

Zonal rotor analysis of the subcellular localization of α -glycerophosphate dehydrogenase, α -naphthyl palmitate and β -naphthyl laurate hydrolases in the mucosa of the guinea-pig small intestine

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Synopsis. Whole homogenate of guinea-pig small intestine mucosa was analysed by centrifugation in a zonal rotor. The results indicate that FAD-linked α -glycerophosphate dehydrogenase is localized in the mitochondria and that NAD-linked α -glycerophosphate dehydrogenase is a soluble phase enzyme. An enzyme hydrolysing α -naphthyl palmitate at an acid pH was localized in the lysosomes and was activated by 0.1% Triton X-100 and by freezing and thawing. An alkaline hydrolase acting on β -naphthyl laurate was localized in the 'microsomes'. The possibility of this enzyme being different from α -naphthyl acetate hydrolase is discussed.

Introduction

Recently, Gee *et al.* (1974) have examined the subcellular localization of α -glycerophosphate dehydrogenase in the liver and kidney of several species of mammal. In addition to soluble phase and mitochondrial enzyme a variable amount of activity, depending on species and tissue, was localized in the peroxisomes. This evidence for a peroxisomal α -glycerophosphate dehydrogenase stimulated us to examine the localization of the enzyme in the small intestine. In addition we studied the localization of enzymes hydrolysing α -naphthyl palmitate and β -naphthyl laurate, substrates with potential for histochemistry and which may be suitable as artificial substrates for lipolytic enzymes.

In the past the fractionation of mucosal homogenates has often proved difficult. For example, Hubscher *et al.* (1965) comment on the heavy cross-contamination of subcellular fractions due to the binding by mucus. Similarly, Porteous (1974) has discussed the problems of separating nuclei from brush borders. Happily, guinea-pig mucosal

homogenates appear to be free from these drawbacks. Hubscher *et al.* (1965) and Wrigglesworth & Pover (1966), using differential pelleting (Reid & Williamson, 1974), have achieved reasonably good separations into N, ML, P, S and N, M, L, P, S fractions respectively (fraction nomenclature according to De Duve *et al.*, 1955). Also, rate zonal or isopycnic centrifugation of guinea-pig homogenate or post-nuclear supernatant give fairly good resolution between organelles (Connock & Kirk, 1973; Connock & Pover, 1970). This is in direct contrast to the situation with the rat where Peters (1972), using the Beaufay type zonal rotor and isopycnic centrifugation in a sucrose gradient, observed similar modal densities for the mitochondria, lysosomes and peroxisomes. A variety of other gradient materials were employed with no greater success. An additional advantage with the guinea-pig is that it is possible to resolve brush borders from the nuclei by a single, low *g*, rate zonal sedimentation using a zonal rotor with a long path length (Connock *et al.*, 1971).

Materials and methods

Preparation of homogenates

Adult guinea-pigs of either sex were killed by a blow on the head. The small intestine was removed and flushed out repeatedly with ice cold 10% (w/w) sucrose. The whole intestine was then gently scraped along the outside with a microscope slide so as to expel the mucosa. The mucosa from one complete small intestine (about 200 mg protein) was suspended in 10% ice cold sucrose to give a volume of about 30 ml. The suspension was homogenized by five strokes in a Potter-Elvehjem homogenizer with a tight fitting glass impregnated Teflon pestle rotating at approximately 1500 r.p.m. The homogenate was examined by dark-field microscopy to ensure sufficient cell breakage had been achieved.

Subcellular fractionation

Analytical centrifugation was carried out in an MSE aluminium B XIV zonal rotor. Several rate sedimentation separations were carried out and also a separation which approached closely or achieved isopycnic conditions ($g_{\min} = 1.6 \times 10^5 g \text{ hr}$). The durations and speeds of the centrifugations are given in the figure captions as also are details of the gradients employed. In all experiments 20 ml homogenate was loaded on to linear density gradients of sucrose in water resting on a cushion of 60% w/w sucrose. The homogenate was loaded by a perfusion pump at the rate of 2.75 ml/min; this procedure was adopted to minimize the broadening of the sample band. For loading, the rotor was spun at 2200 r.p.m. and the centrifuge bowl was cooled (the latter resulted in greater viscosity of gradients than in our previous experiments (Connock & Kirk, 1973; Connock *et al.*, 1974) leading to the need for larger centrifugal forces for comparable sedimentations).

An overlay of 20 ml 5% sucrose was then added by perfusion. The rotor was run up to speed. Unloading was done at 2200 r.p.m. by peripheral displacement with 60% sucrose, and approximately 20 fractions of 25 ml were collected, the first fraction representing sample volume plus overlay. The densities of the fractions were estimated from refractive index measurements.

The fractions and a sample of the original homogenate were analysed for their content

of various markers for subcellular organelles. DNA was used as a marker for the nuclei*, succinate dehydrogenase for the mitochondria, alkaline phosphatase for the brush borders, catalase for the peroxisomes. Acid phosphatase, aryl sulphatase and β -glucuronidase were used as markers for the lysosomes. In rat liver (De Duve *et al.*, 1955) β -glucuronidase is not an ideal marker for lysosomes since it is present also in some constituent of the microsomal fraction (presumably the endoplasmic reticulum membranes). However, this is not the case for the guinea-pig small intestine mucosa. In the microsomal fraction isolated from this tissue, Wrigglesworth & Pover (1966) found a lower relative specific activity for β -glucuronidase than for either acid phosphatase or acid deoxyribonuclease. Similarly, after isopycnic density gradient centrifugation of post-nuclear supernatant, Connock & Pover (1970) found less β -glucuronidase than acid phosphatase, aryl sulphatase, or acid ribonuclease at the light ('microsomal') end of the gradient. These findings support the use of β -glucuronidase as a lysosomal marker in this tissue.

Non-specific esterase and glucose-6-phosphatase were used as markers for the 'microsomes'.† In rat liver, non-specific esterase, assayed against a variety of substrates including α -naphthyl acetate, is localized equally with glucose-6-phosphatase in the microsomal fraction (Underhay *et al.*, 1956). Detailed analysis of the rat liver microsomal fraction (Beaufay *et al.*, 1974) has shown that esterase and glucose-6-phosphatase can be assigned to the same subcellular site (referred to as the group C component of the microsomal fraction). Histochemical findings (Lojda, 1974; Pearse, 1967) support its use as a marker for the endoplasmic reticulum. Glucose-6-phosphatase was assayed less often than esterase because its activity is relatively low in the intestine and because the assay is complicated by the presence of non-specific acid and alkaline phosphatases. It is probable that there are additional components of the 'microsomes' that do not carry either esterase or glucose-6-phosphatase activity. The fate of these components in the fractionation experiments reported is, unfortunately, unknown.

Biochemical analyses

All enzymes (except catalase) were assayed under zero order kinetics and incubations terminated within the linear range of the reaction. Lysosomal acid hydrolases were assayed after treatment with Triton X-100 (at a final concentration of 0.1%), as also was catalase (concentration 1%).

(a) The following estimations were carried out as previously reported (Connock & Pover, 1970; Connock *et al.*, 1971 & 1974):

Acid phosphatase was assayed by measuring the fluoride-sensitive hydrolysis of α -naphthyl phosphate in the presence of EDTA, alkaline phosphatase (α -naphthyl phosphate as substrate with no exogenous Mg^{2+}), glucose-6-phosphatase, DNA, catalase, aryl sulphatase (*p*-nitrocatechol sulphate as substrate).

