Independent biosynthesis of soluble and membrane-bound alkaline phosphatases in the suckling rat ileum

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Enzymically active intestinal alkaline phosphatase exists in both soluble and membrane-bound forms in the suckling rat. Antiserum prepared against purified soluble alkaline phosphatase (anti-AIP) was shown to be monospecific when assessed by Ouchterlony double-diffusion analysis and immunoelectrophoresis. The two forms of alkaline phosphatase were antigenically identical and possessed similar affinities for anti-AIP. To study the biosynthesis of the two forms, 14-day-old rats were injected intraperitoneally with [^3H]leucine. The labelling kinetics of alkaline phosphatase, extracted from supernatant and brush-border membrane fractions with anti-AIP, was followed over 20 h. Incorporation of [^3H]leucine into membrane-bound alkaline phosphatase was rapid, reaching a plateau at 6 h. The soluble enzyme showed slower incorporation of label and maximal radioactivity was not reached until 12 h after labelling, a lag of 6 h behind the membrane-bound enzyme. Soluble alkaline phosphatase could not have been a precursor of the membrane form, as there was no early peak of radioactivity in the soluble form. To determine if the soluble enzyme was irreversibly derived from the membrane enzyme, a newly developed technique of labelling brush-border membrane proteins *in vivo* by intraluminal injection of diazotized [^{125}I]iodosulphanilic acid was used. The appearance of ^{125}I in soluble and membrane alkaline phosphatase was then monitored over a 7 h period, encompassing the lag between maximal leucine labelling of the two forms. The results failed to show either a proportional transfer of radioactivity from membrane to soluble alkaline phosphatase or an absolute increase in radioactivity of the soluble form during degradation of brush-border alkaline phosphatase. Therefore there does not appear to be a serial precursor/product relationship between the soluble and membrane-bound forms of suckling-rat intestinal alkaline phosphatase.

Certain hydrolases of the intestinal brush border can occur in the supernatant as well as the membrane fraction after homogenization of mucosa. Hynie & Zbarsky (1973) found that up to 39% of intestinal alkaline phosphatase was in a soluble form in suckling-rat small bowel. Similarly, Galand & Forstner (1974) found 44% of maltase activity in the supernatant fraction of suckling rat gut homogenates. The soluble proportion fell to 7% after

weaning, and after treatment of suckling rats with cortisol. Seetharam *et al.* (1977) confirmed these findings and showed that this phenomenon was more marked in the ileum, that it persisted to a lesser degree in adult rats, and that the soluble enzymes corresponded to their brush-border but not their lysosomal counterparts. The cellular location of these soluble enzymes was not determined. Forstner & Forstner (1979) have shown that developmental changes in soluble maltase are not coincident with those of lysosomal marker enzymes, indicating the lack of dependence between the soluble enzyme phenomenon and the endocytic-lysosomal apparatus of the villous cell.

The genetic relationship of these two intestinal alkaline phosphatases is uncertain. They may arise

Abbreviation used: anti-AIP, antiserum monospecific for intestinal alkaline phosphatase.

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from two different gene codes, and the changes which occur with maturation could represent a change in gene expression. To clarify their relationship, we purified the two forms from the suckling-rat ileum (Yedlin *et al.*, 1981). Comparison of the purified hydrolases revealed similar kinetics for multiple substrates and similar amino acid compositions, suggesting that they originated from the same gene code. However, the membrane enzyme differed from the soluble enzyme in a number of ways. First, the membrane enzyme was incorporated readily into synthetic liposomes. Second, it possessed a different carbohydrate composition, with more mannose and less fucose than the soluble enzyme. Third, the principal isoelectric points of membrane alkaline phosphatase were more acidic than those of the soluble form. The soluble enzyme was not an artefact of proteolysis during enzyme purification. It was concluded that the soluble form lacked the hydrophobic anchor-piece necessary for membrane incorporation.

If soluble and membrane-bound alkaline phosphatase were derived from the same gene code, they may be biosynthetically related in one of two different ways: synthesis could proceed in parallel from a common precursor (e.g. nascent peptide), or alternatively they could have a serial precursor/product relationship. The different carbohydrate compositions of the two forms suggests that the latter is unlikely (Yedlin *et al.*, 1981), but such evidence is not conclusive.

Therefore we have further studied the relationship between the two forms of intestinal alkaline phosphatase in the suckling rat by using highly specific antibody prepared against soluble alkaline phosphatase. The kinetics of incorporation of [^3H]-leucine *in vivo* into each form of alkaline phosphatase have been elucidated. We have also examined the possibility that soluble alkaline phosphatase is derived from the membrane-bound form, by using a newly developed method of labelling with diazotized [^{125}I]iodosulphanilic acid. We conclude that soluble alkaline phosphatase is neither a precursor nor a product of the membrane enzyme.

