnitine acetyl transferase according to Markwell et al. [23]. Other enzymes were assayed as follows:

### Palmitoyl-CoA synthetase (E.C.6.2.1.3)

**Method 1.** Activity was determined by converting the palmitoyl-CoA produced into palmitoyl-[Me<sup>3</sup> H] carnitine with excess of carnitine palmitoyl transferase [13].

The assay system consisted of, in a final volume of 0.25 ml: 25 mM Tris (pH 7.4), 5 mM dithiothreitol, 75  $\mu\text{M}$ -CoA, 11.25 mM-ATP, 15 mM  $\text{MgCl}_2$ , 1.2 mM-potassium palmitate, 1.5 mg bovine serum albumin (Fraction V), 40 mM (-)-[Me<sup>3</sup> H] carnitine (2.7  $\mu\text{Ci}/\mu\text{mol}$ ), excess of carnitine palmitoyl transferase (approx. 650  $\mu\text{g}$  protein), and 1% Triton X-100. The reaction was started with 15 to 55  $\mu\text{g}$  protein and after 10 minutes at 37 °C was stopped with 0.1 ml of 5.5 M HCl. Palmitoyl-[Me<sup>3</sup> H] carnitine was extracted with butan-1-ol, washed twice with butanol saturated water and the radioactivity measured by liquid scintillation counting in a Phillips LSA liquid scintillation analyser in a scintillation cocktail consisting of Triton X-100: xylene (1:2) containing 0.5% PPO and 0.01% POPOP.

**Method 2.** Palmitoyl-CoA synthetase was also determined by measuring the direct formation of [1-<sup>14</sup>C] palmitoyl-CoA [22]. The assay system consisted of, in a final volume of 0.25 ml: 25 mM Tris-HCl buffer pH 7.4, 5 mM dithiothreitol, 550  $\mu\text{M}$  CoA, 2.5 mM ATP, 2.5 mM  $\text{MgCl}_2$ , 4 mM potassium [1-<sup>14</sup>C] palmitate (56  $\mu\text{Ci}/\mu\text{mol}$ ) and 1.5 mg bovine serum albumin. The reaction was started with 5 to 50  $\mu\text{g}$  protein and after 10 minutes at 37 °C was stopped with 1 ml Dole reagent, 0.35 ml water and 0.6 ml of n-heptane. Extraction of the [1-<sup>14</sup>C] palmitoyl-CoA was carried out as described by Bar-Tana et al. [4], except that the lower phase was washed three times with 0.6 ml portions of n-heptane. The radioactivity in a 0.5 ml sample of the lower phase was measured by liquid scintillation counting in 10 ml of scintillant as above.

**Carnitine palmitoyl transferase** (E.C.2.3.1.21) was determined by measuring the incorporation of labelled carnitine into palmitoyl carnitine [24]. The assay system consisted of, in a final volume of 1 ml: 0.5 mM ( $\pm$ )-palmitoyl carnitine, 0.12 mM CoA, 10 mM Tris buffer (pH 7.5), 5 mM reduced glutathione, 0.25 mM (-)-[Me<sup>3</sup> H] carnitine (2.67  $\mu\text{Ci}/\mu\text{mol}$ ). The reaction was started by the addition of 0.25–1 mg protein and after 10 minutes at 37 °C was stopped by the addition of 0.1 ml of 5.5 M HCl. Extraction and subsequent counting of labelled palmitoyl carnitine was as for the palmitoyl-CoA synthetase assay (Method 1).

**Phosphatidate phosphatase** (E.C.3.1.3.4.) was determined by measuring the release of inorganic phosphate [8]. The assay system consisted of, in a final volume of 0.5 ml: 60  $\mu\text{mol}$  maleate buffer pH 6.0 and 3.0  $\mu\text{mol}$  phosphatidic acid. The reaction was started by the addition of 50 to 250  $\mu\text{g}$  protein and after incubation at 37 °C for 40 minutes was stopped by the addition of 1 ml of 13.3% trichloroacetic acid. The amount of inorganic phosphate released was estimated according to the method of Baginski et al. [3], and was corrected for the amount lib-

erated from the enzyme preparation in the absence of substrate. In the absence of enzyme, no inorganic phosphate was liberated from phosphatidic acid.

**Butyryl-CoA synthetase** (E.C.6.2.1.2.). Butyryl-CoA synthetase was determined by measuring the rate of formation of [1-<sup>14</sup>C] butyryl-CoA [30]. The assay system consisted of, in a final volume of 0.25 ml, 50 mM potassium phosphate (pH 7.7), 5.9 mM dipotassium ATP, 9.8 mM  $\text{MgCl}_2$ , 1 mM CoA, 0.1% Triton X-100 and 6.4 mM potassium [1-<sup>14</sup>C] butyrate (0.90  $\mu\text{Ci}/\mu\text{mol}$ ). The reaction was started by addition of enzyme (less than 40  $\mu\text{g}$  mitochondrial protein) and after 4.5 min at 37 °C the tubes were transferred to a water bath at 95 °C for 5 min. In control tubes the 37 °C incubation was omitted and CoA was added only after the heat inactivation of the enzyme. 0.9 ml of distilled water was added to each tube. After centrifugation (2000 g x 10 min) a 0.9 ml aliquot was transferred to a column of internal diameter 0.5 cm containing 270 mg (dry weight) of Dowex-1X-8 resin (chloride form, 200–400 mesh). The column was washed with 22 ml of 2 M formic acid (to remove unreacted potassium [1-<sup>14</sup>C] butyrate) and eluted with 5 ml of 2 M HCl. The radioactivity in a 2.5 ml aliquot of the latter was measured in a Phillips LSA liquid scintillation counter in a scintillation cocktail consisting of 8 ml Triton X-100; toluene (1:2) containing 0.5% PPO and 0.01% POPOP.