(b) Protein (samples and standards normalized for sucrose concentration) was measured according to Lowry *et al.* (1951).

* DNA was only analysed in the fractions from the isopycnic separation. Previous experiments showed that the nuclei, like the brush borders, virtually reached the gradient/cushion interface in all the rate sedimentation experiments. (Connock, Elkin & Pover, unpublished data).

† 'Microsomes' is used here as an operational term (Roodyn, 1967) for the components of the microsomal fraction.

(c) β -Glucuronidase was assayed according to Michell *et al.* (1970) with *p*-nitrophenyl- β -D-glucuronide as substrate.

(d) Succinate dehydrogenase was assayed as previously described (Connock *et al.*, 1971) except that the reaction was stopped and the reduced INT estimated according to Prospero (1974).

(e) Non-specific esterase or α -naphthyl acetate esterase. The method was based on that of Ravin & Seligman (1953): 10 mg substrate was dissolved in acetone (10 ml) which was then added to 100 ml buffer as described by Ravin and Seligman. The buffer was 0.825% w/v sodium diethyl barbiturate which had then been adjusted to pH 7.4 with 0.2 N HCl. 3 ml substrate buffer was added to 50 μ l appropriately diluted enzyme sample. Controls contained no enzyme. Incubation was at 37°C for 30 min. Hydrolysis was terminated by placing the tubes in a water bath at 90–95°C for 4–4.5 min. Tubes were cooled to below 35°C and to each was added 3 ml 0.4 M acetate buffer, pH 5.0, containing 0.04 M EDTA. (This addition was found to stabilize subsequent colour development.) To each tube was now added 5 ml diazo salt solution (Fast Red ITR). The solution was made up according to Robinson (1972) as follows: a diluent was made of 0.034 N HCl containing 2.18% w/v sodium acetate $3\text{H}_2\text{O}$, 2.06% w/v sodium diethyl barbiturate, and 4.0% sodium lauryl sulphate. Just before use, the diazo salt (Fast Red ITR) was added and dissolved in the diluent in a dark bottle at a concentration of 200 mg/100 ml. The colour stabilized to a mauve within 5 min and the absorbance was then read at 550 nm. α -Naphthyl acetate esterase was assayed at pH 7.4 rather than at its optimum pH because above pH 8.0, heat caused noticeable hydrolysis of substrate.

(f) β -Naphthyl laurate hydrolase. The method used was similar to that for α -naphthyl acetate esterase except that the substrate was β -naphthyl laurate at a concentration of 30 mg/10 ml acetone added as above to 100 ml buffer. The buffer was again barbitone but the pH was 8.6. Sometimes Triton X-100 was added to the buffer-substrate mixture to give a final concentration during incubation of 0.1%. 3 ml buffer-substrate was added to 50 to 250 μ l enzyme sample (depending on enzyme activity) and incubation was at 37°C for 30 min. Controls contained an equivalent volume of 30% sucrose instead of enzyme. Incubation was terminated as above except that tubes containing no Triton were adjusted to 0.1% by addition of a small volume of concentrated Triton X-100 before heating. Tubes were then treated as for α -naphthyl acetate esterase except that they were centrifuged and the absorbance read at 500 nm (since a red-brown colour was obtained).

(g) α -Naphthyl palmitate hydrolase at pH 5. The method was similar to that for α -naphthyl acetate esterase except that the substrate was α -naphthyl palmitate, the buffer was 0.1 M acetate pH 5.0, and the buffer substrate usually contained Triton X-100 to give an incubation concentration of 0.1%. 3 ml buffer substrate was added to 250/500 μ l of enzyme sample. The assay then proceeded as for β -naphthyl laurate hydrolase except that on cooling a different buffer was added. This was 1 ml of a 1:1 freshly mixed cocktail of 0.1 M EDTA and the acetate/EDTA buffer used at this stage for the naphthyl and acetate assays. To each tube was added 4.5 ml of diazo salt solution as described for α -naphthyl acetate esterase. Tubes were then stored in the dark at 22 to 30°C, centrifuged and absorbance measured (550 nm) 100 to 130 min after the addition of the diazo salt solution. α -Naphthyl palmitate hydrolase was also assayed at pH 6.5

(NP 6.5). The assay was similar to that for α -naphthyl acetate esterase but substrate concentration was 14 mg/10 ml acetone in 100 ml of pH 6.5 barbitone buffer. Triton X-100 was usually included as for α -naphthyl palmitate hydrolase (pH 5). Incubation and termination of hydrolysis was as for α -naphthyl palmitate hydrolase (pH 5). On cooling the assay proceeded as for α -naphthyl acetate esterase.