Materials and methods

Animals

Pregnant albino Wistar rats were obtained from National Laboratory Animal Co., O'Fallon, MO, U.S.A. After delivery, all litters were made equal in size, to ensure uniform growth rates.

Preparation of brush borders and soluble fraction

At the desired age, suckling rats were stunned and decapitated, and the small bowel was immediately removed and rinsed with cold iso-osmotic saline (0.9% NaCl). The distal half of the small bowel (i.e.

the ileum) was cut into small pieces. This was homogenized at 4°C in 2 mM-Tris/HCl, pH 7.4, containing 50 mM-mannitol, with five up-and-down strokes of a Potter-Elvehjem-type size B tissue grinder (A. H. Thomas Co., Philadelphia, PA, U.S.A.) with Teflon pestle rotating at 900 rev./min. The method of Schmitz *et al.* (1973) was used to isolate brush-border fragments, which were then resuspended in 10 mM-Tris/HCl, pH 7.4, containing 0.2 mM-MgCl₂ (hereafter called Tris/Mg buffer) before further treatment. The supernatant fraction remaining after isolation of brush borders was then spun at 105 000 g for 60 min and the resultant supernatant fraction used as the source of soluble alkaline phosphatase.

Enzyme and protein assays

Alkaline phosphatase was assayed by the method of Forstner *et al.* (1968) at pH 9.2 and acid phosphatase was assayed by the method of Henning & Plattner (1974) at pH 4.8, with *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO, U.S.A.) as substrate in both cases. One unit of activity is the amount of enzyme that hydrolyses 1 μmol of substrate in 1 min at 37°C. Samples for alkaline phosphatase and acid phosphatase assays were always pretreated with 0.1% Nonidet P40 (BDH Chemicals, Poole, Dorset, U.K.). Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin (97–99% pure; Sigma) as standard.

Polyacrylamide-gel electrophoresis

Disc gel electrophoresis was performed by the method of Davis (1964), with or without 0.1% Triton X-100 (Fisher Scientific Co., Pittsburgh, PA, U.S.A.). Total acrylamide concentration was 5% (acrylamide:bisacrylamide 38:1). On each gel 0.1 unit of soluble or solubilized membrane alkaline phosphatase was loaded and electrophoresed at 2 mA per gel. After electrophoresis, gels were stained for alkaline phosphatase activity at room temperature with a solution of 2 mM- β -naphthyl acid phosphate, 1 mM-tetrazotized *o*-dianisidine, and 0.4 mM-MgCl₂ in 7.5 mM-sodium barbital buffer, pH 9.4. The *o*-dianisidine was added last. All chemicals were obtained from Sigma.

Detergent solubilization of membrane fractions

Membrane alkaline phosphatase was solubilized from either brush borders or total membrane preparations (i.e. the 105 000 g pellet of intestinal homogenates). In each case solubilization was performed at 4°C for 16 h in Tris/Mg buffer. The following non-ionic detergents were used in various concentrations: Nonidet P40, Triton X-100, Emulphogen and Empigen BB (Marchon, White-

haven, Cumbria, U.K.). Solubilized material was collected after centrifugation at 45 000 *g* for 15 min.

Ethanol precipitation of proteins

Both detergent-solubilized membrane proteins and soluble tissue proteins were precipitated with 70% (v/v) ethanol precooled to -15°C . Precipitation was performed in precooled containers in a freezer room at -15°C , for 30 min. The precipitated proteins were collected by centrifugation at 45 000 *g* for 15 min. The pellet was redissolved in Tris/Mg buffer containing 0.1% Nonidet P40 and denatured protein removed by repeat centrifugation.

Preparation and characterization of antisera

Antiserum was prepared against purified soluble intestinal alkaline phosphatase in rabbits as described in detail by Yedlin *et al.* (1981). Immunization was performed over a 6-week period by using Freund's complete adjuvant. Antiserum was adsorbed with the supernatant fraction from the suckling-rat duodenum before use. Anti-AIP was monospecific when assessed by Ouchterlony double-diffusion analysis (Fig. 1*a*) and immunoelectrophoresis (Fig. 1*b*). Titration of anti-AIP against the two forms of ileal alkaline phosphatase (Fig. 2) revealed a similar titre and affinity for each form. Anti-AIP did not cross-react with rat bone or liver alkaline phosphatase or with rat acid phosphatase. There was no evidence of precipitation of other brush-border hydrolases (sucrase, maltase and lactase) during the titration studies.

