Analytical subcellular fractionation studies on enterocytes from the jejunum and ileum of the rat and some properties of brush-border alkaline phosphatase

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Summary

1. Enterocytes, isolated from the proximal jejunum and distal ileum of the rat, were homogenized and their organelles separated by isopycnic centrifugation on continuous sucrose density gradients. The distributions of marker enzymes for the principal organelles, RNA and protein were determined in the sucrose gradients and related to the activities per enterocyte.

2. In the jejunum the modal equilibrium densities of the various organelles were: brush borders (1.20), lysosomes (1.20), peroxisomes (1.19), mitochondria (1.17) and basal-lateral membranes (1.13). These values were not significantly different in the ileum. The activities of brush-border enzymes, soluble and mitochondrial malate dehydrogenase, soluble and membrane-associated lactate dehydrogenase and particulate protein content, however, were greater in the jejunal than the ileal enterocytes.

3. Detergent exposed latent alkaline phosphatase activity in jejunal enterocytes and indicated that this enzyme is present not only in the brush border but also in the basal-lateral membrane and soluble fractions of the cell.

4. Isolated jejunal brush-border preparations showed latent activities of both alkaline phosphatase and γ-glutamyltransferase whereas the activities of α-glucosidase and leucyl-β-naphthylamidase were not affected by detergent. Mechanical disruption of these preparations suggested the presence of two forms of alkaline phosphatase in the brush border and provides a technique to assess membrane fragility.

Key words: alkaline phosphatase, brush border, enterocyte, ileum, intestine, jejunum, subcellular fractionation.

Introduction

The enterocytes present a barrier through which ingested nutrients must pass to gain access to the intercellular space and hence the blood. These cells, however, are specialized to facilitate this passage, the organelles having specific functions some of which are associated with the terminal processing of nutrients and their transference across this barrier. In the present study the properties and enzyme content of some of these organelles have been compared in enterocytes from the proximal jejunum and distal ileum, by use of an analytical subcellular fractionation technique, and related to the functional importance of these cells.

Alkaline phosphatase is extremely active in the small intestine and yet no specific function has been definitively assigned to this enzyme. The subcellular localization of alkaline phosphatase in the jejunal enterocyte has been investigated and some properties of the brush-border component of this and other enzymes have been examined.
Methods

Enterocyte preparation and analytical subcellular fractionation

Adult male Wistar rats, 250–300 g in weight, were fed on a normal diet no. 41B (Oxoid Ltd, London). Enterocytes were isolated from 30 cm segments of proximal jejunum and distal ileum and a postnuclear supernatant fraction was prepared by differential centrifugation (Batt & Peters, 1976a).

Analytical subcellular fractionation was performed in the Beaufay automatic zonal rotor (Beaufay, 1966) as described previously (Peters, 1976). A portion (5 ml) of postnuclear supernatant fraction was layered on to 30 ml sucrose density gradient extending linearly with respect to volume from a density of 1-05 to one of 1-28 and resting on a 4 ml cushion of density 1-32. After centrifugation alkaline phosphatase was assayed fresh and the remainder of the gradient then stored at −20°C. Results are expressed in the form of frequency–density histograms, the pooling and averaging of distributions being performed by computer (Leighton, Poole, Beaufay, Baudhuin, Coffey, Fowler & de Duve, 1968).

Preparation of brush borders

Brush borders were prepared from isolated jejunal enterocytes. The enterocytes were prepared as described previously (Batt & Peters, 1976b) up to and including the second wash with solution 2. The pellet was then resuspended in 20 ml of a solution containing Tris (5 mmol/l) and EDTA (5 mmol/l), pH 7-4 (Tris/EDTA solution) (Milton, Critchley & Tovell, 1966) and disrupted in a medium-sized Dounce homogenizer (Kontes Glass Co., Vineland, New Jersey, U.S.A.) with 10 strokes of a loose-fitting pestle (type A) and then 20 strokes of a tight-fitting pestle (type B). The homogenate was centrifuged at 800 g for 10 min at 4°C in an MSE 4L centrifuge (Measuring and Scientific Equipment Ltd, Crawley, Sussex, U.K.) and the pellet washed three times by resuspension in 20 ml of Tris/EDTA solution with three strokes of the type A pestle and recentrifugation. The final pellet was then washed with 20 ml and resuspended in 10 ml of sorbitol (Koch–Light Laboratories Ltd) (0-3 mol/l) containing EDTA (1 mmol/l), pH 7-4, and ethanol (22 mmol/l) (sorbitol VE medium).