**Propionyl-CoA synthetase** (E.C.6.2.1.). The assay system was the same as that for butyryl-CoA synthetase except that the fatty acid was 6.5 mM potassium [1-<sup>14</sup>C] propionate (0.44  $\mu\text{Ci}/\mu\text{mol}$ ). The optimum assay conditions for the butyryl- and propionyl-CoA synthetases were investigated. In order to ensure that the enzyme activities were linear with incubation time and quantity of mitochondrial protein, it was found necessary to reduce each considerably in relation to those employed by others. Triton X-100 was needed at a final concentration of 0.1%. Although preincubating the enzyme in 0.1% Triton X-100 (0 °C for 20 min) with a final concentration of 0.016% activated the enzyme, the activity could be increased still further by using a final concentration of 0.1% but without preincubation.

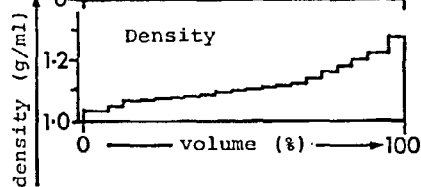
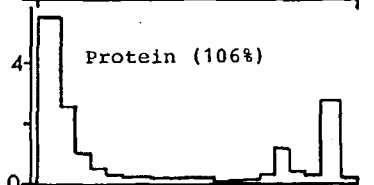
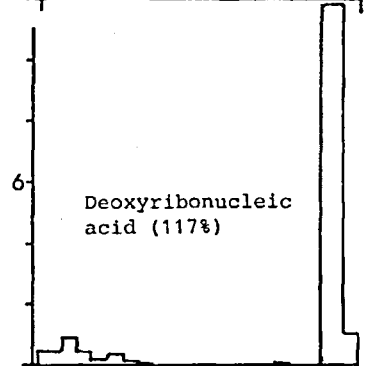
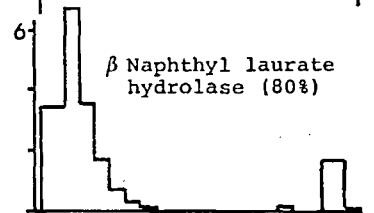
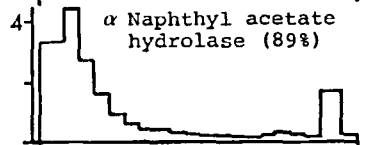
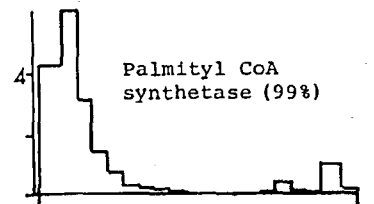
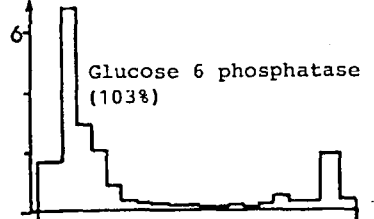
**Presentation of results.** Data for distribution of enzymes in subcellular fractions are presented in the form of histograms as described by de Duve [11]. The ordinates represent the relative concentration of the enzyme in the fraction; this is defined as: the concentration of the biochemical in the fraction divided by the concentration if it were distributed uniformly throughout the total analysed volume unloaded from the rotor. The abscissae are divided according to the % volume of the fractions. The data in the figures represent the results of experiments in which all the unknowns and markers were assayed. The fractionation conditions selected for these experiments were based on the results of preliminary experiments in which incomplete lists of markers and unknowns were analysed. The results of the major experiments are consistent with those of the preliminary ones.

## Results

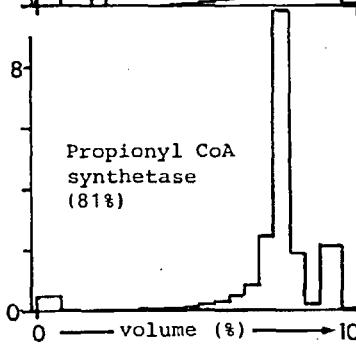
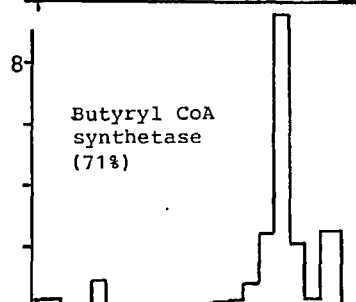
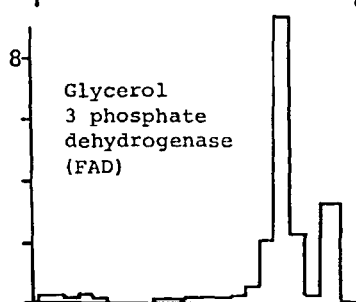
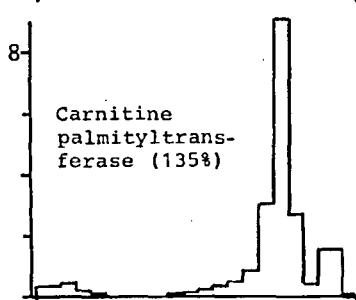
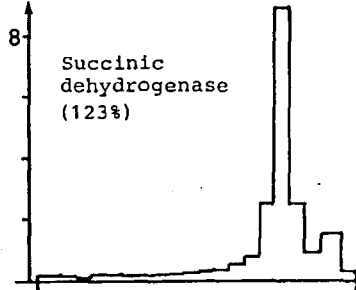
A sample of whole homogenate was briefly centrifuged into a long shallow sucrose density gradient resting on a short steep sucrose gradient. The distribution of markers is depicted in Figure 1 and was essentially as previously reported [37]. Five

**Fig. 1.** Distribution of enzymes, protein, and DNA after centrifugation of 20 ml homogenate on 350 ml of linear sucrose gradient (13% to 35% w/w) resting on a gradient of 100 ml (40% to 50%). Centrifugation was for 45 minutes at 16000 rpm in an MSE B-XIV zonal rotor. The broken line for phosphatidate phosphatase = enzyme assayed after preincubation with 10 mM EDTA + 10 mM NaF (91%). (Palmitoyl-CoA synthetase assayed by method 1.)—% figures represent recovery of material from the gradient.