[^3H]Leucine-labelling studies

For this, 47 14-day-old non-starved suckling rats from five litters were each injected intraperitoneally with 50 μCi of [^3H]leucine (specific radioactivity 45.7 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) contained in 0.25 ml of 50 mM phosphate-buffered iso-osmotic saline, pH 7.4. Animals were returned to their mothers and then killed at the following time points: 10, 20, 30 and 60 min, 2, 3, 4, 6, 8, 12, 16 and 20 h. Ileal homogenates were prepared in 18 ml of buffer as described above. A sample (0.5 ml) of homogenate was centrifuged at 105 000 *g* for 60 min to allow determination of the total amount of enzyme in the soluble and membrane fraction of each animal's ileum. The remaining 17.5 ml was used to make brush-border and soluble fractions. After solubilization of brush-border proteins in Nonidet P40, both the soluble fraction and detergent-solubilized brush-border proteins were precipitated by ethanol as described above. Precipitated proteins were resolubilized in 0.1% Nonidet P40 in Tris/Mg buffer. Alkaline phosphatase was then isolated by immunoprecipitation.

Fig. 1. (a) Ouchterlony double-diffusion analysis of anti-AIP against various forms of intestinal alkaline phosphatase, and (b) immunoelectrophoresis of ileal supernatant fraction by the method of Grabar & Williams (1953)

(a) The central well contains anti-AIP. Commencing from the top well and moving clockwise, the peripheral wells contain: ileal supernatant fraction, purified soluble alkaline phosphatase, purified membrane alkaline phosphatase, Nonidet-P40-solubilized ileal brush-border proteins, ileal supernatant fraction and Nonidet-P40-solubilized ileal total membrane fraction. Immunodiffusion was performed in 0.8% agarose containing 50 mM-sodium barbital, pH 8.6, and 0.1% Nonidet P40. (b) Supernatant fraction (105 000 *g*) from the ileum containing 0.9 unit of alkaline phosphatase activity was placed in the top well. The same amount of protein from a duodenal supernatant fraction was placed in the other well. Electrophoresis was performed towards the anode for 2 h. A 1:4 dilution of anti-AIP was then placed in the trough. Histochemical staining for alkaline phosphatase (see the Materials and methods section) showed concentration of enzymic activity in the precipitin line. The support medium was the same as in Fig. 1(a).

Immunoprecipitation of alkaline phosphatase

Alkaline phosphatase was isolated from brush-border and soluble fractions by immunoprecipitation from 1 ml samples containing a known

Fig. 2. Titration curve of anti-AIP against various forms of ileal alkaline phosphatase

▼, Purified soluble alkaline phosphatase; ■, ileal supernatant fractions; □, Nonidet-P40-solubilized ileal brush-border proteins. The concentration of alkaline phosphatase in each case was 1.4 units/ml. For further details see the Materials and methods section.

amount of alkaline phosphatase activity, in the range 5–10 units. To determine the amount of antibody required to precipitate at least 98% of enzyme activity, pilot studies were performed with 50 μ l of sample and 50 μ l of diluted anti-AIP. Generally a titre of 1:8 or 1:16 was required. Sample/anti-AIP mixtures were then incubated at 37°C for 2 h and maintained at 4°C overnight. Visible immune pellets were collected by centrifugation at 1200 *g* for 10 min, and the supernatant fraction was assayed to ensure that greater than 98% enzyme activity had been precipitated. Immune pellets were washed three times in Tris/Mg buffer containing 0.1% Nonidet P40. Non-specific trapping in the immune pellet of radioactive proteins other than alkaline phosphatase was determined by adding non-radioactive concentrated alkaline phosphatase to the immune-supernatant fraction in an amount equal to the original enzyme activity. Precipitation was repeated with the same amount of anti-AIP and the pellets were washed as described above.

To determine the incorporation of ^3H into alkaline phosphatase, washed immune pellets were resuspended in 50 μ l of water and then transferred to scintillation vials containing 0.5 ml of NCS Tissue Solubilizer (0.6 M; Amersham/Searle, Arlington Heights, IL, U.S.A.) After solubilization at room temperature for 16 h, 10 ml of a toluene-based scintillation fluid was added, each litre of which contained 6 g of 2,5-diphenyloxazole and 75 mg of

1,4-bis-(5-phenyloxazol-2-yl)benzene (New England Nuclear). Radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrophotometer (model 3320) until 10000 counts were accumulated or 50 min had elapsed. Background radioactivity was 15.7 c.p.m. Samples with the lowest radioactivity gave at least twice this number of counts. Counting efficiency determined by the channels-ratio method was uniform, in the range 40.5–42.5%. For soluble alkaline phosphatase, non-specific trapping of radioactivity was always less than 4.6% of total radioactivity. When alkaline phosphatase was immunoprecipitated from detergent-solubilized brush borders, non-specific trapping was fairly constant, being no higher than twice background. This represented 2–27% of radioactivity in alkaline phosphatase, depending on the time point. Preliminary studies showed that if ethanol precipitation of fractions was omitted before immunoprecipitation, non-specific trapping could reach 5 times background.

