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SUBCELLULAR LOCALIZATION OF MONOGLYCERIDE ACYLTRANSFERASE, XANTHINE OXIDATION, NADP: ISOCITRATE DEHYDROGENASE AND ALKALINE PHOSPHATASE IN THE MUCOSA OF THE GUINEA-PIG SMALL INTESTINE

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Abstract—1. Rate dependent and isopycnic banding in a zonal rotor were used to analyse the subcellular sites of enzymes in homogenates of guinea-pig small intestinal mucosa.

2. The results demonstrate the following localizations: monoglyceride acyltransferase—microsomal; xanthine oxidase and dehydrogenase—soluble phase, and NADP: isocitrate dehydrogenase—soluble phase and mitochondrial.

3. Alkaline phosphatase is confined to brush borders and is absent from the basolateral plasma membrane. A variable proportion of the activity, up to 40%, is on brush borders which during homogenization break up into particles of reduced density and slow sedimentation rate.

INTRODUCTION

Microperoxisomes are assumed to be a special type of peroxisome and are apparently ubiquitous in mammalian tissues (Novikoff *et al.*, 1973; Hruban *et al.*, 1972). In contrast to the majority of the peroxisomes of the liver and kidney, microperoxisomes have no core, are almost invariably smaller (Novikoff *et al.*, 1973) and, in addition, have been much less extensively investigated.

It is possible that in some tissues enzymes originally reported as "microsomal" or "mitochondrial" are in reality partially or wholly microperoxisomal or peroxisomal. Recent studies of rat liver provide several instances of this including palmitoyl-CoA synthetase (Lippel *et al.*, 1970; Krisans & Lazarow, 1978), carnitine acetyltransferase (Norum & Bremer, 1967; Markwell *et al.*, 1977), dihydroxyacetone phosphate acyltransferase (La Belle & Hajra, 1972; Jones & Hajra, 1977), and those enzymes responsible for the β-oxidation of palmitoyl-CoA (Lazarow, 1977).

Most previously reported fractionation studies of the mammalian intestine have ignored the microperoxisomes. Conscious of possible erroneous localizations resulting from this and also of the need to identify peroxisomal enzymes in tissues additional to mammalian liver, we have determined the localization of possible microperoxisomal enzymes in the mucosa of both the guinea-pig small intestine (Temple *et al.*, 1976; Martin *et al.*, 1979) and the goldfish intestine (Temple *et al.*, 1979). In the goldfish intestine 10–20% of the carnitine acetyltransferase is microperoxisomal but the other enzymes studied (including xanthine oxidase, xanthine dehydrogenase and NADP linked isocitrate dehydrogenase) are apparently absent from these organelles. We now report the localization of the latter three enzymes in the guinea-pig small intestinal mucosa. We also studied monoglyceride acyltransferase. None of these enzymes were found to have a microperoxisomal component.

MATERIALS AND METHODS

Chemicals and enzymes

These were mostly bought or prepared as described previously (Martin *et al.*, 1979). [9,10-^{3}H]palmityl-(−)-carnitine was prepared using [9,10-^{3}H]palmitic acid. Before use it was diluted with palmityl-(−)-carnitine, similarly prepared (Sanchez *et al.*, 1973). Alumina and DL-lactic acid were purchased from BDH Chemicals Ltd, Poole, Dorset, U.K. Cytochrome *c* and NADP were from Bohringer, (BCL), Bell Lane, Lewes, East Sussex, U.K. Antimycin A, DL-isocitric acid (sodium salt, type I), homovanillic acid, 1-monopalmitylglycerol, *p*-nitrophenyl phosphate (ditris salt), peroxidase (type II from horseradish) and xanthine were from Sigma Chemical Co., Ltd. Glycollic acid was purchased from Hopkin and Williams, P.O. Box 1, Romford, Essex, U.K.

Preparation of homogenate and subcellular fractionation

The procedures employed were as described previously (Temple *et al.*, 1976). Subcellular fractions were brought to the same sucrose concentration before analysis.