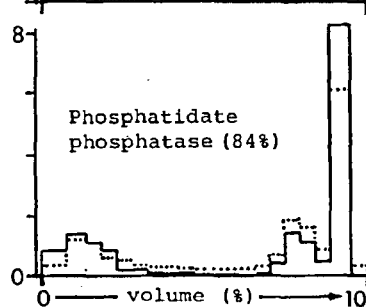
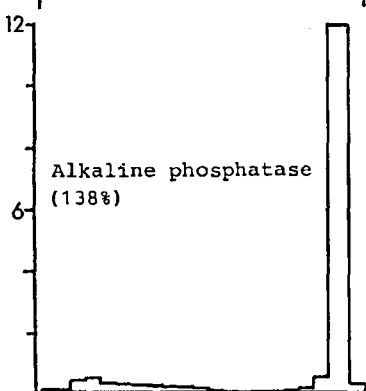
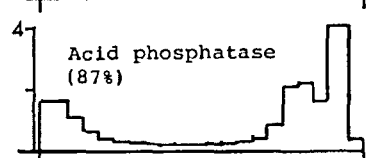
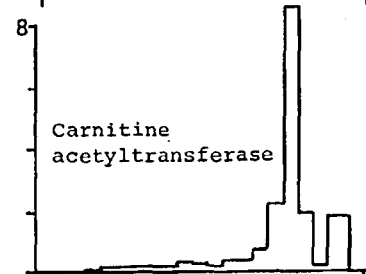
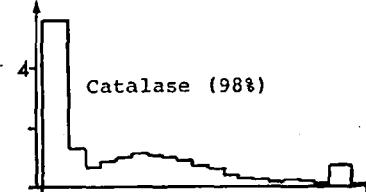
relative concentration in fraction



relative concentration in fraction



relative concentration in fraction



0 — volume (%) — 100

distinct patterns of distribution were observed. Pattern I is characterised by  $\beta$ -naphthyl laurate and  $\alpha$ -naphthyl acetate hydrolases and glucose-6-phosphatase and shows that "microsomes" have barely moved into the gradient. Pattern II is exemplified by catalase and indicates that intact peroxisomes have moved into the middle of the shallow gradient while soluble catalase has remained in the sample layer. Pattern III is typified by the mitochondrial markers succinic dehydrogenase and  $\alpha$ -glycerophosphate dehydrogenase which show a large peak of activity in fraction 16 at the junction between the shallow and steep gradients. Pattern IV has a large peak of activity in fraction 19 and is common for both DNA (marker for nuclei) and alkaline phosphatase (marker for brush borders). The lysosomal marker acid phosphatase has an intermediate pattern with peaks at the top of the gradient and also in fractions 17 and 19. Several of the unknowns which were examined have distributions clearly similar to those of the subcellular markers used. Palmityl-CoA synthetase distribution corresponds to pattern I, while the distributions of propionyl- and butyryl-CoA synthetases, and of carnitine palmityl and acetyl transferases correspond to pattern III. On the basis of these distributions it would appear that palmityl-CoA synthetase is "microsomal" while the shorter chain synthetases and the carnitine transferases are mitochondrial. Phosphatidate phosphohydrolase distribution is complex. A peak of activity at the top of the gradient indicates a soluble, "microsomal" or lysosomal localisation while the peak in fractions 16 + 17 supports a lysosomal localisation and that in the penultimate fraction (19) a nuclear or brush border site. The inclusion of non-specific phosphatase inhibitors (fluoride and EDTA) during incubation has little effect on the overall distribution of the enzyme. All the enzymes analysed are well represented in fraction 19 indicating that this fraction contains those cells which remained wholly or partially intact during homogenisation and centrifugation.

In order to examine these provisional subcellular allocations more closely a sample of homogenate was centrifuged into a linear sucrose gradient so as to approach isopycnic distribution of markers. The results of such an experiment are shown in Figure 2. Palmityl-CoA synthetase again has a distribution very similar to that of "microsomal" markers  $\alpha$ -naphthyl acetate and  $\beta$ -naphthyl laurate hydrolases and glucose-6-phosphatase. These enzymes are spread out in the gradient with a median density between 1.160 and 1.195 g/ml. The propionyl- and butyryl-CoA synthetases and the carnitine acyl transferases have distributions very similar to that of the mitochondrial marker with a large peak at a density of about 1.18 g/ml. However carnitine acetyl transferase activity in the catalase rich fractions is slightly greater than the activity of the other mitochondrial enzymes. Phosphatidate phosphohydrolase again has a complex distribution supporting the idea of microsomal and brush border localisations.

So as to analyse the "microsomal" localisation of palmityl-CoA synthetase a sample of whole homogenate was pretreated with pyrophosphate and then centrifuged to equilibrium in a sucrose gradient. Amar-Costesec et al. [2] have employed pyrophosphate treatment of liver microsomal fraction as an analytical tool. Pyrophosphate brings about the release of proteins and ribosomes from endoplasmic reticulum vesicles. This loss causes a specific shift in the equilibrium density of endoplasmic reticulum membranes. Other components of liver micro-

somal fractions are very little affected by pyrophosphate treatment.