Latent brush-border enzyme activity

Freshly prepared jejunal brush borders in sorbitol VE medium were used to determine the percentage latent brush-border enzyme activities. The enzyme was assayed with the appropriate buffered substrate in sorbitol VE medium with (total activity) and without (free activity) Triton X-100 (100 mg/100 ml).

Latent brush-border enzyme (%) = total activity − free activity total activity × 100

Alkaline phosphatase activity in fresh brush-border preparations was also determined with the appropriate buffered substrate in sorbitol VE medium containing digitonin (Koch–Light Laboratories Ltd, Colnbrook, Bucks., U.K.) at concentrations between 0-015 and 0-3 mmol/l or Triton X-100 (100 mg/100 ml). Apparent activity was expressed as a percentage of total activity, assayed in the presence of Triton.

Brush-border membrane mechanical fragility

A sample (8 ml) of freshly prepared brush borders in sorbitol VE medium was placed in a medium-sized Dounce homogenizer, cooled in ice, and disrupted with 200 strokes of a tight-fitting type B pestle. At intervals during homogenization a sample (0-2 ml) was taken, diluted to 1 ml with sorbitol VE medium and both total and free alkaline phosphatase activities were determined as described above. In order to demonstrate the differential effects of homogenization on total and free activities apparent activity was expressed as a percentage of initial total activity.

The proportion of membrane-bound total brush-border enzyme activity was determined by taking a sample of the brush-border preparation before and after homogenization with 200 strokes of the type B pestle. This was centrifuged at 100 000 g for 60 min at 4°C with an 8 × 10 ml angle-head rotor in an MSE Superspeed 40 ultracentrifuge. The supernatant was decanted and stored on ice. Sorbitol VE medium (2 ml) was added to the pellet, which was gently resuspended with five strokes of the type B pestle. These samples were stored at −20°C before assay of brush-border enzymes, the appropriate buffered substrate in the presence of Triton X-100 (100 mg/100 ml) being used.

Sedimentable brush-border enzyme (%) = activity in pellet activity in pellet + supernatant × 100

Recovered brush-border enzyme (%) = activity in pellet + supernatant activity in brush-border sample × 100
Analytical techniques

Marker enzymes for the principal subcellular organelles were assayed as described previously (Batt & Peters, 1976a). Alkaline phosphatase was assayed with 4-methylumbelliferyl phosphate (0.15 mmol/l) in diethanolamine/HCl buffer (0.1 mol/l), pH 9.2, containing MgCl₂ (50 mmol/l). γ-Glutamyltransferase was assayed with γ-glutamyl naphthylamide (0.15 mmol/l) in ammediol (2-amino-2-methylpropan-1,3-diol) HCl (0.05 mol/l), pH 8.5, containing glycylglycine (14.3 mmol/l). RNA was assayed by the method of Prasad, Mouchell, Koniuch & Oberleas (1972) with calf-liver RNA (Sigma type IV) as a standard. Protein was assayed by a modification (Peters, Batt, Heath & Tilleray, 1976) of the method of Hiraoka & Glick (1963) with bovine serum albumin (Armour Pharmaceutical Co., Chicago, U.S.A.) as a standard.

Results

Analytical subcellular fractionation

Fig. 1 compares the relative activities and density gradient distributions of the principal marker enzymes, RNA and protein in extracts of jejunal and ileal enterocytes.

In the jejunum, α-glucosidase activity has a small soluble component and a particulate component with a modal density of 1.20. This distribution is almost identical with those of leucyl-β-naphthylamidase and γ-glutamyltransferase (not shown). The soluble component of α-glucosidase activity is similar in the ileal to that of the jejunal enterocytes. The particulate component, however, is selectively diminished in the ileum, but with a similar density distribution to that of the jejunal cells. Leucyl-β-naphthylamidase and γ-glutamyltransferase activities in the ileum (not shown) have similar distributions to that of α-glucosidase but with relatively less soluble enzyme.

5'-Nucleotidase in the jejunum has a completely different distribution pattern from that of α-glucosidase, most of the enzyme activity being particulate at the less-dense region of the gradient with a modal density of 1.17. There is, in addition, a shoulder of activity which has moved deeper into the gradient. In the ileum this enzyme has a similar distribution with enhanced activities throughout the gradient, the particulate component having a peak at density 1.12 and a definite shoulder at density 1.19.