[^3H]Leucine incorporation into soluble or membrane alkaline phosphatase was calculated for each animal, after correction for non-specific trapping and background. Incorporation was calculated in two ways: (a) c.p.m. of ^3H per unit of enzyme activity; and (b) c.p.m. in the total mass (i.e. c.p.m. per unit of activity $\times$ total units in each fraction) of ileal soluble or brush-border-bound alkaline phosphatase. The first method fails to account for variations in the total mass of enzyme and for a possible difference in catalytic efficiency (units of enzyme activity per mol of enzyme protein) between the two forms.

Labelling studies with [^{125}I]iodosulphanilic acid

[^{125}I]Iodosulphanilic acid (>1000 Ci/mmol; New England Nuclear) was converted into the diazonium salt according to the instructions provided by New England Nuclear and used immediately. The use of this agent *in vivo* to label brush-border membrane proteins of adult rats has been described in detail elsewhere (Young & Alpers, 1981). Nine 16-day-old rats from a single litter were operated on, under ether anaesthesia; 65 μ Ci of diazotized [^{125}I]iodosulphanilic acid in 0.2 ml of 10 mM-potassium phosphate-buffered iso-osmotic saline, pH 7.4, was injected via a 27-gauge needle into the distal bowel at the jejuno-ileal junction. No obvious peritoneal leak was seen through the small puncture hole. All animals recovered rapidly from the procedure after closure of the abdominal wound with a continuous silk suture. Animals were returned to their mothers and killed after 1, 3 or 7 h, three at each time point. After extensive rinsing of the intestine with phosphate-buffered saline, ileal homogenates were prepared as described above by using eight strokes of the homogenizer. Homogenates were then im-

mediately treated with 70% ethanol as described and precipitated for 10 min. This extracted over 90% of non-protein-bound diazotized [125 I]iodosulphanilic acid (assessed by precipitation with 10% trichloroacetic acid). This made multiple washing of membranes unnecessary and achieved separation of released soluble intracellular proteins from free label, thus stopping the reaction. Ethanol-precipitated homogenate proteins were redissolved in Tris/Mg buffer, and soluble alkaline phosphatase was separated from membrane-bound alkaline phosphatase by centrifugation at 105 000 *g* for 60 min. Supernatant fractions were dialysed overnight at 4°C against Tris/Mg buffer. Membrane proteins were solubilized, precipitated with ethanol and resolubilized in 0.1% Nonidet P40 as described for brush-border membranes. These treatments resulted in over 95% of diazotized [125 I]iodosulphanilic acid being bound to protein, as assessed by trichloroacetic acid precipitation. Alkaline phosphatase was immunoprecipitated from each fraction as described above and incorporation of 125 I measured in a Nuclear-Chicago gamma counter for 10 min. Background was 14 c.p.m., counting efficiency was 71%. Results were expressed as c.p.m. in total mass of soluble or membrane alkaline phosphatase.

Uptake of 125 I into the liver was determined by counting radioactivity of 1 ml samples of a 10% (w/v) liver homogenate prepared in 50 mM-mannitol/10 mM-Tris/HCl (pH 7.4).

Results

In the 14-day-old rat, 55% of small-bowel alkaline phosphatase activity was found in the ileum. Of this ileal enzyme, $64.9 \pm 6.7\%$ (mean $\pm$ S.D.) was found in the 105 000 *g* supernatant fraction. That is, in the ileum the pool of soluble enzyme is twice the size of that of the membrane-bound enzyme, based on enzyme activity. In the jejunum, only $10.9 \pm 4.3\%$ was soluble. Fig. 3 shows the results of polyacrylamide-gel electrophoresis of ileal supernatant and membrane-bound alkaline phosphatase. The membrane (i.e. brush-border) form migrated slowly, with a mean R_f of 0.23, and would not enter the gel in the absence of non-ionic detergent. The supernatant form entered the gel whether or not detergent was present, with a mean R_f of 0.44 in the presence of detergent. The slow form was not seen in the ileal supernatant fraction (105 000 *g*), nor was the fast form seen in washed pellets. Hence centrifugation at high speed for 1 h was a satisfactory means of separating the two.