The effect of pyrophosphate treatment of intestinal homogenate can be deduced by comparing Figures 2 and 3. The major influence of pyrophosphate is to shift the distribution of  $\alpha$ -naphthyl acetate hydrolase,  $\beta$ -naphthyl laurate hydrolase, glucose-6-phosphatase and palmityl-CoA synthetase to low density regions of the gradient (region 'b', Fig. 3). The median density for these enzymes is now 1.14 to 1.15 g/ml (compared with 1.16 to 1.195 g/ml with untreated homogenate). This result clearly indicates that these enzymes are mainly localised on the endoplasmic reticulum membrane. The low density peak of  $\text{Na}^+/\text{K}^+$ -ATPase activity (marker for plasma membrane fragments [21]) has a modal density of about 1.15 g/ml in both experiments (Figs. 2 and 3). This is in agreement with the results of Lewis et al. [21] and indicates that in common with hepatic plasma membrane [2] intestinal plasma membrane is unaffected by pyrophosphate treatment.

Pyrophosphate appears to cause a shift in the distribution of a proportion of the DNA. With untreated homogenate brush borders and nuclei equilibrate in the same dense region of the gradient (Fig. 2). After pyrophosphate a substantial portion of DNA is found at lower densities (region 'd' and the low density part of region 'e' in Fig. 3). Electron microscopy shows that the outer nuclear membrane bears ribosomes and may be similar to the endoplasmic reticulum membrane and therefore this effect of pyrophosphate might be expected. On the other hand some nuclei could have been damaged by the pyrophosphate treatment. Prolonged pyrophosphate treatment ruptures liver peroxisomes [23] and the brief treatment employed in the present experiment has marginally increased the proportion of soluble catalase recovered from the gradient. Pyrophosphate may also have damaged the mitochondria to some extent since a higher proportion of mitochondrial marker activity is found in the dense region of the gradient (region 'e', Fig. 3). This activity might however be due to incompletely homogenised cells. The distribution of propionyl- and butyryl-CoA synthetases and of carnitine acetyl and palmityl transferases is very similar to that of the mitochondrial markers. The distribution of phosphatidate phosphohydrolase is complex. Such a pattern could be explained by appropriate combination of localisations in a microsomal component and in brush borders.

In a further experiment we tested the possibility that carnitine acetyl transferase activity may be present in the peroxisomes. A sample of homogenate was first submitted to a rate centrifugation according to the experiment described in Figure 1. The distribution of markers and of carnitine acetyl transferase was very similar to that reported in Figure 1. Aliquots of catalase rich fractions from the middle of the shallow gradient (equivalent to fractions 5 to 12 in Fig. 1) were combined and centrifuged to equilibrium on a second gradient. The distribution of carnitine acetyl transferase in this second gradient was similar to that of the mitochondrial marker. If peroxisomal carnitine acetyl transferase is present, it must either represent less than a few percent of the mitochondrial activity or not be detectable by the methods we employed. Unfortunately, like Markwell et al. [23] and Kahonen [19] we could not compute true recoveries for this enzyme because of the very high soluble acetyl-CoA hydrolase activity in the homogenate. Because of this the unlikely possibility that peroxisomal carnitine acetyl

