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SPATIAL AND TEMPORAL DIFFERENTIATION OF ALKALINE PHOSPHATASE ON THE INTESTINAL VILLI OF THE MOUSE

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The villi of the small intestine are clothed by a constantly renewed population of epithelial cells that are produced in the crypts and extruded at the villi tips (14, 15). During its life of about 1½ days, each epithelial cell undergoes differentiative changes that include a lengthening and narrowing of its microvilli, with the result that the free surface of apical cells is very much greater than that of those cells near the bases of the villi (1).

Alkaline phosphatase is bound to the membranous coat of the microvilli (5, 8, 12). In the mouse at about 16–18 days of age, the microvilli shift from the short, broad configuration of infancy to the long, narrow, mature form (23), and at the same time the phosphatase activity of the duodenum rises about 20-fold (17). This indication that the increase of phosphatase activity may be related to the expansion of the microvillar membrane, rather than *de novo* synthesis of enzyme, is supported by the finding that actinomycin D, puromycin, and other drugs that interfere with the synthesis of RNA or protein bring about an elevation of phosphatase activity not only in developmental stages, when phosphatase normally rises (19), but also in adults, when a constant level ordinarily is maintained (20). Since puromycin exerts an activity-enhancing effect under conditions of complete suppression of mitosis, the elevation of phosphatase activity does not depend on the production of new cells (20).

The rise of phosphatase activity that occurs in the juvenile mouse is accompanied by a change of characteristics most readily expressed as rate of hydrolysis of phenylphosphate (PhP) relative to that of beta glycerophosphate (bGP) (18). At 11 days this PhP/bGP ratio is below 0.8, at 20 days above 3.0. The change reflects the appearance of two new isozymes, one having a ratio of about 6.0, the other a ratio of approximately 2.0 (21); both continue to be present in the duodenum in adult life. It has been proposed recently that, as cells glide up the villi, the configuration of their microvilli is altered by a mechanism that simultaneously converts the bound phosphatase from one isozymic form to another (20). Presumably the rate at which this chemo-structural differentiation goes forward is regulated by a labile protein, the phosphatase-enhancing effect of actinomycin D or puromycin being to eliminate the restraining effect of this postulated regulator.

This hypothesis predicts that the phosphatase isozyme of higher PhP/bGP ratio will be found at the villi tips, and the isozyme of lower ratio at the villi bases. We now have been able to demonstrate the correctness of this hypothesis by means of a technique, recently introduced by Dahlquist and

Nordstrom (6), that involves freezing a piece of intestine flat and sectioning it parallel with the wall, so that cross-sections of villi may be collected in small groups from tips to the cryptal region.

MATERIALS AND METHODS

Two substrains of Swiss mice, characterized by high and low phenylphosphatase activities in the duodenum (22), were used in this investigation. Adult males were fasted for 12–18 hr, and sacrificed by decapitation. For duodenum, the first 1.5–2.0 cm segment posterior to the common bile duct was taken; for jejunum, a 2.0 cm piece from ⅔ of the distance between pylorus and iliocecal junction. The segment was cleaned of adhering pancreas or mesentery, and a short piece was cut from each end for conventional homogenization and assay. The remaining piece was slit open, washed in fresh cold 0.9% NaCl, and arranged over a rectangular hole cut in a piece of heavy filter paper to which the edges of the moist tissue adhered. Care was taken to avoid stretching the tissue. The tissue was frozen in less than 1 min by bringing the serosal surface into contact with a presectioned block of frozen 0.1% agar (6). The block then was trimmed to approximately 4 × 6 mm and sectioned at 12 μ in a cryostat microtome set at −18°C. Sections were collected in four groups of 8–10 sections each; the last section in each group was mounted on a slide, fixed in cold 90% ethanol, and stained with Mayer's hemalum. To verify the localization of phosphatase, complete series of sections from duodena of both high and low strains were prepared and stained by a coupling technique utilizing naphthyl AS-MX phosphate as substrate (2).

The number of sections that could be cut from a single preparation ranged from 40 to 45 for duodenum, and from 30 to 35 for jejunum. Each group of sections was collected in a small prechilled test tube, covered with Parafilm, and stored at −24°C.