Acid phosphatase activity was distributed differently along the intestine from alkaline phosphatase; 75% of activity was found in the distal half of the gut. The amount of activity released into the supernatant fraction by homogenization was

Fig. 3. Polyacrylamide-disc-gel electrophoresis of membrane (a) and soluble (b and c) fractions of ileal homogenates, stained for alkaline phosphatase activity

Enzyme activity was detected in these Tris/glycine gels as described in the Materials and methods section. Gels and samples for (a) and (b) contained 0.1% Triton X-100. No detergent was present in (c). The R_f values for alkaline phosphatase are: (a), 0.23 (b) 0.044, (c) 0.50. The slightly dense area which is seen behind the dye front in gels (a) and (b) is due to interaction between Triton X-100 and a component of Bromophenol Blue.

$33.6 \pm 1.6\%$ (mean $\pm$ S.E.M.) in the ileum and $31.9 \pm 3.6\%$ in the jejunum.

When untreated ileal supernatant proteins were fractionated on Sephadex G-200, alkaline phosphatase activity was eluted with a molecular weight of 97 000, in close agreement with the findings of Yedlin *et al.* (1981) for the purified enzyme.

Immunoprecipitation of these fractions showed radioactivity to be proportional to the amount of enzymically active alkaline phosphatase present (Fig. 4). No other peaks of radioactivity were observed, indicating that anti-AIP did not precipitate enzymically inactive fragments of the alkaline phosphatase molecule or other radioactive proteins.

Fig. 4. Immunoprecipitation of alkaline phosphatase from ileal supernatant proteins separated on Sephadex G-200

Ileal supernatant fractions from three rats, each labelled for 12 h with 50 μ Ci of [3 H]leucine, were prepared as described in the Materials and methods section, pooled, and 1 ml was applied to a Sephadex G-200 column (50 cm $\times$ 1 cm). Fractions (0.75 ml) were eluted with Tris/Mg buffer at a flow rate of 6.3 ml/h per cm^3 . After assay of fractions for alkaline phosphatase, they were pooled in pairs and an equal volume of anti-AIP diluted 1:32 was added (sufficient to precipitate all alkaline phosphatase activity). Radioactivity of the immunoprecipitate was determined as described in the Materials and methods section. ●, Enzyme activity; ○, radioactivity.

This latter observation further confirmed the mono-specificity of anti-AIP.

Solubilization of alkaline phosphatase

The effect of solubilization of alkaline phosphatase with Nonidet P40 is shown in Table 1. Regardless of whether brush-border or total membrane preparations were used, a high concentration of detergent was needed to solubilize 95% of the enzyme activity. With Nonidet P40, a 1% concentration solubilized 86.5% of alkaline phosphatase activity from the brush-border preparations. With Triton X-100, Empigen BB or Emulphogen at the same detergent/protein ratio of 100:1 (w/w), 81.5%, 81.0% and 75.0% solubilization respectively was achieved. Detergent had no effect on soluble alkaline phosphatase activity, but it did give a small increase in membrane alkaline phosphatase activity. With Nonidet P40, the mean increase was 9.9% when solubilizing total membranes and 15.2% when solubilizing brush borders. The percentage increase was not concentration-dependent between 0.1 and 5.0% detergent and presumably resulted from better access of the substrate to the enzyme.

Some 99% of alkaline phosphatase activity was precipitated by 70% ethanol at -15°C . Recovery after resolubilization exceeded 95%. Even though it was necessary to solubilize initially with 5% Nonidet P40, the ethanol-precipitated material could be resolubilized with as little as 0.1% Nonidet P40. This low detergent concentration simplified enzyme and protein assays and minimized non-specific trapping of other proteins during immunoprecipitation. Pre-treatment of samples with ethanol did not effect electrophoretic mobility of alkaline phosphatase, except that sharper bands were obtained (results not shown).

Table 1. Solubilization of alkaline phosphatase from suckling rat ileal mucosa by Nonidet P40

The total membrane preparation was collected by centrifugation for 60 min at 45 000 g of pooled ileal homogenates from 15 suckling rats. Homogenates were prepared in 50 mM-mannitol/2 mM-Tris/HCl (pH 7.4). Ileal brush borders were prepared as described in the Materials and methods section from the same homogenate. Solubilization of these membrane preparations was performed overnight in Tris/Mg buffer at 4°C . Insoluble material was removed by centrifugation for 60 min at 45 000 g, and solubilization of alkaline phosphatase was determined by assaying supernatant fractions in triplicate. Control values were obtained by remixing pellet and supernatant fractions for each detergent concentration. The values in parentheses refer to the detergent concentration (v/v).