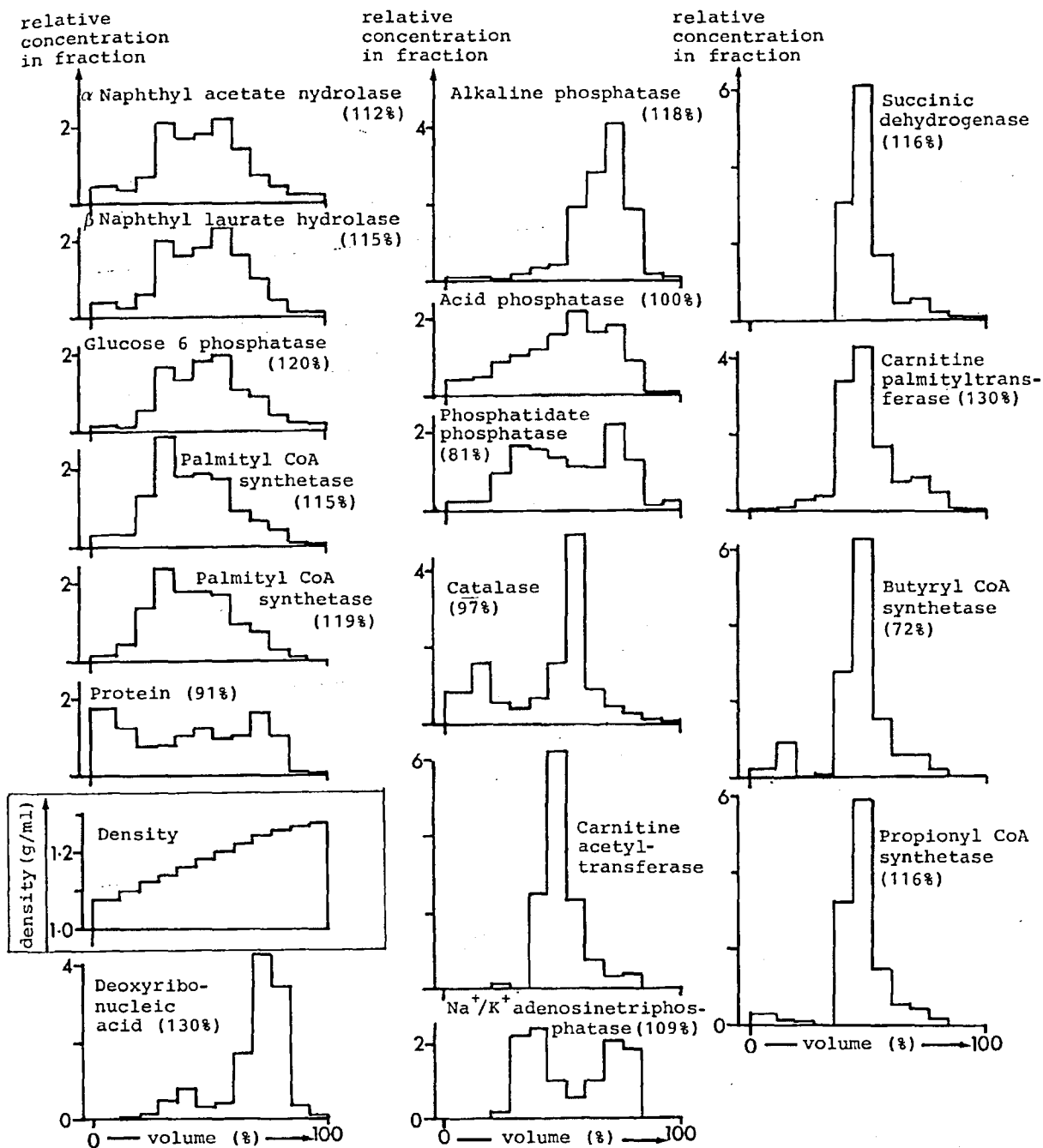


Fig. 2. Distribution of enzymes, protein and DNA after centrifugation of 20 ml of homogenate on 300 ml linear sucrose gradient (30% to 60%) in a M.S.E. B-XIV zonal rotor. Centrifugation was at 25000 rpm

for 12 h (Palmityl-CoA synthetase "upper" = assay method 1, "lower" = assay method 2).

transferase has been selectively inactivated cannot be excluded.

In summary we have applied three independent fractionation conditions to guinea pig small intestinal homogenate. Under each of these regimes all the unknowns we examined (except phosphatidate phosphatase) consistently behaved as though they were localised in a single identifiable organelle. Phosphatidate phosphatase appears to be localised to at least two subcellular sites.

## Discussion

### *Palmityl-CoA synthetase*

Early investigations localised palmityl-CoA synthetase in the "microsomal" fraction of intestinal homogenates [35]. These microsomal fractions were certainly heterogeneous and the subcellular site of the enzyme was not elucidated. De Jons and Hulsmann (1970) [12] found no evidence for mitochondrial palmityl-CoA synthetase in subcellular fractions of rat jejunum and concluded that the enzyme was "microsomal". More recently Hulsmann and Kupershoek-Davidov (1976) [17] made an extensive investigation of enzymes involved in glycerolipid synthesis in the small intestine of the rat. They obtained results indicating that most palmityl-CoA synthetase was "microsomal" but that low activity was present in the mitochondria. Their evidence for a mitochondrial localisation is weakened by the fact that only two subcellular fractions were analysed, some fractions being discarded so that recoveries could not be computed, and because only three markers were employed. If mitochondrial palmityl-CoA synthetase activity exists in guinea pig intestine we would particularly expect to detect it in fractions containing fast moving particles isolated by rate zonal sedimentation (Fig. 1). The centrifugation time here is only 45 min and enzyme analyses are done soon after sacrifice of the animal, in addition there is a good separation of mitochondria from microsomes. By comparing palmityl-CoA synthetase distribution with that of markers (Fig. 1) we can find no evidence of mitochondrial activity. It seems unlikely that we have lost activity during fractionation procedures because our recoveries are reasonably close to 100% (average recovery for the five reported analyses = 106%, range 80 to 120%). In other tissues mitochondrial palmityl-CoA synthetase is thought to be localised in the outer membrane [1]. Thus, if our mitochondria were stripped for their outer membranes it could be argued that we would not detect putative palmityl-CoA synthetase in our "mitochondrial fractions" because our markers are on the inner membrane. It is very unlikely that our mitochondria are substantially damaged because homogenisation was relatively gentle in a Potter-Elvehjem homogeniser and because only relatively low centrifugal forces were employed so as to avoid damage by hydrostatic pressure [40]. Lewis et al. [21] found that even with homogenisation in a rotating blade blender a high proportion of guinea pig enterocyte mitochondria retain their outer membrane. A mixed population of damaged and undamaged mitochondria exhibit several peaks of activity for inner membrane markers after isopycnic centrifugation [40] (also Fig. 2, ref. [21]). Our mitochondria consistently show a single peak, after isopycnic centrifugation [9, 10, 37] indicating their structural integrity.

The results we obtained after density perturbation indicate that within the wide spectrum of microsomal membrane components it is the endoplasmic reticulum that carries most or all of the palmityl-CoA synthetase. We have provided the first firm fractionation evidence for this widely assumed localisation.

### *Propionyl-CoA and butyryl-CoA synthetases*

In a study of a large number of tissues from guinea pig and rat Scholte and Groot [33] concluded that these enzymes were mitochondrial in rat intestine. In that study only two fractions were isolated, a single centrifugation was done producing a particulate fraction (called mitochondrial) and a soluble fraction. Markers were employed for only mitochondria and soluble phase. Clearly for a more certain localisation of these particulate enzymes more refined fractionation is required. Our results demonstrate that, in contrast to long chain acyl-CoA synthetase, these short chain acyl-CoA synthetases are almost exclusively mitochondrial. Very little soluble activity was found so that the intestine appears to be similar to kidney and brain [14, 29] in this respect. Soluble activity in some tissues appears to be due to mitochondrial rupture and release of matrix enzymes [14]. Lack of appreciable soluble activity in our experiments is a further indication of the structural integrity of the mitochondria in our homogenates.