The frozen sections were homogenized by vigorous mechanical shaking after addition of 0.5 ml of distilled water; the preparation was refrozen, then diluted to 1.0 ml and shaken again. For the most active samples, further dilution was necessary. Duodenal activity on phenylphosphate (PhP) was determined by adding 0.1 ml of enzyme preparation to a tube containing 0.2 ml of 300 mM PhP, 0.25 ml of 0.2 M carbonate–bicarbonate buffer at pH 9.8, and 0.05 ml of 0.1 M $MgCl_2$; for jejunum, 60 mM PhP and pH 9.4 buffer were used. After 5 min incubation at 37.5°C, during which time zero order kinetics prevail, the reaction was stopped by the addition of 0.2 ml of diluted Folin-Ciocalteu reagent (see reference 13) and 0.32 ml of 20% Na_2CO_3. The color, which corresponds to released phenol, was read after 10 min at 660 mμ in a Coleman Jr. spectrophotometer.

Both duodenal and jejunal activities on beta glycerophosphate (bGP) were determined by adding 0.05 ml of a suitable enzyme dilution to a 7 mm tube containing 0.1 ml of 120 mM bGP, 0.125 ml of 0.2 M carbonate-bicarbonate buffer at pH 9.4, and 0.025 ml of 0.01 M $MgCl_2$. The reaction was stopped after 5 min by the addition of 0.1 ml of 10% trichloroacetic acid, the released phosphorus then being measured according to the Fiske-Subbarow technique (11). Color also was read after 10 min at 660 mμ in a Coleman Jr. spectrophotometer. Protein content was determined by a semi-micro modification of the method of Lowry et al. (16).

As a check on the validity of the results obtained by these procedures, small pieces of tissue cut from both ends of the sectioned piece were ground in a glass homogenizer and assayed by the methods described above. In all cases activities manifested by these controls agreed well with the average of the activities obtained on the corresponding series of sections.

RESULTS

Inspection of the fixed sections showed that the lowermost section of the C level (third group) fell at the mouths of the crypts. Thus the C level sections represent the villi bases, the B level the

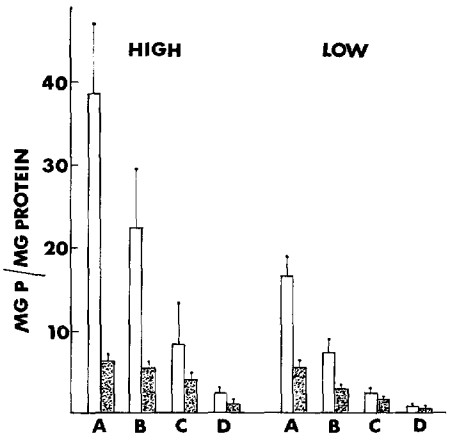

FIGURE 1 Specific activity of alkaline phosphatase along the length of the villi in the duodenum of mice of high- and low-activity strains. Ordinate represents amount of P released per mg protein per 30 min; when PhP is used as substrate, released phenol is converted to its equivalent weight of P by dividing by 3. Open columns, activity with PhP; stippled columns, with bGP; vertical lines represent standard deviation.

TABLE I

Distribution of PhP/bGP Ratios in the Duodenum

Level	PhP/bGP (mean and range)	
	High-activity strain*	Low-activity strain*
A	6.09 (5.33–7.74)	3.17 (2.47–4.18)
B	4.11 (3.18–4.95)	2.39 (1.68–2.86)
C	2.36 (1.63–3.25)	1.53 (1.08–1.79)
D	2.15 (1.71–2.73)	1.24 (1.00–1.64)

* 6 animals were used of each strain.

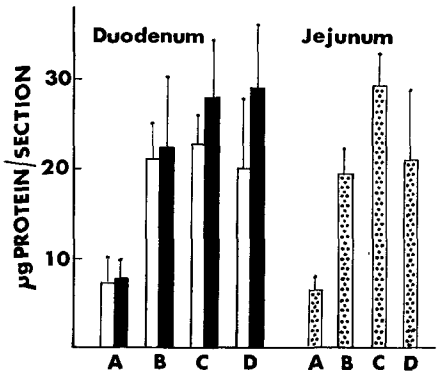

FIGURE 2 Protein content per section in duodenum and jejunum. For duodenum, open columns represent high-activity strain; filled columns, low-activity strain.

mid-region, and the A level the tips, with the D level comprising the crypts.

In the duodenum, the specific activity of alkaline phosphatase declines strikingly from the villi tips to the crypts (Fig. 1). In both high- and low-activity strains this gradient manifests itself with both substrates, but is obviously steeper with PhP than with bGP. Consequently the PhP/bGP ratios also fall from level to level (Table I). Evidently phosphatase varies not only quantitatively but also qualitatively along the length of the villus.