	Detergent/protein ratio (w/w)	Alkaline phosphatase activity (units/ml)		Alkaline phosphatase solubilized (%)
		Control	Supernatant	
Total membranes	60:1 (5%)	4.0	3.9	95.4
	12:1 (1%)	4.1	3.0	73.5
	1.2:1 (0.1%)	4.0	1.9	46.6
Ileal brush borders	500:1 (5%)	0.60	0.56	94.9
	100:1 (1%)	0.59	0.51	86.5
	10:1 (0.1%)	0.59	0.40	67.9

[3H]Leucine labelling

Fig. 5 shows the results of incorporation of [3H]leucine *in vivo* into the total pools of soluble and membrane alkaline phosphatase. Significant labelling of brush-border alkaline phosphatase occurred as early as 30min and reached a plateau at 6h.

Fig. 5. Patterns of incorporation of [3H]leucine into total pools of brush-border or soluble alkaline phosphatase in the ileum of the 14-day-old rat

Each rat was injected intraperitoneally with 50μCi of [3H]leucine and alkaline phosphatase was immunoprecipitated from soluble and membrane fractions as described in the Materials and methods section. Each point represents the mean incorporation into the total pool of enzyme in the soluble (□) or membrane (●) fractions. Vertical bars indicate S.E.M. Studies continued beyond 20h showed that maximal incorporation of radioactivity into the soluble form occurred at 12–20h. The values for the membrane enzyme for 6–20h were not significantly different (unpaired Student's *t* test), nor were those for 12–20h for the soluble form.

Labelling of soluble alkaline phosphatase proceeded more slowly and reached a plateau at 12h. The close agreement in radioactivity incorporated into the total pool of ileal soluble and ileal membrane alkaline phosphatase at 20h (Fig. 5) does not reflect similar specific radioactivities. At 20h there were 45.4 c.p.m./unit of membrane alkaline phosphatase and 23.9 c.p.m./unit of soluble alkaline phosphatase. Radioactivity in the total pools of soluble or membrane enzymes was similar, as there was almost twice as much soluble as membranous enzyme (64.9% was soluble). If the results were plotted as c.p.m./unit of alkaline phosphatase activity rather than as shown in Fig. 5, no change in relative patterns of [3H]leucine incorporation was seen.

Labelling with diazotized [125I]iodosulphanilic acid

Table 2 shows the results of labelling *in vivo* of ileal alkaline phosphatase with diazotized [125I]-iodosulphanilic acid. Radioactivity incorporated into the membrane alkaline phosphatase pool reached a maximum at 3h, owing to continuation of the labelling reaction. The subsequent fall between 3 and 7h presumably resulted from turnover of the membrane form, as mean total enzyme activity remained constant (Table 3). Over the 7h of study, radioactivity in the soluble pool, expressed as a proportion of total radioactivity in both pools, fell from 23.9 to 16.6%. A fall in actual radioactivity in the soluble enzyme pool occurred between 3 and 7h, during turnover of the membrane enzyme.

This labelling technique did not label the membrane form exclusively, as some labelling of the soluble enzyme was observed. However, membrane alkaline phosphatase was at least 4 times more radioactive than soluble enzyme, there being almost twice as much of the latter. Some non-protein-bound diazotized [125I]iodosulphanilic acid was able to cross suckling-rat ileal mucosa. For the liver, maximum radioactivity was observed at 1h (mean

Table 2. Incorporation of radioactivity into alkaline phosphatase after labelling of ileal mucosa *in vivo* with diazotized [125I]iodosulphanilic acid

Nine 16-day-old rats from a single litter each received 65μCi of diazotized [125I]iodosulphanilic acid (ISA) by intraluminal injection into the ileum (see the Materials and methods section). Three animals were killed at each time point, and ileal homogenates were separated into soluble and membrane fractions by centrifugation at 105 000 *g* for 60 min. Alkaline phosphatase was extracted from soluble and Nonidet-P40-solubilized membrane fractions with anti-AIP (see the Materials and methods section). Radioactivity is expressed as c.p.m. incorporated into the total enzyme pool in each fraction, as means ± S.E.M. derived from two determinations in each of three animals.