### *Carnitine acyl transferases*

In rat liver carnitine acetyl transferase has been shown to reside at three subcellular sites, in peroxisomes, mitochondria and microsomes [23]. On the other hand in rat kidney the enzyme is exclusively mitochondrial [23]. As far as we know ours is the first study of the localisation of this enzyme in intestine. Our results indicate that here, as in kidney, the enzyme is probably wholly mitochondrial. Similarly carnitine palmityl transferase has previously not been studied in intestine and its localisation is also mitochondrial. In rat liver van Tol and Hulsmann [39] concluded that some carnitine palmityl transferase was microsomal. This localisation has not been substantiated by others. Extensive fractionation studies by Hoppel and Tomec [16] and by Markwell et al. [23] indicate that the enzyme is solely mitochondrial in rat liver.

Carnitine palmityl transferase is generally believed to function in fatty acid oxidation and therefore its spatial separation from palmityl-CoA synthetase in the intestine is noteworthy. Brindley [5] has discussed the possibility that microsomal acyl-CoA synthetase is not rate limiting for intestinal glycerolipid synthesis, and it is possible that the microsomal enzyme could supply activated fatty acid for extra microsomal oxidation. In this context it is interesting that peroxisomes would be spatially well situated to receive this acyl-CoA [25], while on the other hand mitochondria contain carnitine palmityl transferase. The source of acyl-CoA for hepatic peroxisomal oxidation is not known. No acyl-CoA synthetase has been reported for liver peroxisomes and carnitine palmityl transferase is absent from these organelles [23].

### *Phosphatidate phosphohydrolase*

Our experiments suggest a microsomal and brush border localisation. The microsomal activity is possibly not of ER origin in

view of the enzyme distribution after treatment of homogenate with pyrophosphate. The presence of some lysosomal activity cannot be ruled out. Other studies support a microsomal localisation (possibly in Golgi apparatus) in the intestine [32]. In rat liver Sedgwick and Hubscher [34] concluded that the enzyme active on aqueous suspensions of phosphatidate was present in mitochondria, microsomes and lysosomes. This enzyme probably has a complex localisation in most tissues. A further complication is that inorganic phosphate (Pi) can be liberated from phosphatidate without direct dephosphorylation. Tzur and Shapiro [38] have shown that with a preparation of lyophilized rat liver microsomes Pi production greatly exceeds diacyl glycerol formation from phosphatidate. Thus phospholipases and lipases can contribute to Pi production from phosphatidate.

Phospholipases are known to be active in brush borders and might contribute to Pi detected in our assays. Final Pi release by such an indirect route would depend on non-specific alkaline phosphatase activity and therefore should be inhibited by EDTA. In our experiments inclusion of EDTA did not greatly inhibit Pi production from phosphatidate (see Fig. 1,Q) by brush border rich fractions. We conclude that there may be a genuine specific enzyme in this organelle. It is unlikely that the phosphatidate hydrolase we detected plays a significant role in triglyceride synthesis since it appears that for this it is membrane bound phosphatidate that is used [5]. Nevertheless the activities may play a role in overall lipid metabolism in the intestine.