Malate dehydrogenase has a distinct distribution in the jejunum with a particulate component of modal density 1.17, separated clearly from those of α-glucosidase and 5'-nucleotidase, and a smaller soluble component. Both components, especially the particulate enzyme activity, are reduced in the ileal enterocytes. Lactate dehydrogenase activity, in contrast, appears to be mainly soluble with markedly less enzyme in the ileum than in the jejunum. RNA is similarly found almost exclusively in the soluble fractions with very little entering the gradient in either the jejunum or ileum.

There is very little soluble N-acetyl-β-glucosaminidase activity in the jejunum or ileum, with the enzyme occurring as a particulate component of modal density 1.20. Catalase has a negligible soluble component in the jejunum and a distinct particulate component of modal density 1.19. In the ileal enterocytes there is an increase in soluble catalase activity but a reduction in the particulate component, the latter having a similar density distribution to that of the jejunum. Protein is mainly recovered in the soluble fractions with a distinct particulate component of modal density 1.17 corresponding to mitochondria in both jejunum and ileum. Although the amount of soluble protein is similar in enterocytes from both regions, there is much less particulate protein in the ileal cells.

Alkaline phosphatase localization

In order to differentiate between components of alkaline phosphatase activity, which appears to have more than one subcellular localization, experiments were performed in which the enzyme was assayed both in the presence and absence of detergent (Triton X-100, 100 mg/100 ml). The results of such an experiment with jejunal enterocytes are presented in Fig. 2. Alkaline phosphatase activity assayed in the presence of Triton X-100 has a large soluble component and a broad distribution throughout the gradient with a peak of modal density 1.10 and a shoulder at density 1.19. When detergent is omitted from the assay the distribution is markedly different with a major particulate component of modal density 1.19 similar to that of α-glucosidase. There is, in addition, a shoulder of activity corresponding approximately with the peak of 5'-nucleotidase activity, and a small soluble component.

When the Triton-activated component of alkaline phosphatase is calculated the distribution is almost identical with that of lactate dehydro-
Fig. 1. Isopycnic centrifugation of postnuclear supernatant fraction from jejunal (——) and ileal (-----) enterocytes. Graphs, of averaged data from between two and four experiments, show the relative frequency–density distributions of six marker enzymes, RNA and protein. Frequency is defined as that portion of the total recovered activity present in an individual fraction divided by the density span covered by that fraction. Relative frequency was derived by multiplying the frequency data from ileal enterocytes by the relative specific activity (munits/mg of DNA) of the ileal compared with the jejunal cells. Maximum relative frequency for ileal RNA was 16. The percentage recoveries for the jejunum and ileum respectively, with the relative specific activities in parentheses, are: α-glucosidase 101, 76 (0.41); 5'-nucleotidase 91, 109 (2.1); malate dehydrogenase 88, 78 (0.33); lactate dehydrogenase 107, 95 (0.31); RNA 76, 76 (1.0); N-acetyl-β-glucosaminidase 80, 71 (1.1); catalase 87, 74 (0.72); protein 74, 87 (0.73).

Latent brush-border enzyme activity

There was a large latent component of alkaline phosphatase activity (75%) in jejunal brush-border preparations. γ-Glutamyltransferase activity was also found to be latent, but to a lesser extent (27%), whereas both α-glucosidase and leucyl-β-naphthylamidase had negligible latent components (7%; 0%).

Fig. 3 demonstrates the effect of digitonin on alkaline phosphatase activity in jejunal brush-border preparations. With increasing concentrations of detergent there was a progressive enhancement of activity, a plateau being reached at
Subcellular fractionation of enterocytes

Alkaline phosphatase (+ Triton)

Alkaline phosphatase (no Triton)

Alkaline phosphatase (Triton-activated)

5'-Nucleotidase

a-Glucosidase

Lactate dehydrogenase

FIG. 2. Isopycnic centrifugation of postnuclear supernatant fraction from jejunal enterocytes. Graphs, from a representative experiment, show the frequency-density distributions of alkaline phosphatase, assayed with and without Triton, and three marker enzymes. Frequency is defined as that portion of the total recovered activity present in an individual fraction divided by the density span covered by that fraction. The percentage recoveries are: alkaline phosphatase, with Triton, 49; without Triton, 58; Triton-activated, 42; 5'-nucleotidase, 85; a-glucosidase, 65; lactate dehydrogenase, 46.