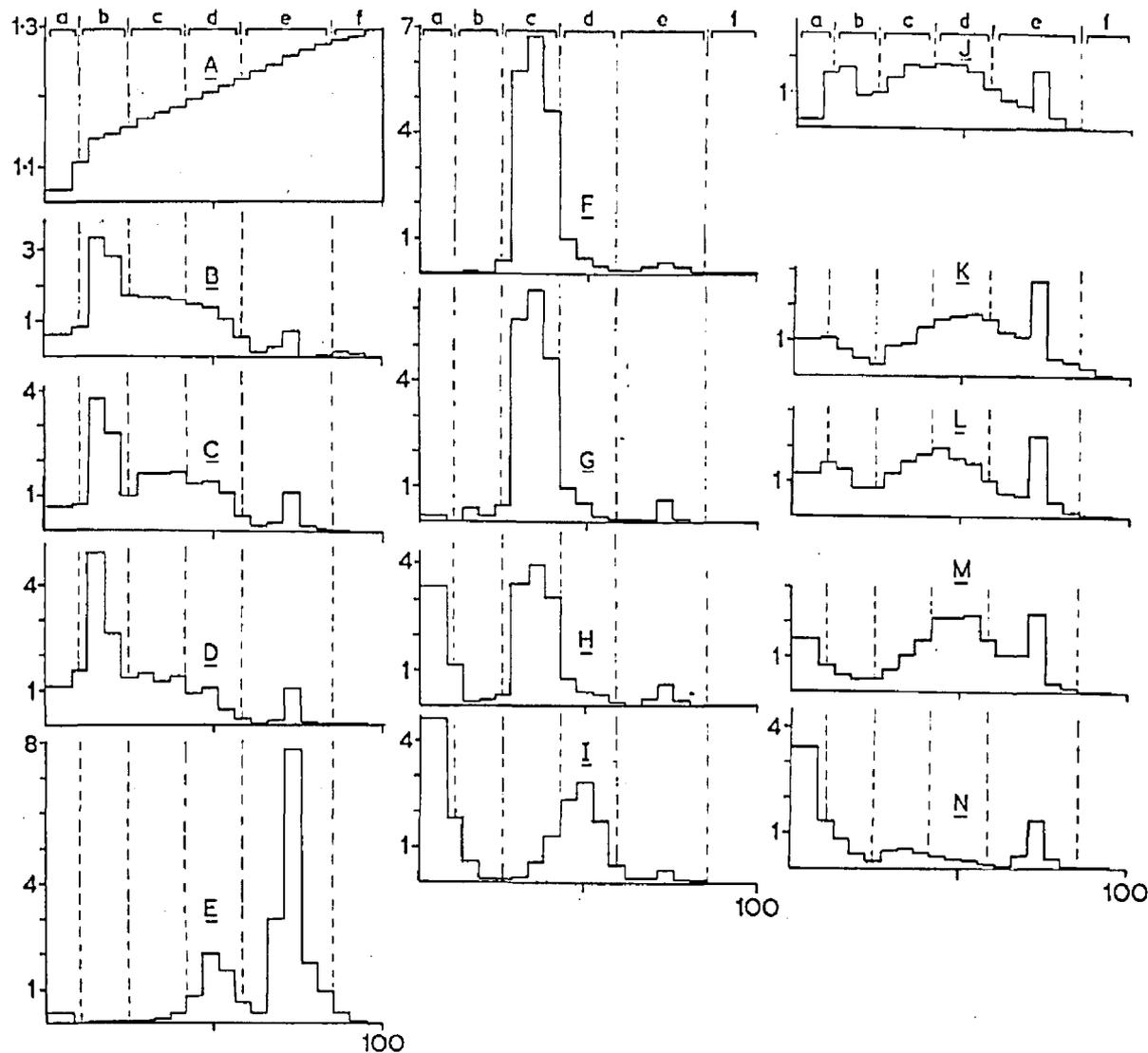
(h) α -Glycerophosphate dehydrogenase. The method used was that of Gardner (1974) except that the substrate concentration was doubled and the enzyme reaction terminated and colour development obtained by the method of Prospero (1974). This assay depends on the reduction of INT (2-iodophenyl-3-(*p*-nitrophenyl)-5-phenyl-tetrazolium chloride) by the reduced FAD of the dehydrogenase. In addition we developed an estimation for NAD-linked α -glycerophosphate dehydrogenase. The procedure was similar to that for the FAD enzyme except that the incubation medium contained NAD and PMS (phenazine methosulphate). The latter functioning to carry electrons from reduced NAD to INT. This procedure will detect both FAD- and NAD-linked enzymes. Thus mitochondrial fractions exhibited considerable activity in the NAD-linked assay. However, the omission of NAD alone from the incubation medium, with mitochondrial fractions, failed to decrease the optical densities measured. We, therefore, concluded that the α -glycerophosphate dehydrogenase activity of the mitochondrial fractions is due to the FAD-linked enzyme alone. The assay procedures were as follows: (1) FAD-linked α -glycerophosphate dehydrogenase. 200 to 300 μ l of enzyme sample was incubated in a total volume of 900 μ l according to Gardner. The enzyme reaction was stopped by the addition of 4.5 ml of stopping reagent as in the assay of succinate dehydrogenase. (2) NAD-linked α -glycerophosphate dehydrogenase. 100 μ l NAD (2.49 mg/ml), 5 μ l of 2% w/v PMS, 160 μ l 0.5 M α -glycerophosphate (neutralized by HCl) or 160 μ l water for controls, 80 μ l of 0.4 M potassium phosphate buffer pH 7.6 containing 0.01 M KCN, and 250 μ l 0.4% INT were mixed. To this was added 200 μ l of enzyme sample and incubation carried out at 37°C for 30 min. The assay was terminated as described above.

Results

After prolonged density gradient centrifugation of whole homogenate, so as to approach isopycnic conditions, enzyme markers for subcellular components were distributed as shown in Fig. 1. From these distributions the gradient can be divided into several regions each of which has characteristic components. These regions are: (a) containing soluble phase enzymes and the bulk of the protein in the homogenate; (b) relatively enriched in 'microsomes' as indicated by the peak of α -naphthyl acetate esterase activity; (c) containing most of the mitochondria (marker succinate dehydrogenase); (d) containing most of the microperoxisomes (marker catalase); (e) containing most of the brush border fragments (marker alkaline phosphatase); analyses of DNA indicated that the nuclei were also in this region; (f) very dense region of the gradient containing only traces of any marker.

Lysosomal markers β -glucuronidase and acid phosphatase are broadly distributed with activity in region (a), and spanning regions (c), (d) and (e). In region (e) both enzymes show a relative enrichment in fraction 15 (density = 1.25 g/ml) which is characterized by its high brush border content. This peak of lysosomal activity is prob-

Figure 1. Distribution of enzymes and protein after centrifugation of 28 ml whole homogenate on 450 ml of linear sucrose gradient (30% to 60% w/w). Centrifugation was for 12 hr at 25000 rpm. (A) Density; (B) α -naphthyl acetate esterase (83%); (C) β -naphthyl laurate hydrolase with 0.1% Triton X-100 (80%); (D) β -naphthyl laurate hydrolase minus Triton X-100 (62%); (E) alkaline phosphatase (88%); (F) succinate dehydrogenase (105%); (G) FAD-linked α -glycerophosphate dehydrogenase (76%); (H) NAD-linked α -glycerophosphate dehydrogenase (80%); (I) catalase (99%); (J) α -naphthyl palmitate hydrolase (pH 5) minus Triton X-100 (86%); (K) α -naphthyl palmitate hydrolase (pH 5) with 0.1% Triton X-100 (91%); (L) β -glucuronidase (81%); (M) acid phosphatase (91%); (N) protein (87.1%) with the ordinate = $10^{-1} \times$ mg protein in the fraction. Ordinates (except for A and N) represent the concentration of material in the fraction divided by its concentration if it were homogeneously distributed throughout the gradient. The abscissae are divided according to the % volume of each fraction. The vertical columns labelled a to f represent regions of the gradient discussed in the text. % figures represent the recovery of material from the gradient relative to the original homogenate.



ably due to the presence of incompletely homogenized cell fragments and also partly to lysosomes located in cytoplasm still attached to the brush borders.

Each of the enzymes whose subcellular localization is unknown has a distribution similar to a particular marker. Thus β -naphthyl laurate hydrolase, whether assayed with or without Triton X-100, has a distribution like that of α -naphthyl acetate esterase. α -Naphthyl palmitate hydrolase (pH 5) assayed with or without Triton is like acid phosphatase and β -glucuronidase. FAD-linked α -glycerophosphate dehydrogenase is very similar to succinic dehydrogenase, while NAD-linked α -glycerophosphate dehydrogenase is similar to succinate dehydrogenase but in addition shows considerable activity in the soluble phase region (a); the activity in the mitochondrial region can be accounted for by FAD-linked enzyme (see *Materials and methods*). Thus we may provisionally allocate α -naphthyl palmitate hydrolase (pH 5) to the lysosomes, β -naphthyl laurate hydrolase to the 'microsomes', FAD-linked α -glycerophosphate dehydrogenase to the mitochondria, and NAD-linked α -glycerophosphate dehydrogenase to the soluble phase.

To test these allocations further, we carried out rate sedimentation experiments. In these (e.g. Fig. 2G) the rotor contained a long, shallow, low-density gradient resting on a cushion of a short, steep, high-density gradient which in turn rested on a layer of 60% sucrose. The long, shallow, gradient was designed to analyse the sedimentation rate of slow moving particles, while the steep gradient was employed to bring about some separation (mainly on the basis of density) of those fast moving particles reaching the cushion before the end of the centrifugation period. Four experiments were conducted at successively increasing centrifugal force in order to analyse each of the components of major interest (lysosomes, microsomes, peroxisomes and mitochondria).