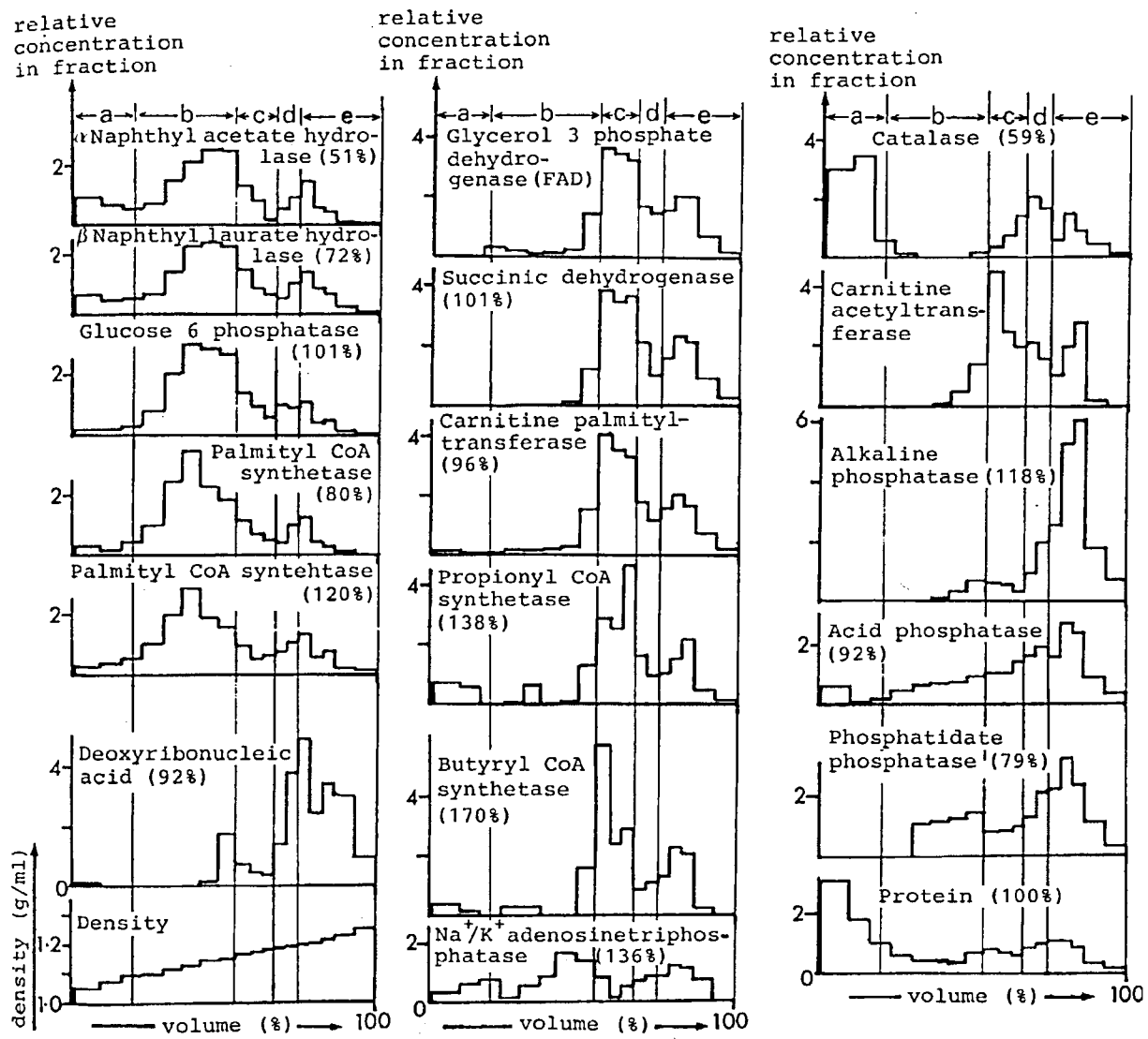


Fig. 3. Distribution of enzymes, protein and DNA after centrifugation of 27 ml of pyrophosphate treated homogenate on a 450 ml linear sucrose gradient (15% to 50%). Centrifugation was at 25000 rpm for

12 h in a M.S.E. B-XIV zonal rotor. Vertical columns labelled "a" to "e" represent regions of the gradient discussed in the text (Palmityl-CoA synthetase "upper" = assay method 1, "lower" = assay method 2).

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*Note added in proof:* Krisans and Lazarow (J. Cell. Biol. 79, 210a) have recently reported the detection of rat liver peroxisomal palmitoyl-CoA synthetase activity.