Time (h)	10 ⁻³ × [125I]ISA-labelled alkaline phosphatase (c.p.m.)		[125I]ISA bound to supernatant alkaline phosphatase (%)
	Membrane	Supernatant	
1	3.61 ± 0.24	1.13 ± 0.17	23.9
3	9.38 ± 0.52	2.31 ± 0.16	19.7
7	5.34 ± 0.13	1.06 ± 0.21	16.6

Table 3. Total alkaline phosphatase activity in soluble and membrane fractions of suckling-rat ileal mucosa labelled with diazotized [125 I]iodosulphanilic acid

See Table 2 for details. Results are means $\pm$ S.D. for three rats at each time-point.

Time (h)	Mass of alkaline phosphatase (units/ileum)		Ratio soluble/membrane
	Membrane	Soluble	
1	15.82 $\pm$ 2.64	28.6 $\pm$ 2.0	1.8
3	16.60 $\pm$ 2.46	27.6 $\pm$ 3.6	1.7
7	17.04 $\pm$ 0.94	31.0 $\pm$ 3.8	1.8

496.6 $\times 10^3$ c.p.m. per liver). This represented 3% of the total dose. By trichloroacetic acid precipitation, 70.8% of radioactivity was protein bound. A similar observation has been made in adult rats (Young & Alpers, 1981).

Discussion

The results indicate that there are important biosynthetic differences between the two immunologically identical but structurally different forms of intestinal alkaline phosphatase found in the ileum of the suckling rat. The pool of soluble enzyme is twice the size of that of membrane-bound enzyme, based on enzyme activity in supernatant and membrane fractions. This is likely to be true for enzyme protein mass also, as the two forms have similar specific activities for *p*-nitrophenyl phosphate and have similar molecular weights (Yedlin *et al.*, 1981). When suckling rats were injected with [3 H]leucine, radioactivity appeared more rapidly in the pool of membrane enzyme; maximal radioactivity in the soluble pool was not reached until about 6 h after the maximum in membrane alkaline phosphatase (Fig. 5). The absence of an early peak of radioactivity in the soluble pool means that the soluble enzymically active alkaline phosphatase is most unlikely to be a precursor of membrane alkaline phosphatase.

However, it was possible that soluble alkaline phosphatase was derived from the brush-border form without loss of enzyme activity, by action of luminal proteinases, with or without bile. The enzymically active soluble protein would subsequently have to be taken up intact by the villous cell. For a number of reasons it seemed that solubilization of brush-border alkaline phosphatase *in vivo* would not account for this soluble form of the enzyme. We have shown that the two glycoproteins have considerably different isoelectric points and carbohydrate compositions: in particular, the soluble form possessed more fucose and less mannose (Yedlin *et al.*, 1981). Such differences in sugar composition would require extensive modification of carbohydrates after membrane release, involving addition as well as removal of

sugars. In addition, after labelling *in vivo* with [3 H]leucine, the specific radioactivity of the soluble enzyme (c.p.m./unit) only reached half that achieved in the membrane enzyme. Furthermore, radioactivity in the membrane pool of alkaline phosphatase did not decline rapidly as it rose in the soluble pool. For these reasons we decided to study more directly the possibility that soluble enzyme was a derived product of the membrane enzyme.

Diazotized [125 I]iodosulphanilic acid has been used in low concentrations to label plasma-membrane proteins of platelets and erythrocytes without affecting cell function (Sears *et al.*, 1971; George *et al.*, 1976). When diazotized [125 I]iodosulphanilic acid was tested as a label for adult-rat intestinal brush-border proteins *in vivo*, we found that principal proteins were labelled proportionally to their relative presence in the membrane (Young & Alpers, 1981). In the present study some non-protein-bound label was absorbed, with subsequent labelling of intracellular soluble proteins, including alkaline phosphatase. Such labelling must have occurred *in vivo*, because it could not be prevented by immediate extraction of homogenates with ethanol to remove label that had not reacted. This labelling *in vivo* of soluble enzyme accounted for less than 24% of total alkaline phosphatase labelled and thus did not interfere with the interpretation of results. The rationale for labelling brush-border alkaline phosphatase by this method was that radioactivity would be transferred to the soluble enzyme pool, if it were derived from the brush-border form. Provided that soluble alkaline phosphatase was turned over (or degraded) more slowly than membrane alkaline phosphatase, then a rise in radioactivity would be seen in the soluble enzyme pool as it fell in the membrane pool.