At relatively low centrifugal force (Fig. 2) the lysosomes and mitochondria begin to move into the gradient. α -Naphthyl palmitate hydrolase (pH 5) distribution resembles that of lysosomal markers aryl sulphatase, β -glucuronidase and acid phosphatase more closely than it does that of any other marker. α -Naphthyl palmitate hydrolysis assayed at pH 6.5 appears to give a slightly different distribution with a greater proportion of activity localized in a slow moving component.

At greater centrifugal forces (Fig. 3), lysosome distribution is broader and the microsomes begin to move into the gradient. The distribution of FAD-linked α -glycerophosphate dehydrogenase and succinate dehydrogenase are again very similar and that of NAD-linked α -glycerophosphate dehydrogenase indicates soluble phase activity. α -Naphthyl acetate esterase and β -naphthyl laurate hydrolase have a similar distribution, as do α -naphthyl palmitate hydrolase (pH 5) (with 0.1% Triton X-100 in the incubation medium) and acid phosphatase. Fig. 4 shows the results of a similar experiment in which α -naphthyl palmitate hydrolase activity at pH 5 was compared with that at pH 6.5. Although the distribution of both activities resembles that of acid phosphatase more closely than that of other markers, once again α -naphthyl palmitate hydrolase (pH 5) is more similar to acid phosphatase than is α -naphthyl palmitate hydrolase (pH 6.5).

Larger centrifugal forces cause 'microsomes' to move well into the gradient (Figs. 5, 6). β -Naphthyl laurate hydrolase distribution corresponds with that of microsomal markers α -naphthyl acetate esterase and glucose-6-phosphatase, all other markers having reached the dense end of the gradient. However, both α -naphthyl acetate

esterase and β -naphthyl laurate hydrolase have a peak of activity at the dense end of the gradient not reflected so well by glucose-6-phosphatase (Fig. 5).

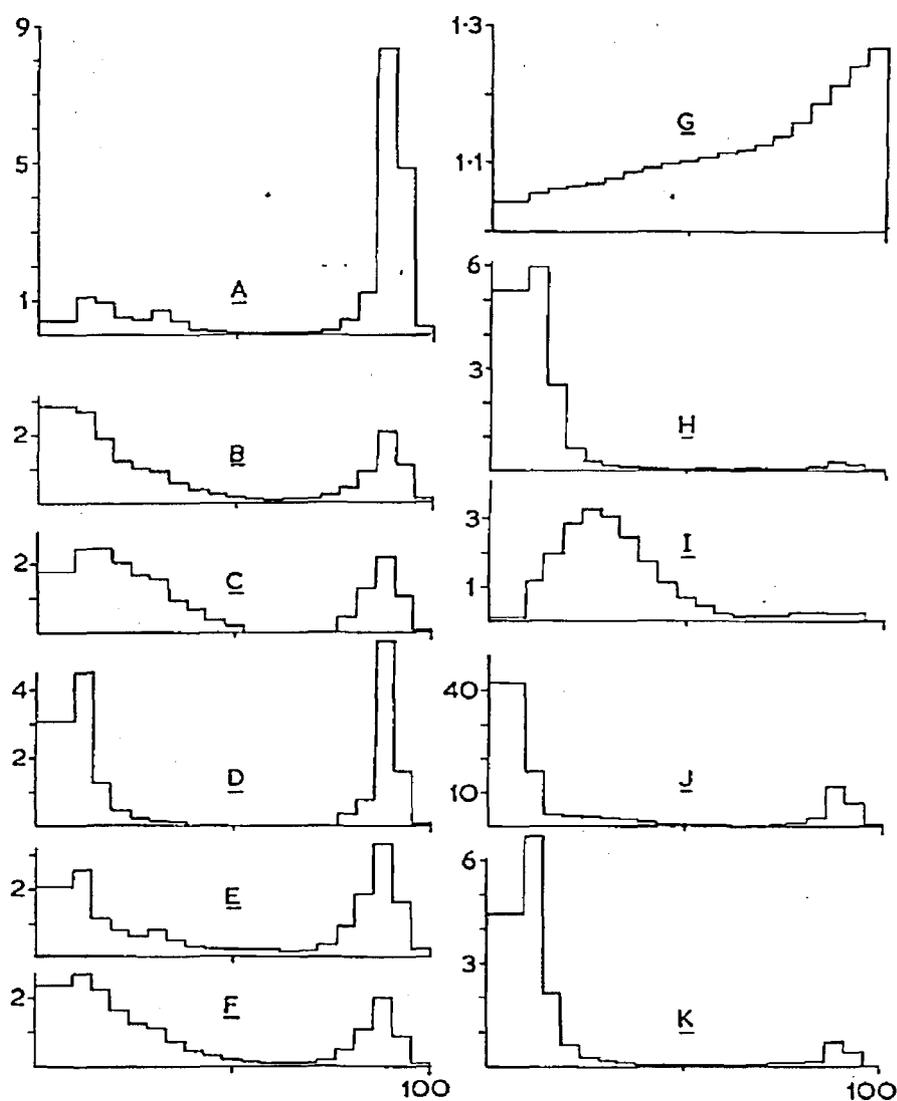


Figure 2. Distribution of enzymes and protein after centrifugation of 20 ml homogenate on 350 ml of linear sucrose gradient (13% to 30% w/w) resting on a gradient of 100 ml 40% to 50% w/w sucrose. Centrifugation was for 15 min at 12000 rpm. (A) Alkaline phosphatase (95%); (B) acid phosphatase (85%); (C) aryl sulphatase (78%); (D) α -naphthyl palmitate hydrolase (pH 6.5) with 0.1% Triton X-100 (47%); (E) α -naphthyl palmitate hydrolase (pH 5) with 0.1% Triton X-100 (75%); (F) β -glucuronidase (93%); (G) density; (H) catalase (119%); (I) succinate dehydrogenase (81%); (J) protein (89%) with ordinate = mg protein in fraction; (K) α -naphthyl acetate esterase (76%). Ordinates (except G and J) and abscissae as Fig. 1.