## References

- [1] Aas, M.: Organ and subcellular distribution of fatty acid activating enzymes in the rat. *Biochim. Biophys. Acta.* 231, 32-47 (1971).
- [2] Amar-Costesec, A., M. Wibo, D. Thines-Sempoux, H. Beaufay, and J. Berthet: Analytical study of microsomes and isolated subcellular membranes from rat liver. IV Biochemical, physical and morphological modifications of microsomal components induced by Digitonin, EDTA and pyrophosphate. *J. Cell. Biol.* 62, 717-745 (1974).
- [3] Baginski, E. S., P. P. Foa, and B. Zak: Microdetermination of inorganic phosphate, phospholipids and total phosphate in biological materials. *Clin. Chem.* 13, 326-332 (1967).
- [4] Bar-Tana, J., G. Rose, and B. Shapiro: The purification and properties of microsomal palmitoyl-coenzyme A synthetase. *Biochem. J.* 122, 353-363 (1971).
- [5] Brindley, D. N.: Intracellular phase of fat absorption. In: D. H. Smyth (ed.): *Biomembranes*, vol. 4B. Plenum Press, London 1974.
- [6] Carter, H. E., and P. K. Bhattacharyya: Improvements in the synthesis of DL-carnitine. *J. Amer. Chem. Soc.* 75, 2503-2504 (1953).
- [7] Chase, J. F. A., and P. K. Tubbs: Specific inhibition of mitochondrial fatty acid oxidation by 2-bromopalmitate and its coenzyme A and carnitine esters. *Biochem. J.* 129, 55-65 (1972).
- [8] Coleman, R., and G. Hubscher: Metabolism of phospholipids V. Studies of phosphatidic acid phosphatase. *Biochim. Biophys. Acta* 56, 479-490 (1962).
- [9] Connock, M. J., P. R. Kirk, and A. P. Sturdee: A zonal rotor method for the preparation of microperoxisomes from epithelial cells of guinea pig small intestine. *J. Cell. Biol.* 61, 123-133 (1974).
- [10] Connock, M. J., and W. F. R. Pover: Catalase particles in the epithelial cells of the guinea pig small intestine. *Histochem. J.* 2, 371-380 (1970).
- [11] de Duve, C.: General principles. In: D. B. Roodyn (ed.): *Enzyme Cytology*. Academic Press, London and New York 1967.
- [12] de Jong, J. W., and W. C. Hulsmann: A comparative study of palmitoyl-CoA synthetase activity in rat liver, heart and gut mitochondrial and microsomal preparations. *Biochim. Biophys. Acta* 197, 127-135 (1970).
- [13] Farstad, M.: A palmitoyl-CoA synthetase stimulating factor of particle free supernatants. *Biochim. Biophys. Acta.* 146, 272-283 (1967).
- [14] Groot, P. H. E., H. R. Scholte, and W. C. Hulsmann: Fatty acid activation: specificity, localisation, and function. *Adv. Lip. Res.* 14, 75-125 (1976).
- [15] Hess, R., W. Staubli, and W. Riess: Nature of the hepatomegalic effect produced by ethyl-chlorophenoxyisobutyrate in the rat. *Nature (Lond.)* 208, 856-858 (1965).
- [16] Hoppel, C. L., and R. J. Tomec: Carnitine palmityl transferase. Location of two enzymatic activities in rat liver mitochondria. *J. Biol. Chem.* 247, 832-841 (1972).
- [17] Hulsmann, W. C., and R. Kurpershoek-Davidov: Topographic distribution of enzymes involved in glycerolipid synthesis in rat small intestinal epithelium. *Biochim. Biophys. Acta.* 450, 288-300 (1976).
- [18] Jones, C. L., and A. K. Hajra: The subcellular distribution of acyl-CoA: dihydroxyacetone phosphate acyl transferase in guinea pig liver. *Biochem Biophys. Res. Commun.* 76, 1138-1143 (1977).
- [19] Kahonen, M. T.: Effect of clofibrate treatment on carnitine acyl transferases in different subcellular fractions of rat liver. *Biochim. Biophys. Acta.* 428, 690-701 (1976).
- [20] Lazarow, P. B., and C. de Duve: A fatty acyl-CoA oxidising system in rat liver peroxisomes: enhancement by clofibrate, a hypolipidemic drug. *Proc. Natl. Acad. Sci. U.S.A.* 73, 2043-2046 (1976).
- [21] Lewis, B. A., A. Elkin, R. H. Michell, and R. Coleman: Basolateral plasma membranes of intestinal epithelial cells. *Biochem. J.* 152, 71-84 (1975).
- [22] Lloyd-Davies, K. A., and D. N. Brindley: Palmitate activation and esterification in microsomal fractions of rat liver. *Biochem. J.* 152, 39-49 (1975).
- [23] Markwell, M. A. K., E. J. McGroarty, L. L. Bieber, and N. E. Tolbert: The subcellular distribution of carnitine acyl transferases in Mammalian liver and kidney. A new peroxisomal enzyme. *J. Biol. Chem.* 248, 3426-3432 (1973).
- [24] Norum, K. R.: Palmitoyl-CoA: carnitine palmityl transferase purification from calf liver mitochondria and some properties of the enzyme. *Biochim. Biophys. Acta.* 89, 95-108 (1964).
- [25] Novikoff, P. M., and A. B. Novikoff: Peroxisomes in absorptive cells of mammalian small intestine. *J. Cell. Biol.* 53, 532-566 (1972).
- [26] Peters, T. J.: Analytical subcellular fractionation of jejunal biopsy specimens: Methodology and characterisation of the organelles in normal tissue. *Clin. Sci. Mol. Med.* 51, 557-574 (1976).
- [27] Peters, T. J., and H. Shio: Analytical subcellular fractionation studies on rat liver and on isolated jejunal enterocytes with special reference to the separation of lysosomes, peroxisomes and mitochondria. *Clin. Sci. Mol. Med.* 50, 355-366 (1976).
- [28] Psenicnik, M., and N. Pipan: Nafenopin-induced proliferation of peroxisomes in the small intestine of mice. *Virchows Arch. Cell Path.* 25, 161-169 (1977).
- [29] Reijnierse, G. L., H. Veldstra, and C. J. van den Berg: Short-chain fatty acid synthases in Brain. *Biochem. J.* 152, 477-484 (1975).
- [30] Reijnierse, G. L. A., H. Veldstra, and C. J. van den Berg: Radioassay of acetyl-CoA synthetase, propionyl-CoA synthetase and butyryl-CoA synthetase in Brain. *Anal. Biochem.* 72, 614-622 (1976).
- [31] Sanchez, M., D. G. Nicholls, and D. N. Brindley: The relationship between palmitoyl-coenzyme A synthetase activity and esterification of sn-Glycerol 3-phosphate in rat liver mitochondria. *Biochem. J.* 132, 697-706 (1973).
- [32] Sarzala, M. G., and P. Wlodawer: Phosphatidate phosphohydrolase in frog intestinal tissue. *Comp. Biochem. Physiol.* 28, 1029-1044 (1969).
- [33] Scholte, H. R., and P. H. E. Groot: Organ and intracellular localisation of short-chain acyl-CoA synthetases in rat and guinea-pig. *Biochim. Biophys. Acta.* 409, 283-296 (1975).
- [34] Sedgwick, B., and G. Hubscher: Metabolism of phospholipids. IX. Phosphatidate phosphohydrolase in rat liver. *Biochim. Biophys. Acta.* 106, 63-77 (1965).
- [35] Senior, J. R., and K. J. Isselbacher: Activation of long chain fatty acids by rat gut mucosa. *Biochim. Biophys. Acta* 44, 399-400 (1960).
- [36] Svoboda, D. J.: Response of microperoxisomes in rat small intestinal mucosa to CPIB, a hypolipidemic drug. *Biochem. Pharmac.* 25, 2750-2752 (1976).
- [37] Temple, N. J., P. A. Martin, and M. J. Connock: Zonal rotor analysis of the subcellular localisation of  $\alpha$ -glycerophosphate dehydrogenase,  $\alpha$ -naphthyl palmitate and  $\beta$ -naphthyl laurate hydrolases in the mucosa of the guinea pig small intestine. *Histochem. J.* 8, 159-173 (1976).
- [38] Tzur, R., and B. Shapiro: Phosphatidic acid metabolism in rat liver microsomes. *Eur. J. Biochem.* 64, 301-305 (1976).
- [39] van Tol, A., and W. C. Hulsmann: The localisation of palmitoyl-CoA: carnitine palmitoyl-transferase in rat liver. *Biochim. Biophys. Acta.* 189, 342-353 (1969).
- [40] Wattiaux, R.: Behaviour of rat liver mitochondria during centrifugation in a sucrose gradient. *Mol. Cell. Biochem.* 4, 21-29 (1974).