The difference in rates of incorporation of [3 H]leucine into soluble and membrane pools of alkaline phosphatase depends on the ratio of synthesis to degradation in each form and does not provide information about synthesis or degradation independently. But if soluble enzyme were derived from brush-border enzyme by an irreversible process, relative degradation rates can be deduced from pool sizes, because the rate of transfer of

enzyme to the soluble pool cannot exceed the synthesis rate of membrane-bound enzyme. Two-way exchange between the pools appears unlikely, as the soluble enzyme lacks the hydrophobic anchor-piece necessary for incorporation into membranes (Yedlin *et al.*, 1981). Because the soluble enzyme pool is twice the size of the membrane pool, the degradation rate of the soluble enzyme would need to be less than (approximately half) that of membrane enzyme to account for the difference in pool sizes, if the two enzyme pools were serially related to each other. There is also direct evidence that the soluble enzyme pool is turned over slowly in the suckling rat. By studying the effect of fat ingestion on intestinal alkaline phosphatase activity in plasma and small-bowel mucosa, we have shown that circulating enzyme arises from the soluble mucosal enzyme (Young *et al.*, 1982). The fractional clearance rate of the plasma enzyme is 0.13 unit/min, based on the plasma half-life of ^{125}I -labelled enzyme. Mean plasma intestinal alkaline phosphatase in suckling rats is 0.014 unit/ml. It can thus be calculated that soluble enzyme is released into the circulation at a rate of approx. 0.1 unit/h. This is only a small fraction of the total soluble pool in the enterocyte, of over 30 units. In fat-fed adult rats, the rate of release is 7.5 units/h and the small-bowel soluble pool is 20–30 units. Thus a much larger fraction of the soluble pool is being released in adult rats.

Despite these arguments suggesting a slow turnover of the soluble pool, the proportion of total ^{125}I -labelled alkaline phosphatase did not increase in the soluble pool at the expense of the membrane pool, during the 6 h period covering the lag between maximal $[^3\text{H}]$ leucine labelling of the two forms. In particular, in the 4 h after maximal labelling of membrane enzyme, total radioactivity fell in the soluble pool (Table 2). As a rise in neither the proportion of radioactivity bound to the soluble form nor its specific radioactivity occurred, the evidence is against the soluble enzyme being derived from the brush-border form.

The labelling experiments described here depend upon the fact that there is not a trivial explanation for the two forms of alkaline phosphatase. They are readily separable by high-speed centrifugation of homogenates, and the soluble enzyme is not derived from the membrane form by proteolysis during homogenization (Yedlin *et al.*, 1981). The supernatant form is clearly soluble, as it will enter polyacrylamide gels in the absence of detergent and as it is eluted from a Sephadex G-200 column at a molecular weight of 97 000. Soluble alkaline phosphatase is not lysosomal because: (a) it is liberated completely by homogenization, whereas most lysosomes remain intact, as determined by acid phosphatase activity; (b) histochemical studies have

not shown activity in lysosomes (Ono, 1975); and (c) the pH optimum is different from that of lysosomal enzymes. However, it is difficult to be certain of the cellular localization of soluble alkaline phosphatase. It may be cytoplasmic, as it is readily released by gentle homogenization. To support this suggestion, alkaline phosphatase has been observed histochemically in the subapical cytoplasm (Ono, 1975) and in the cytosolic fraction of intestinal cells separated on continuous density gradients (Batt & Peters, 1978). But it cannot be discounted that it is contained within fragile intracellular organelles. In particular, there is a possibility that soluble alkaline phosphatase is a secretory glycoprotein, as increased amounts of alkaline phosphatase are found in the non-chylomicron fraction of mesenteric lymph, and in the serum, after fat feeding (Barrowman, 1979).

It is conceivable that the soluble and membrane phosphatases are produced by two different cell types and would not be expected to be biosynthetically related to each other (Forstner & Forstner, 1979). This possibility seems unlikely, for two reasons. First, both forms are present in mucosal cells isolated from the same level of the villus-crypt axis (Young *et al.*, 1982). Second, in the suckling-rat ileum, alkaline phosphatase has been seen both on the membrane and in the apical cytoplasm in the same cells (Ono, 1975).

As the two forms have very similar amino acid compositions (Yedlin *et al.*, 1981) and are antigenically identical (Figs. 1 and 2), it seems most likely that they originate from the same gene code. Their ultimate biosynthetic differences may result from an early event in the biosynthesis of the nascent peptide which leads to diverging synthetic pathways. The principal biochemical difference resides in the absence from soluble alkaline phosphatase of the hydrophobic anchor-piece (Yedlin *et al.*, 1981). This is known to be C-terminal in pig intestinal alkaline phosphatase (Colbeau & Maroux, 1978). Thus a terminal deletion mechanism may be responsible for formation of the two isoenzymes, i.e. the final C-terminal segment of the nascent peptide to be synthesized may be removed. This could occur post-translationally, owing to cleavage by proteinases, or it may result from two separate mRNA species.

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