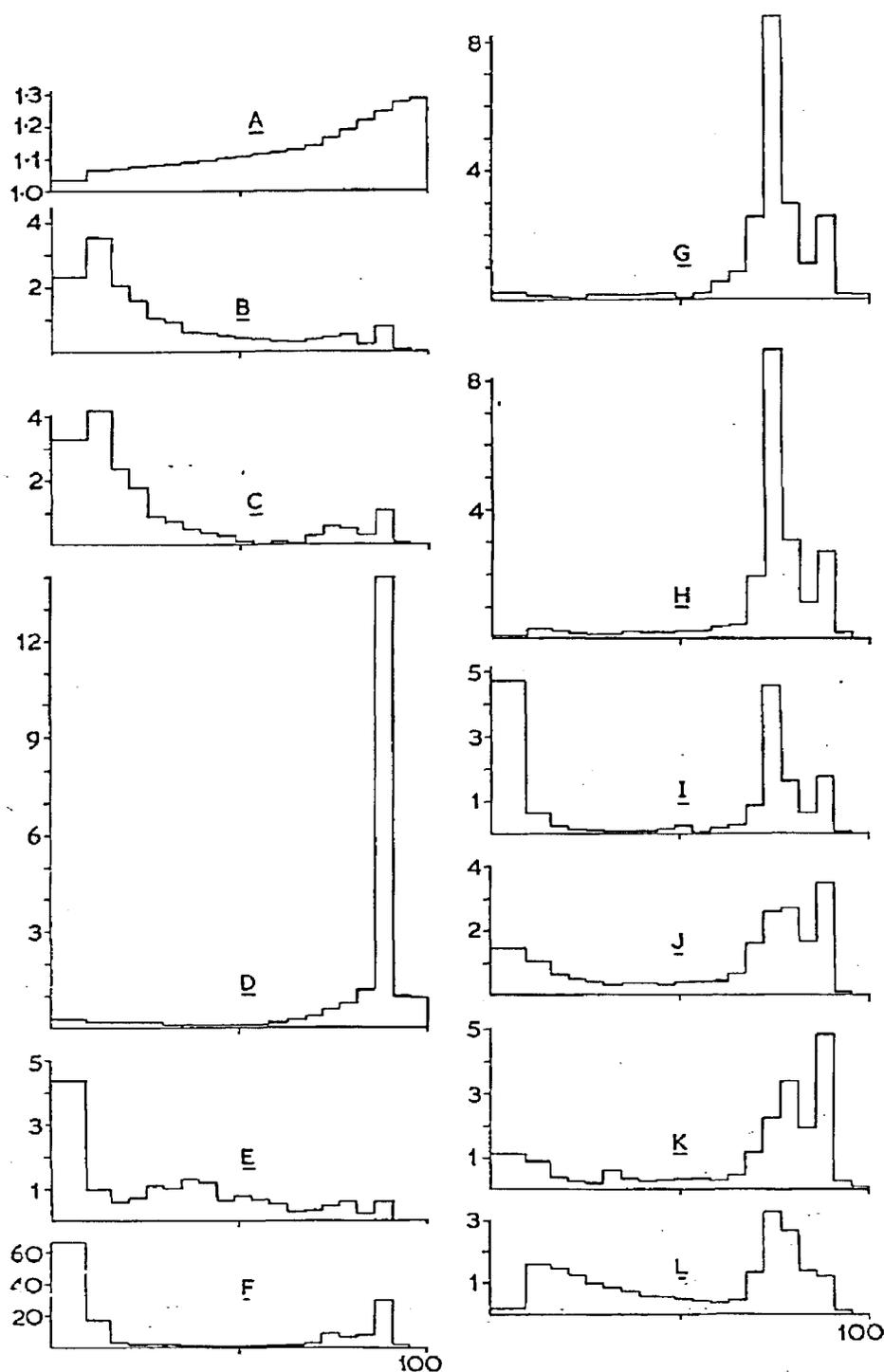


Figure 3. As Fig. 2 but centrifugation at 16000 r.p.m. for 45 min. (A) Density; (B) α -naphthyl acetate esterase (104%); (C) β -naphthyl laurate hydrolase minus Triton X-100 (144%); (D) Alkaline phosphatase (132%); (E) catalase (80%); (F) protein (79%) with ordinate as J Fig. 2; (G) succinate dehydrogenase (165%); (H) FAD-linked α -glycerophosphate dehydrogenase (86%); (I) NAD-linked α -glycerophosphate dehydrogenase (95%); (J) acid phosphatase (99%); α -naphthyl palmitate hydrolase (pH 5) with 0.1% Triton X-100 (77%); (L) α -naphthyl palmitate hydrolase (pH 5) minus Triton X-100 (157%). Ordinates (except A and F) as Fig. 1.

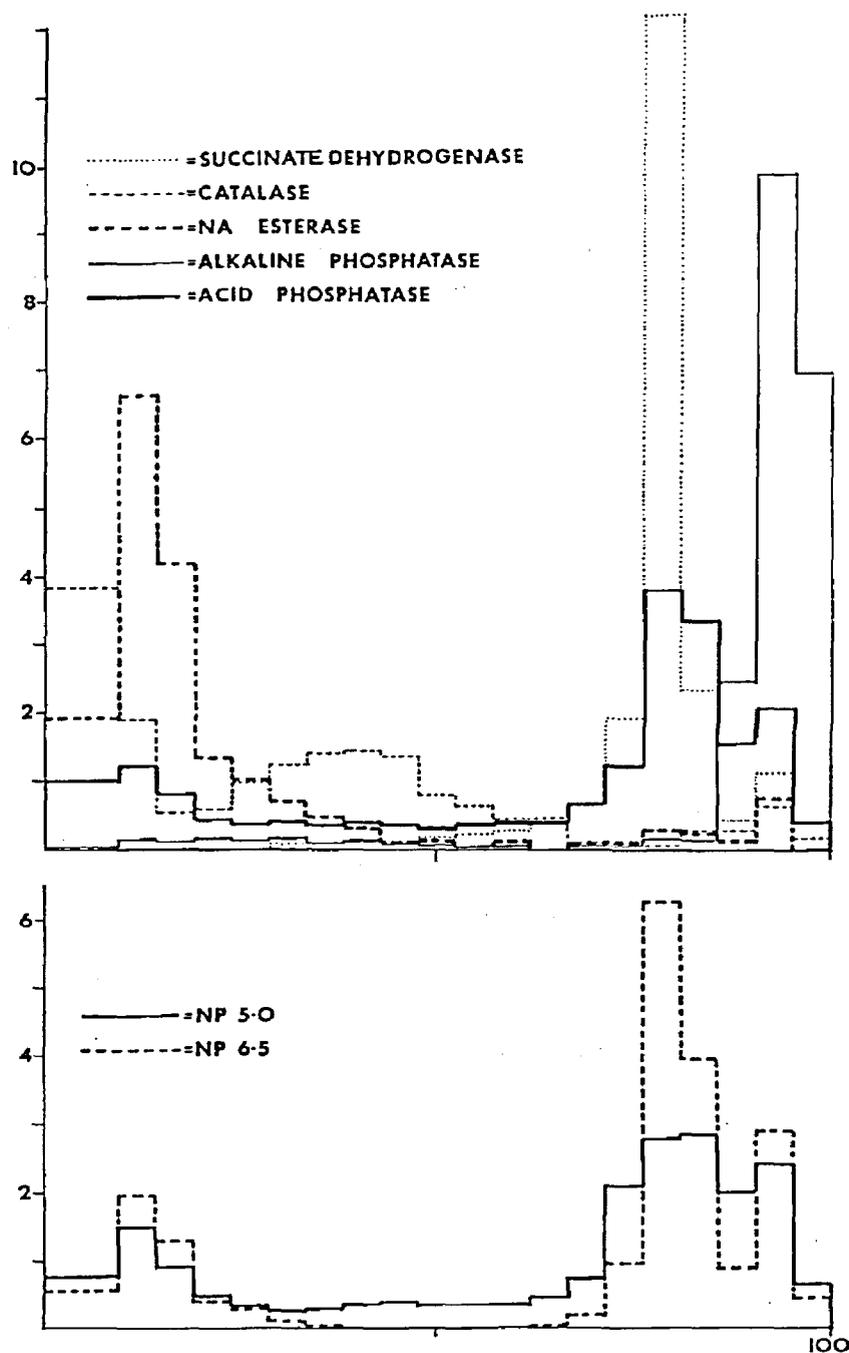


Figure 4. Comparison of α -naphthyl palmitate hydrolase distribution at pH 5 and pH 6.5 after centrifugation at 16000 r.p.m. for 45 min. Gradient as Fig. 3. Note α -naphthyl palmitate hydrolase (pH 5) resembles acid phosphatase in being spread across the whole gradient. α -Naphthyl palmitate hydrolase at pH 5 and pH 6.5 were assayed with 0.1% Triton X-100 in the incubation medium. Ordinates and abscissae as Fig. 1. Recoveries were as follows: acid phosphatase (95%), alkaline phosphatase (109%), α -naphthyl acetate esterase (NA esterase) (78%), succinate dehydrogenase (78%), catalase (88%), α -naphthyl palmitate hydrolase at pH 5 (NP5) with 0.1 Triton X-100 (78%), α -naphthyl palmitate dehydrogenase at pH 6.5 (NP6.5) with 0.1% Triton X-100 (70%).