Biochemical analyses

The following were assayed as described previously: alkaline phosphatase and acid phosphatase (substrate for both, 1-naphthyl phosphate) (Connock *et al.*, 1974), glucose-6-phosphatase (Connock *et al.*, 1972), 1-naphthyl acetate esterase (non-specific esterase) and succinate dehydrogenase (Temple *et al.*, 1976), NADP: isocitrate dehydrogenase (with 6.6 mM isocitrate and 0.071% w/v Triton X-100) and NAD: xanthine dehydrogenase (with 0.26 mM xanthine and no Triton X-100) (Temple *et al.*, 1979). Other assays were as follows: K^+ (potassium stimulated) *p*-nitrophenyl phosphatase (Garrahan *et al.*, 1969; Murer *et al.*, 1976), catalase (Baudhuin *et al.*, 1964), NADH cytochrome *c* reductase (enzyme sample preincubated 5 min at 0°C with 32 μg/ml antimycin A where indicated) (Donaldson *et al.*, 1972), protein (Lowry *et al.*, 1951) and DNA (Burton, 1956). L-α-hydroxyacid oxidase (12.5 and 52 mM DL-lactate, and 5.5 and 24 mM glycollate as substrates) and xanthine oxidase (with 0.74 mM xanthine) were assayed by methods dependent on measuring the production of hydrogen peroxide (Guilbault *et al.*, 1968; Gaunt &

De Duve, 1976). Incubations were for 60 min and allowances were made for the production of hydrogen peroxide independent of exogenous substrate. Xanthine oxidase only was detectable. Monoglyceride acyltransferase was assayed essentially according to Short *et al.* (1974). The incubation was for 6 to 10 min in a medium which included 2 mM 1-monopalmitylglycerol, 70 μM CoA, 100 μM [9,10-^{3}H]palmityl-(−)-carnitine (0.11 μCi/μmol) and up to 60 μg sample protein. To reduce quenching due to chloroform the final column eluate was evaporated to dryness and then redissolved in a small volume of 5% w/v Triton X-100. An aliquot was measured by liquid scintillation counting in 10 ml of Triton X-100: xylene scintillant (Martin *et al.*, 1979). Incubations for all enzyme assays were at 37°C except NADH cytochrome *c* reductase (20°C) and catalase (0°C).

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 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 ordinate for density is expressed as g/ml. The abscissae are divided according to the percentage volume of the fractions.

RESULTS

Figure 1 depicts the distribution of enzymes after isopycnic fractionation of homogenate in a sucrose gradient contained in a BXIV zonal rotor. Markers used were catalase for microperoxisomes, succinate dehydrogenase for mitochondria, alkaline phosphatase for brush borders, acid phosphatase for lysosomes, 1-naphthyl acetate esterase, NADH cytochrome *c* reductase and glucose 6-phosphatase for microsomes (ER component), DNA for nuclei and K^+, *p*-nitrophenyl phosphatase for basolateral plasma membranes of enterocytes. Microperoxisomes have equilibrated in region "d" at a lower density than the nuclei (region "e") but higher than the mitochondria (region "c"). The microsomes span regions "c" and "d" and the more dense part of region "b". The distribution of monoglyceride acyltransferase resembles that of the microsomal markers. Xanthine oxidase and dehydrogenase and most of the isocitrate dehydrogenase have remained in the sample layer indicating that they are soluble phase. Some of the isocitrate dehydrogenase is distributed similarly to the mitochondrial marker.

Brief centrifugation of homogenate into a shallow sucrose gradient (Fig. 2) gave a separation of organelles dependent on their rate of sedimentation. NADH cytochrome *c* reductase revealed some mitochondrial activity (discontinuous line) which was mostly inhibited by preincubation of each sample with antimycin A (continuous line). The distributions of monoglyceride acyltransferase and of isocitrate dehydrogenase confirm that the former is microsomal and the latter mainly soluble phase and partially mitochondrial.

The plasma membrane marker used here was K^+ stimulated *p*-nitrophenyl phosphatase. This enzyme carries out the second part of the Na^+-K^+ stimulated