Brush-border membrane mechanical fragility

The effect of mechanical stress on both total and free alkaline phosphatase activities is shown in Fig. 4. Homogenization of the jejunal brush-border preparations resulted in a progressive decrease in total enzyme activity whereas the free activity remained unaltered. Table 1 shows the percentage sedimentable (100 000 g × 60 min) and recovered activities of four enzymes in the brush-border preparations before and after the homogenization with 200 strokes of the type B pestle. These results indicate that virtually all the enzyme activity is less than 100% as digitonin, in contrast to Triton X-100, appears to be inhibitory at the higher concentrations.

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associated with membrane both before and after this treatment.

Discussion

In this study analytical subcellular fractionation has been used to compare directly the properties and enzyme content of the individual organelles in enterocytes from the jejunum and ileum of the rat. Some properties of the brush-border component of alkaline phosphatase have been investigated and used to develop a technique to study brush-border membrane fragility.

Comparison between the organelles of jejunum and ileum

In the sucrose density gradients, extracts from jejunal enterocytes showed similar density distributions for α-glucosidase, leucyl-β-naphthylamidase and γ-glutamyltransferase, enzymes which have been localized to the brush border (Holt & Miller, 1962; Lewis, Elkin, Michell & Coleman, 1975; Peters, 1976; Peters & Shio, 1976). These enzymes appear to have much smaller extra-brush-border components in the rat than in the human jejunum (Peters, 1976), a finding which may be related to the use of whole mucosal homogenates in the latter study. The ileal enterocytes had a much lower activity of brush-border α-glucosidase than the jejunal cells. The equilibrium densities of the brush borders, however, were virtually identical in the jejunum and ileum (1.20), indicating that the components responsible for the greater transport capacity (Batt & Peters, 1976b) and brush-border enzyme activities in the jejunal enterocytes do not markedly affect brush-border density.

The distribution of 5′-nucleotidase in gradients of jejunal enterocytes suggests a predominantly basal–lateral membrane localization, consistent with previous findings in the rat (Douglas, Kerley & Isselbacher, 1972; Peters & Shio, 1976) and man (Peters, 1976). The shoulder of activity further in the gradient approximates to the distribution of the brush-border enzymes. A similar finding in man (Peters, 1976) is thought to indicate that 5′-nucleotidase in an intrinsic brush-border enzyme. This component is more prominent in the ileal enterocytes and the basal–lateral membranes are slightly less dense than those of the jejunal cells.

Malate dehydrogenase activity was found to be three times higher in jejunal than ileal enterocytes due to a greater activity of both cytoplasmic and mitochondrial components of this enzyme. This finding is consistent with a greater potential for the production of energy-rich compounds for biosynthetic reactions and active transport in the jejunal cells.

The distributions of lactate dehydrogenase and RNA in both the jejunum and ileum were found to be similar and to be almost exclusively localized to the soluble fractions. In the jejunum, however, there is more soluble lactate dehydrogenase activity than in the ileum and a significant distribution through the gradient, the latter probably representing binding of enzyme to membranes, particularly the endoplasmic reticulum (de Duve, Wattiaux & Baudhuin, 1962; Peters, 1975). Previous workers
(Porteus & Clark, 1965; Peters, 1970) have found significant amounts of sedimentable RNA. In these studies, however, differential pelleting was used rather than density-gradient centrifugation, the latter technique minimizing artifactual adsorption of organelles to each other.

The equilibrium density of the lysosomes assessed by the distribution of N-acetyl-β-glucosaminidase was identical in the jejunum and ileum with a modal density of 1·20. As most of the other enzymes studied show a marked difference in activity between the jejunal and ileal enterocytes, it is of interest that lysosomal enzymes should be relatively active in ileal cells. The reason for this is not known, although lysosomes have been specifically implicated in the absorption of dietary folate (Hoffbrand & Peters, 1969), vitamin B₁₂, intrinsic factor complex (Strauss & Wilson, 1960) and other macromolecules (Clarke & Hardy, 1969; Walker & Isselbacher, 1974) and are believed to play a crinophagic role in the transport and secretion of cell-coat material (Ginsel, Daems, Emeis, Vio & van Gemund, 1973). The modal density of catalase activity, a peroxisomal marker enzyme, was also identical in the jejenum and ileum with a value of 1·19.