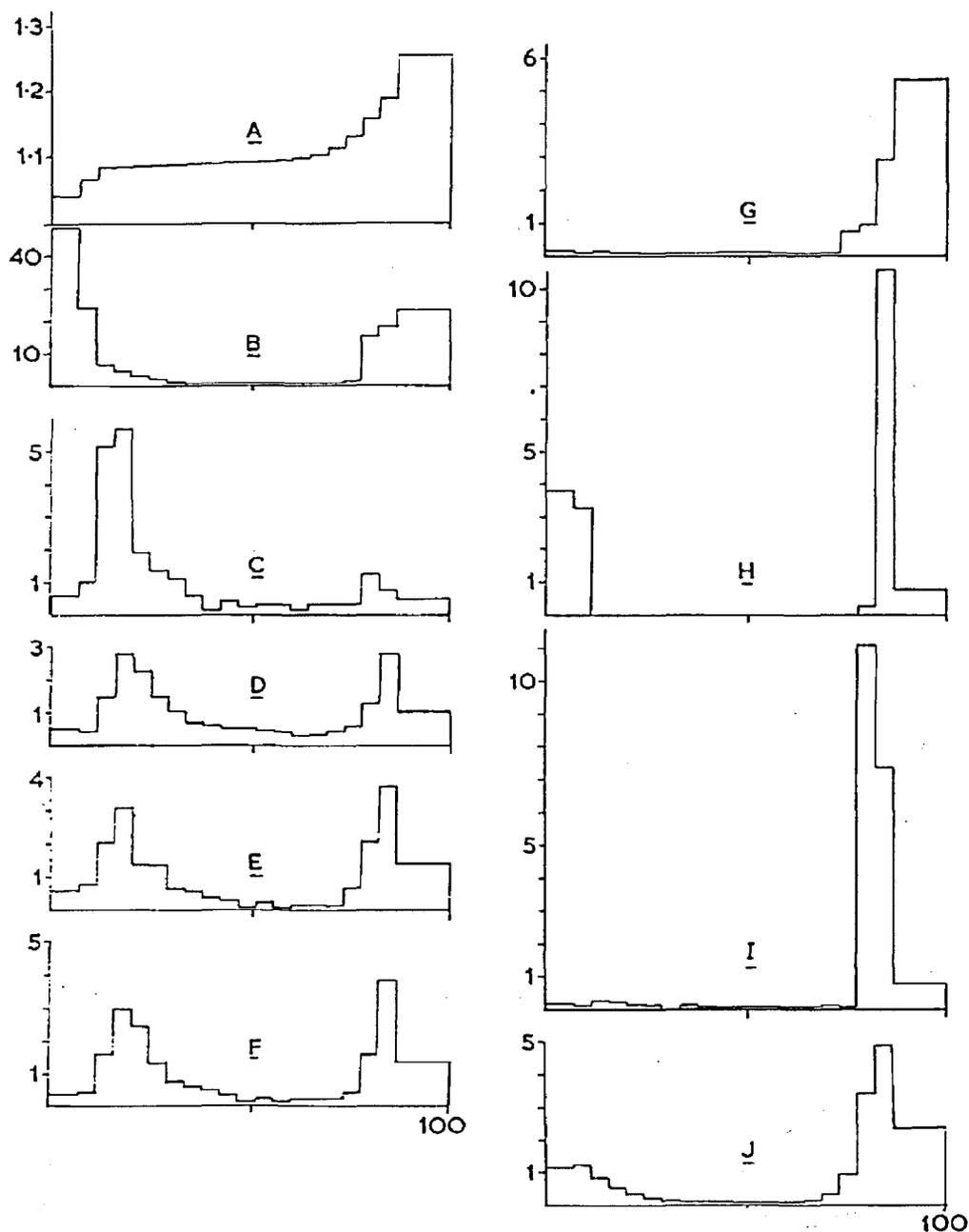


Figure 5. Distribution of enzymes and protein after centrifugation of 20 ml homogenate on 400 ml of linear sucrose gradient (13% to 30%) resting on a gradient of 60 ml 30% to 50% w/w sucrose. Centrifugation was for 130 min at 27000 r.p.m. (A) Density; (B) protein (89%) with ordinate as J Fig. 2; (C) glucose-6-phosphatase (111%); (D) α -naphthyl acetate esterase (112%); (E) β -naphthyl laurate hydrolase minus Triton X-100 (109%); (F) β -naphthyl laurate hydrolase with 0.1% Triton X-100 (86%); (G) alkaline phosphatase (98%); (H) catalase (159%); (I) succinate dehydrogenase (114%); (J) acid phosphatase (109%). Ordinates (except A and B) and abscissae as Fig. 1.