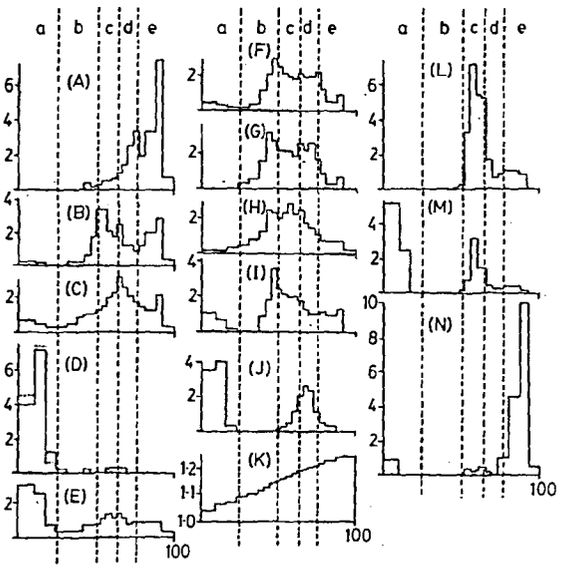

Fig. 1. Distribution of markers and enzymes after isopycnic banding. 23 ml of homogenate was loaded onto 450 ml of a 15–50% (w/w) linear sucrose gradient. Centrifugation was for 13.5 hr at 25,000 rpm in a BXIV aluminium zonal rotor. Twenty fractions were unloaded and analysed for the following: (A) alkaline phosphatase (94%); (B) K^+ *p*-nitrophenyl phosphatase (79%); (C) acid phosphatase (98%); (D) xanthine oxidase (continuous line, 120%) and xanthine dehydrogenase (discontinuous line, 168%); (E) protein (115%); (F) 1-naphthyl acetate esterase (85%); (G) glucose-6-phosphatase (76%); (H) NADH cytochrome *c* reductase (102%); (I) monoglyceride acyltransferase (102%); (J) catalase (128%); (K) density; (L) succinate dehydrogenase (95%); (M) isocitrate dehydrogenase (98%); (N) DNA (138%). Vertical columns labelled "a" to "e" represent regions of the gradient discussed in the text. Percentage figures represent the recovery of activity from the rotor relative to that loaded.

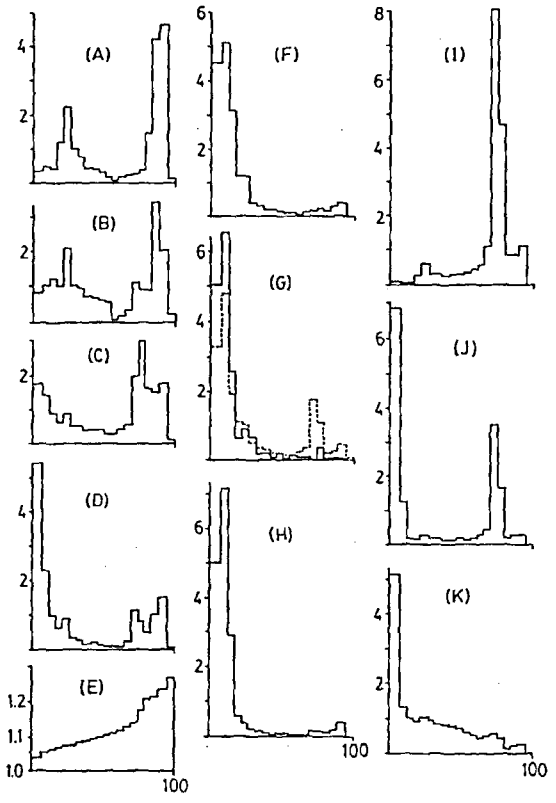

Fig. 2. Distribution of markers and enzymes after rate-dependent banding. 15 ml of homogenate was loaded onto 350 ml of 13–30% (w/w) linear sucrose gradient resting on 100 ml of 40–50% (w/w) linear sucrose gradient. Centrifugation was for 45 min at 16,000 rpm in an aluminium BXIV zonal rotor. Twenty fractions were unloaded and analysed for the following: (A) alkaline phosphatase (85%); (B) K^+ *p*-nitrophenyl phosphatase (108%); (C) acid phosphatase (95%); (D) protein (97%); (E) density; (F) 1-naphthyl acetate esterase (60%); (G) NADH cytochrome *c* reductase. Analyses included (continuous line, 107%) or excluded (discontinuous line, 90%) antimycin A. (H) monoglyceride acyltransferase (64%); (I) succinate dehydrogenase (90%); (J) isocitrate dehydrogenase (101%); (K) catalase (111%).

ATPase reaction (Garrahan *et al.*, 1969). A reason for assaying K^+ *p*-nitrophenyl phosphatase rather than Na^+-K^+-ATPase is that *p*-nitrophenyl phosphate is more resistant than ATP to hydrolysis by non-specific phosphatases.

In both experiments (Figs 1 and 2) K^+ *p*-nitrophenyl phosphatase revealed a complex distribution suggesting a large contribution from the subcellular fragments containing the brush borders plus another carrier of the enzyme. The latter migrates slowly causing it to be spread across the long, shallow gradient in Fig. 2 but equilibrates at a density of 1.155 g/ml (junction of regions "b" and "c", Fig. 1). This carrier is therefore a light membrane, presumably basolateral plasma membranes unattached to brush borders. The possibility of a minor lysosomal component cannot be discounted.