The jejunal enterocytes consist of much more particulate protein than the ileal enterocytes, a finding which reflects the principal differences between these cells. These are the greater activities of enzymes associated with both the brush borders and the mitochondria in the jejunal cells consistent with the relative importance of the proximal small intestine in the handling of the dietary constituents.

**Alkaline phosphatase localization**

The subcellular localization of alkaline phosphatase activity appears to be more complex than previously described. Alkaline phosphatase is thought, by preparative subcellular fractionation procedures, to be predominantly localized to the brush-border membrane (Forstner, Sabesin & Isselbacher, 1968; Douglas et al., 1972; Fujita, Ohta, Kawai, Matsui & Nakao, 1972). In the present analytical study, in the presence of detergent alkaline phosphatase was found to have a large soluble component and a spread of particulate activity through the gradient. By assaying the enzyme both in the presence and absence of detergent it is apparent that there are three main components to the enzyme activity. In the absence of Triton X-100 there is a large brush-border and a small basal-lateral-membrane component. Triton X-100 apparently activates some brush-border activity but largely basal-lateral membrane and soluble enzyme activities, suggesting that these components of alkaline phosphatase are within a hydrophobic environment, probably surrounded by lipid and unavailable to the synthetic substrate used in the assay unless detergent is added. Differential centrifugation experiments (R. M. Batt & T. J. Peters, unpublished results) have confirmed that a greater proportion of alkaline phosphatase activity in the soluble fraction is latent, suggesting that this enzyme, whether in membrane or not, is surrounded by lipid. In the previously reported studies this enzyme has been assayed without detergent so that the Triton-activated components would not be detected, probably explaining the apparent predominantly brush-border localization.

In the present study alkaline phosphatase has been shown to be present in the enterocyte not only in the brush-border but also in the basal-lateral membranes and the soluble fractions of the cell; however, it remains to be determined whether these reflect different isoenzymes. The presence of more than one isoenzyme of alkaline phosphatase has been demonstrated previously in the small intestine of both the mouse and the rat (Moog & Grey, 1967; Saini & Done, 1972); however, there is no indication of subcellular localization. In the present study the experiments on membrane fragility indicate that there may be two components of this enzyme associated with the brush border alone.

**Brush-border membrane fragility**

The results for percentage of latent enzyme in the brush-border preparations demonstrate the accessibility of the active sites of these enzymes to their respective substrates. The demonstration of a large latent component to alkaline phosphatase activity indicates that in the brush border the active site of a large proportion of this enzyme is not exposed at the membrane surface. This was confirmed by finding a progressive enhancement of enzyme activity with increasing digitonin concentration.

These and previous studies are consistent with brush-border alkaline phosphatase being an intrinsic membrane protein. Disaccharidases are removed from the microvillus membrane much more readily than alkaline phosphatase with either papain or detergent (Eichholz, 1968; Critchley, Howell & Eichholz, 1975; Louvard, Maroux, Vannier & Desnuelle, 1975). Thus carbohydase activity, as reflected by α-glucosidase in the present
study, appears to be exposed at the membrane surface and readily released, whereas both alkaline phosphatase and γ-glutamyltransferase appear to have buried components suitably situated to play a possible role in carrier-mediated transport.

The experiments on the effect of mechanical stress on alkaline phosphatase activity in brush-border preparations provide additional information on the nature of this enzyme. The free activity (assayed in the absence of Triton X-100) was unaffected by homogenization, whereas there was a selective and progressive decrease in total (assayed in the presence of Triton X-100) activity. This suggests that there are two distinct components to brush-border alkaline phosphatase activity that have quite different properties and may represent two distinct enzymes. One component, represented by the free activity, has the active site at the membrane surface in a hydrophilic environment, whereas the other component, represented by the latent activity, has the active site buried in the membrane. There is a loss of this latent component with homogenization, probably due to membrane disruption and consequent exposure of the buried enzyme to an aqueous environment. In the absence of detergent this could result in an alteration in the three-dimensional conformation of the protein and thus a loss of enzyme activity.

The susceptibility of the latent enzyme to inactivation by homogenization will depend on membrane integrity so that this technique may prove to be a useful means of assessing brush-border membrane fragility.

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