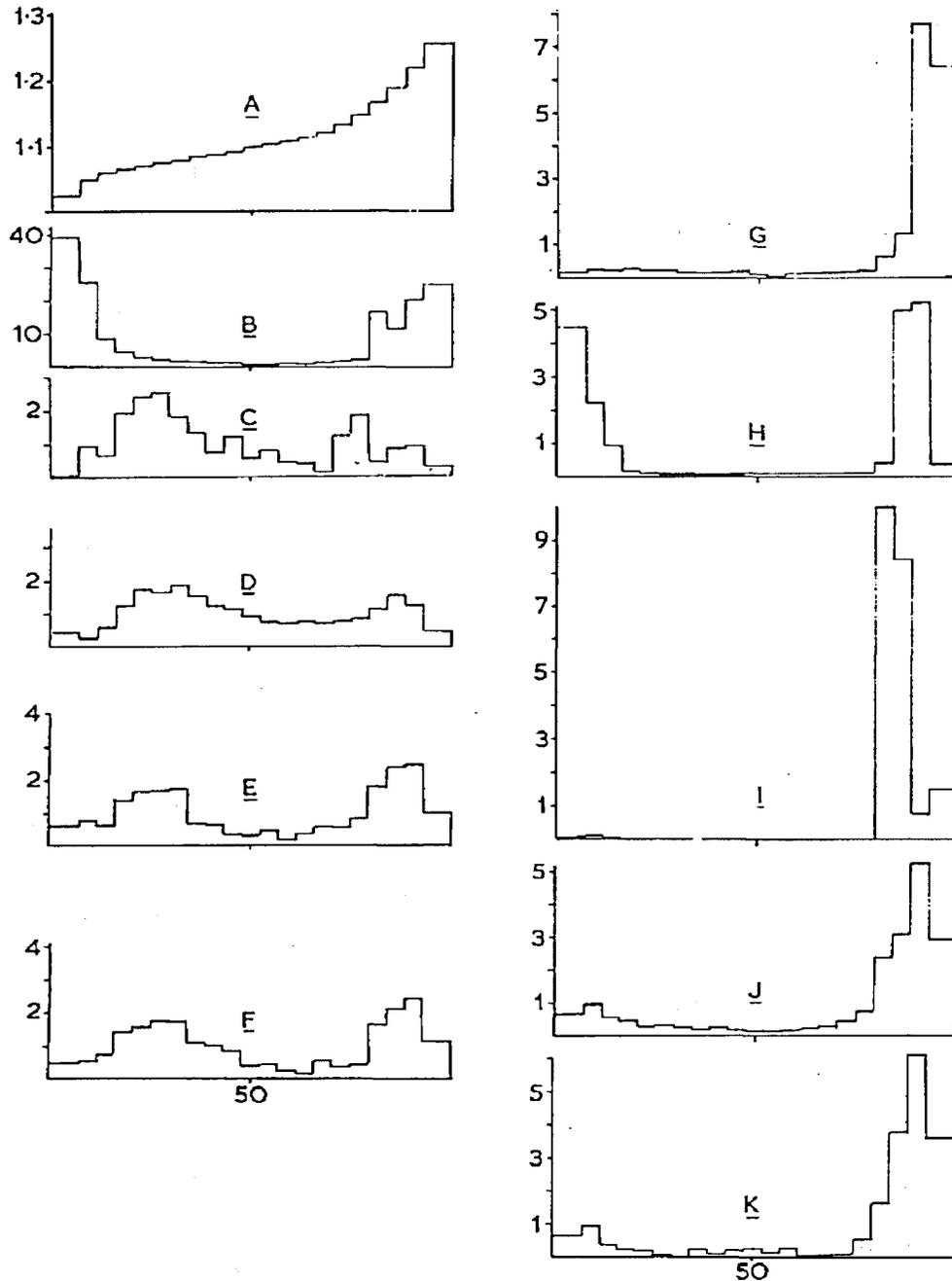


Figure 6. As Fig. 5, but centrifugation at 12 000 r.p.m. for 12 hr. (A) Density; (B) protein (98%) with ordinate as J Fig. 2; (C) glucose-6-phosphatase (58%); (D) α -naphthyl acetate esterase (94%); (E) β -naphthyl laurate hydrolase minus Triton X-100 (66%); (F) β -naphthyl laurate hydrolase with 0.1% Triton X-100 (53%); (G) alkaline phosphatase (110%); (H) catalase (94%); (I) succinate dehydrogenase (86%); (J) acid phosphatase (77%); (K) α -naphthyl palmitate hydrolase (pH 5) with 0.1% Triton X-100 (98%). Ordinates (except A and B) and abscissae as Fig. 1.

Discussion

Evidence presented here indicates that FAD-linked α -glycerophosphate dehydrogenase is a mitochondrial enzyme in the guinea-pig small intestine, and that NAD-linked α -glycerophosphate dehydrogenase is a soluble phase component. In contrast to Gee *et al.* (1974) we could find no α -glycerophosphate dehydrogenase in the peroxisomes. This may be due to the fact that there are two classes of peroxisome organelle (see Novikoff & Novikoff, 1972), namely microperoxisomes which are found in the small intestine (Connock & Kirk, 1973; Connock, 1973) and many other tissues (Novikoff *et al.*, 1973) on the one hand, and the larger peroxisomes found in liver and kidney and investigated by Gee *et al.* on the other. Another explanation may be seen in terms of tissue differences especially in view of the fact that Gee *et al.* report considerable differences in the quantity of peroxisomal α -glycerophosphate dehydrogenase between species and between kidney and liver within one species.

From our results it appears that, in addition to α -naphthyl acetate esterase, there are at least two other enzymes which hydrolyse naphthyl fatty acid esters in the small intestine. Of these β -naphthyl laurate hydrolase appears to be localized in a component of the microsomes. The identification of this component (or for that matter the certain identity of the microsomal component(s) bearing α -naphthyl acetate esterase and glucose-6-phosphatase activities in the small intestine) awaits further analysis.

Intestinal hydrolase activity against α -naphthyl acetate is approximately twenty times greater than that against β -naphthyl laurate. The possibility arises that a single enzyme is responsible for both activities, naphthyl acetate being the preferred substrate. We think this is unlikely for the following reasons: (i) the subcellular distributions of α -naphthyl acetate esterase and β -naphthyl laurate hydrolase seem to differ slightly. (ii) Their pH curves differ slightly (Fig. 7). (iii) Their response at pH 6.0 to acetate and barbitone buffer is different (Fig. 7). (iv) Their response to various esterase inhibitors and activators is different (see Table 1), especially with respect to calcium ions and taurocholate.

Ravin & Seligman (1953) using naphthol esters as substrates found that taurocholate and calcium together activate pancreatic lipase. As we found these chemicals inhibit, β -naphthyl laurate hydrolase is unlikely to be a pancreatic lipase secondarily bound to subcellular structures of the mucosa. Similarly, β -naphthyl laurate hydrolase is unlikely to be the triglyceride hydrolase observed by Di Nella *et al.* (1960) in intestinal mucosa since this enzyme was unaffected by eserine. Further Pope *et al.* (1966) found that monoglyceride hydrolase was strongly inhibited by taurocholate and unaffected by eserine, again in contrast to our results. β -Naphthyl laurate hydrolase is, therefore, unlikely to correspond to this enzyme unless the nature of the substrate determines the effects of these substances.

According to Rona & Takata (1922) lipases are generally inhibited by quinine whereas esterases are unaffected. Our results show a lipase-like inhibition of β -naphthyl laurate hydrolase, but α -naphthyl acetate esterase had esterase-like immunity to this substance. On the present evidence it is not certain whether β -naphthyl laurate hydrolase is a separate enzyme from α -naphthyl acetate esterase and if it is, whether its natural substrates are glycerol esters.

The third hydrolase activity for naphthyl fatty acid esters was measured against α -naphthyl palmitate. Under various centrifugation conditions this enzyme followed

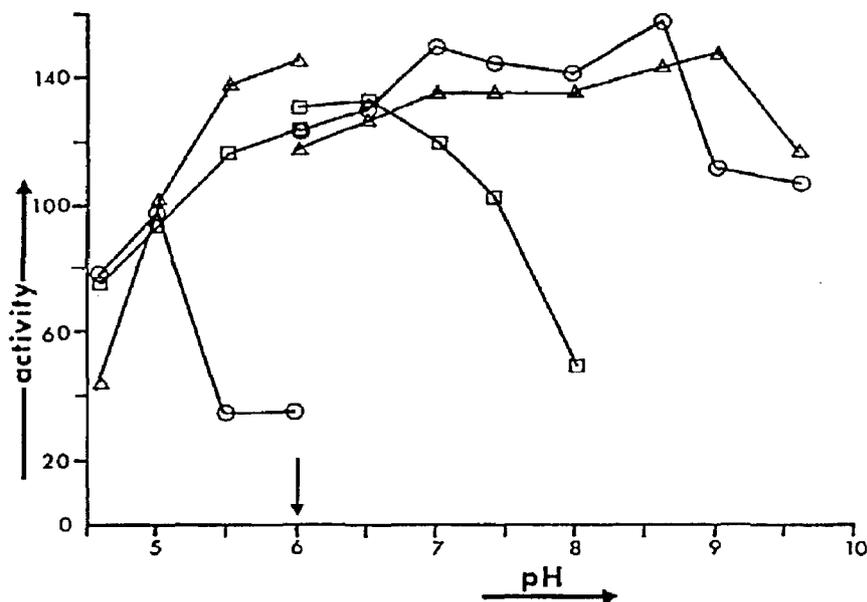


Figure 7. Influence of pH on the activity (arbitrary units for each substrate) of whole homogenate against β -naphthyl laurate(O), α -naphthyl acetate(Δ), and α -naphthyl palmitate(\square). α -Naphthyl palmitate hydrolase was assayed in the presence of 0.1% Triton X-100. The arrow indicates a switch at pH 6 from acetate to barbitone buffer. At each pH controls were run containing α -naphthol and β -naphthol respectively, so that corrections could be made for pH variations in chromogenicity of the end product of the assays.