After rate-dependent banding a substantial part of the alkaline phosphatase resembled the slowest moving peak of K^+ *p*-nitrophenyl phosphatase (Fig. 2). Conceivably, therefore, basolateral plasma membranes carry some alkaline phosphatase activity. This possibility was investigated in a further experiment (Fig. 3).

Rate dependent banding was carried out essentially as in Fig. 2 and a similar distribution of markers, including K^+ *p*-nitrophenyl phosphatase, was obtained (selected distributions shown in Fig. 3, column a). The distributions of xanthine oxidase and dehydrogenase confirmed that both have a soluble phase localization. Two new samples were then prepared by pooling aliquots of several fractions. Both samples were subjected to isopycnic centrifugation (Fig. 3, columns b and c). Each gradient partially resolved K^+ *p*-nitrophenyl phosphatase and alkaline phosphatase into separate peaks with median densities of 1.155 and 1.183 (sample i, Fig. 3b) or 1.173 and 1.195 g/ml, respectively (sample ii, Fig. 3c). This indicates that the slow moving carriers of both enzymes have separate identities whose range of densities overlap to only a minor degree. The distributions of catalase and alkaline phosphatase are largely coincident in these isopycnic gradients. However, the fact that these activities are in separate compartments is indicated by the distributions shown in Fig. 1 and in the gradient from which the samples were obtained (column a, Fig. 3).

DISCUSSION

The localization of xanthine oxidase and dehydrogenase in the guinea-pig small intestinal mucosa indi-

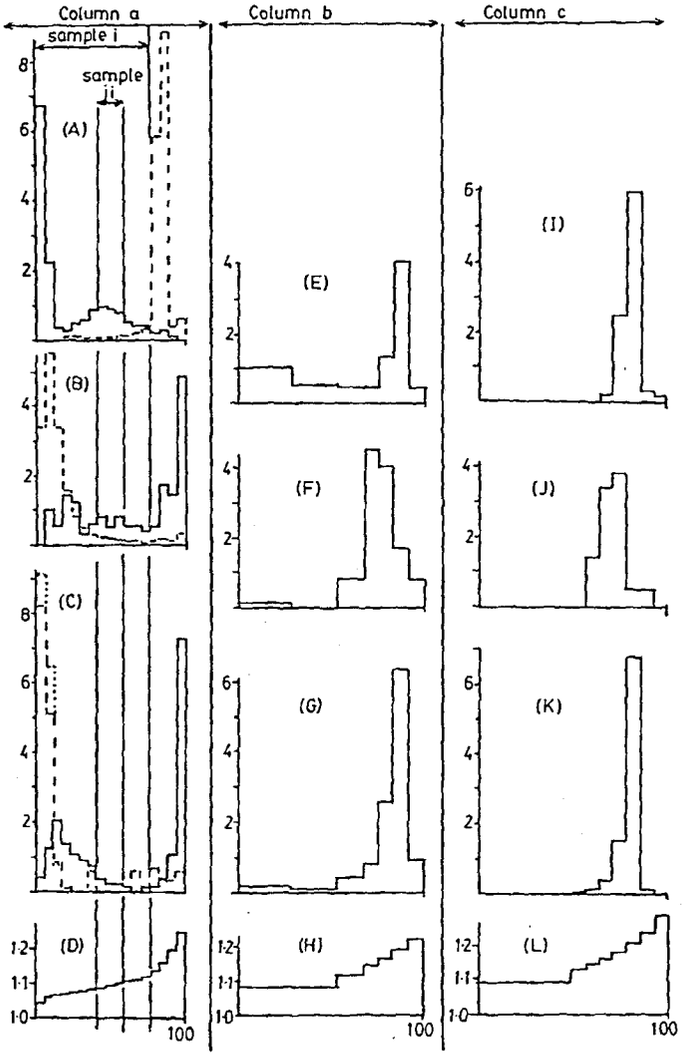

Fig. 3. A rate dependent banding experiment was carried out as described for Fig. 2. Some of the enzyme distributions are shown in column a. Aliquots of equal volume of fractions 1 to 13 (sample i indicated in column a) were combined and 10 ml was loaded on 10 ml of 20% w/w sucrose which rested on 13 ml of a 25 to 50% (w/w) sucrose gradient on a cushion of 3 ml of 55% (w/w) sucrose. The tube was centrifuged at 25,000 rpm for 3 h in a 6 × 38 S.O. (M.S.E.) rotor; the resulting enzyme distributions are shown in column b. In addition aliquots of fractions 8, 9 and 10 (sample ii indicated in column a) were pooled. 20 ml was loaded on a 13 ml gradient and centrifuged as for sample i; the resulting distributions are in column c. (A) Catalase (63%) and succinate dehydrogenase (dashed, 120%). (B) K^+ *p*-nitrophenyl phosphatase (92%) and 1-naphthyl acetate esterase (dashed, 88%). (C) Alkaline phosphatase (61%), xanthine oxidase (dashed, 89%) and xanthine dehydrogenase (dotted, 80%). (D) Density. (E) and (I) catalase (106 and 81%). (F) and (J) K^+ *p*-nitrophenyl phosphatase (67 and 94%). (G) and (K) alkaline phosphatase (67 and 100%). (H) and (L) density.