Table 1. Effect of various compounds on β -naphthyl laurate hydrolase and α -naphthyl acetate esterase activity in pooled microsomal rich fractions from density gradient centrifugation.

Compound	Incubation conc.	β -naphthyl laurate hydrolase	α -naphthyl acetate hydrolase
Calcium (CaCl_2)	10^{-2} M	32.9 ± 3.8 (8)†	73.5 ± 7.1 (7)
*Crude taurocholate (Na salt)	5.8 mg/ml	90.1 ± 5.9 (8)	33.1 ± 4.3 (7)
Calcium + taurocholate (crude)	10^{-2} M and 5.8 mg/ml, respectively	22.6 ± 5.7 (8)	57.7 ± 15.2 (7)
†Pure taurocholate (Na salt)	5.8 mg/ml	88.3 ± 0.1 (4)	86.9 ± 3.3 (4)
Calcium + taurocholate	10^{-2} M and 5.8 mg/ml, respectively	33.9 ± 2.2 (4)	72.6 ± 6.0 (4)
Eserine	10^{-3} M	57.9 ± 6.5 (7)	50.5 ± 2.3 (5)
Quinine (HCl)	10^{-3} M	51.3 ± 5.7 (6)	87.3 ± 2.6 (4)
<i>p</i> -Chloromercuribenzoate (PCMB)	2×10^{-3} M	68.0 ± 4.9 (6)	80.4 ± 13.1 (4)

Notes: Assays were performed as in *Materials and methods*, and the activity is expressed as a percentage of the control value (no addition to incubation medium) \pm standard error.

† number of observations.

* obtained from British Drug House Chemicals Ltd.

‡ obtained from Koch-Light Laboratories Ltd.

closely the distribution of various lysosome markers. Its lysosomal localization is strengthened by the fact that it is activated by Triton X-100 and by repeated freezing and thawing (Fig. 8). The influence of Triton is rather complex. An initial peak of activity is observed at a concentration of 0.1%. This presumably corresponds to the release of structural linked latency since the Triton concentration agrees with that generally found necessary to suppress the latency of lysosomal hydrolases. Higher concentrations of detergent appear to have an inhibitory effect, followed by a further activation at a concentration of about 2%, and a subsequent inhibition. The second peak of activity may be due to the detergent causing increased availability of the hydrophobic substrate.

α -Naphthyl palmitate hydrolase activity was usually assayed at pH 5.0 which is the optimum for many lysosomal enzymes. Fig. 7 shows that, in whole homogenate,

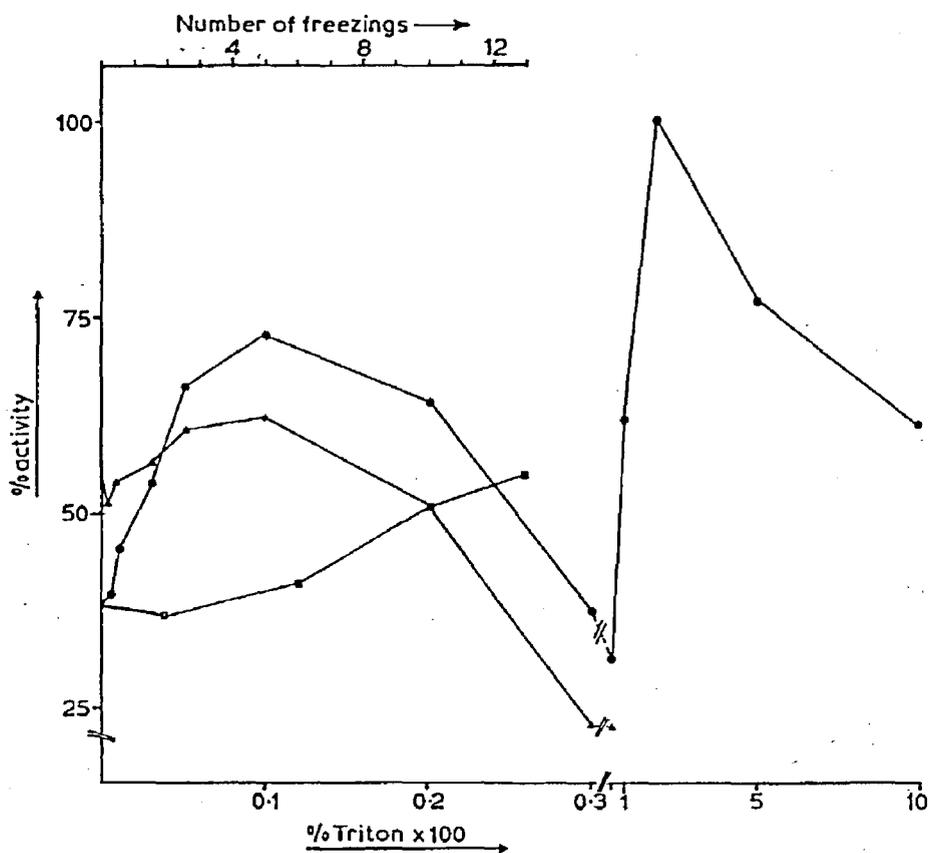


Figure 8. Influence of Triton X-100 and freezing and thawing on the activity of α -naphthyl palmitate hydrolase (pH 5) in whole homogenate. Incubations were reduced to 10 min in order to minimize the effect of incubation on the estimation of free activity. Activities are quoted with reference to the maximum observed (= 100%). ■, repeated freezing and thawing; ●, effect of Triton on unfrozen homogenate; ▲, effect of Triton on 13x frozen homogenate. The influence of Triton on chromogenicity of the diazo dye was checked and found to be negligible. (Aliquots of enzyme were mixed with Triton X-100 to give the concentrations indicated. After at least 5 min at room temperature, buffer substrate was added and incubation started. The addition of buffer substrate diluted the Triton/enzyme mixture fifteen fold.)

activity against α -naphthyl palmitate is greatest at pH 6.5. The subcellular distribution of α -naphthyl palmitate hydrolase (pH 6.5), although essentially lysosomal, appears to reflect more 'microsomal' activity than at pH 5. It is possible that the pH curve is skewed to the alkaline side by the presence of a microsomal enzyme with a pH optimum greater than 6.5.

In general our pH curves for naphthyl fatty acid ester hydrolases are similar to those observed by Mahadevan & Tappel (1968) for lipases. They found a lysosomal optimum pH of 4.2 and a microsomal and soluble phase optimum of 8.6 in rat liver and kidney. Stoffel & Greten (1967) reported a hepatic lysosomal lipase with a pH optimum of 6.5. These parallels of subcellular distribution and pH effect warrant the further investigation of naphtholic esters as substrates for intracellular lipases.

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