cates that all conversion of hypoxanthine to uric acid takes place in the soluble phase. In the goldfish intestine xanthine dehydrogenase is entirely soluble phase while the oxidase was undetectable (Temple *et al.*, 1979). The oxidase of the mammalian liver is an exclusively soluble activity (De Duve & Baudhuin, 1966). The dehydrogenase, though, is partially peroxisomal in the liver and kidney of chicken (Scott *et al.*, 1969) but is wholly soluble in those organs of rat and pig (Tolbert, 1973).

L-α-hydroxyacid oxidase was undetectable; this was also the case in the goldfish intestine (Temple *et al.*, 1979). This enzyme is widely found in reno-hepatic peroxisomes (Masters & Holmes, 1977).

NADP linked isocitrate dehydrogenase is mainly (about two thirds) soluble phase with the remainder in the mitochondria. This localization resembles that in the goldfish intestine (Temple *et al.*, 1979). An additional contribution from the peroxisomes has been detected in Mytilus digestive gland (our unpublished

observations) and in the livers of rat (Leighton *et al.*, 1968) and carp (Goldenberg *et al.*, 1978).

Glyceride synthesis in the intestinal mucosa can proceed via monoglyceride, sn-glycerol-3-phosphate or dihydroxyacetone phosphate (Brindley, 1974). In liver the latter pathway is initiated in peroxisomes and that via sn-glycerol-3-phosphate is mitochondrial and microsomal (Bates & Saggerson, 1979; Jones & Hajra, 1977). The data reported here indicates that the intestinal monoglyceride pathway is microsomal (probably ER) and has no microperoxisomal component. This is in agreement with other fractionation work on the mammalian intestine (Brindley, 1974; Hulsmann & Kurpershoek-Davidov, 1976). Similarly, a histochemical study of the rat intestinal enterocyte localized it mainly in smooth ER (Higgins & Barrnett, 1971).

K^+ *p*-nitrophenyl phosphatase had a complex distribution suggesting at least two carriers. One is a slow moving membrane (density, 1.155 g/ml). The second sediments with alkaline phosphatase suggesting a brush border localization. However, Lewis *et al.* (1975) concluded that the Na^+-K^+ stimulated ATPase in the brush border-rich fractions from guinea-pig enterocytes is due to "tags" of basolateral plasma membrane attached to brush borders. This must also apply to K^+ *p*-nitrophenyl phosphatase since that enzyme carries out the second part of the ATPase mediated reaction (Garrahan *et al.*, 1969). It is possible that lysosomes also carry a part of the K^+ *p*-nitrophenyl phosphatase. If so, this would not be altogether surprising as phagosomes derived from the plasma membrane fuse with primary lysosomes to form secondary lysosomes. Furthermore, Wattiaux *et al.* (1978) observed that highly purified rat liver lysosomes contain significant amounts of enzymes which are otherwise confined to the plasma membrane.

With respect to alkaline phosphatase the experiments described here combined with our earlier observations point to the following conclusions. After isopycnic banding the main peak equilibrated at a density of 1.24 g/ml but there was usually significant activity at a density of 1.18 to 1.21. This was sometimes resolved into a second, smaller peak. In about half of rate-dependent banding experiments a substantial minority of the alkaline phosphatase activity (up to 40%) has a slow rate of migration overlapping with that observed for the microperoxisomes. This activity is largely independent of the basolateral plasma membrane as shown by the differences between its distribution and that of K^+ *p*-nitrophenyl phosphatase after isopycnic banding (Fig. 3, columns b and c). This fact and the wide variation between experiments in both the relative size and rate of migration of the slow moving alkaline phosphatase peak suggests that it probably represents fragments of brush border which are heterogeneous in their size and their ratio of lipid to protein content. This is probably the origin of the low density peak of alkaline phosphatase activity observed in isopycnic banding experiments. In agreement with Lewis *et al.* (1975) and others we conclude that the basolateral plasma membrane of the guinea-pig enterocyte lacks alkaline phosphatase activity.

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