The high specific activities of the villi tips are in part a function of the smaller mass of the tip sections. Fig. 2 shows that the A level sections contain only ⅓ as much protein as the B level sections. This difference is due partly to the uneven length of the villi, and partly to their tendency to taper, although the cytolysis sometimes noted in the extrusion zone may play a role too. When phosphatase activity is expressed per section, A level sections from high strain are found to have only 52% as much PhPase and 38.8% as much bGPase activity as those from the B level (Fig. 3). In the light of the lower protein content, this is not surprising. But it is surprising that different results are obtained with the low-activity strain (Fig. 3): here the bGPase activity of A level is 60% of that of B level ($P < .01$), but the PhPase activity is 83% that of B, and the difference is not significant ($P > 0.2$).

In addition to this difference, the two strains differ in the PhP/bGP ratios observed at the various levels of the villi (Table I). This is perhaps to be expected from the fact that PhP/bGP ratios are generally proportional to specific activity with

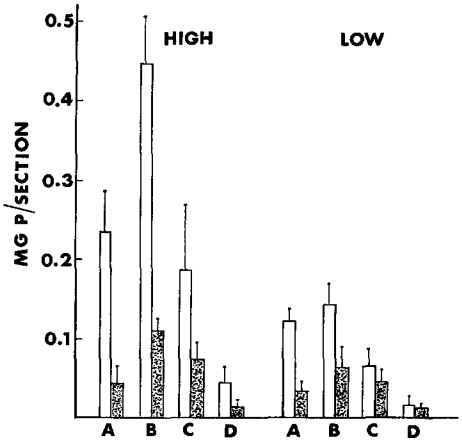

FIGURE 3 Duodenal alkaline phosphatase activity as μg P released per section per 30 min. Open columns, activity with PhP; stippled columns, with bGP.

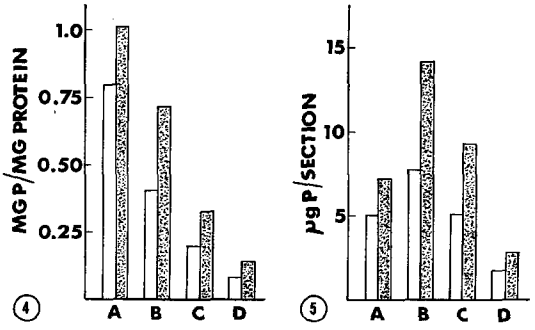

FIGURE 4 (left). Specific activity of alkaline phosphatase along the length of the villus in the jejunum. FIGURE 5 (right). Activity of alkaline phosphatase per section in the jejunum. Open columns show activity per 30 min with PhP; stippled columns, with bGP.

PhP (18), and are lower in low-activity than in high-activity strain mice at all stages of life (22). The degree of change of ratio from crypt to tip is, however, about equal in the two strains: i.e., in high strain the ratio at A level is 2.82 times that at D, in low strain it is 2.56 times.

Since high- and low-activity strains do not differ much in jejunal phosphatase levels, results obtained on the jejuna of two animals of each strain are presented together (Figs. 4, 5). As in duodenum, there is a strong gradient of specific activity from villus tip to crypt with both substrates. Activity per section, on the other hand, is lower at the tip than in the midvillus region, a fact which again reflects the relatively small amount of protein in tip sections (Fig. 2). The difference between A and B levels is, however, considerably greater with bGP than with PhP, with the result that the PhP/bGP ratio of the tip is significantly greater ($P < .001$) than that of the midvillus area (Table II). Although the ratios of the C and D levels are not significantly different from that of B, the evidence does indicate that in the jejunum too the phosphatase changes to some extent as the epithelial cells push out to the villi tips.

TABLE II

Distribution of PhP/bGP Ratios in the Jejunum

Level	PhP/bGP (mean and range)
A	0.73 (0.63–0.80)
B	0.55 (0.50–0.58)
C	0.59 (0.54–0.69)
D	0.66 (0.50–0.74)

DISCUSSION

In butanol extracts of duodenum from predominantly high-activity strain mice, more than 90% of the phosphatase activity is represented by isozymes having PhP/bGP ratios of about 6.0 and about 2.0 (21). It now appears that in the intact duodenum these two isozymes are separated from each other in space and time. The lower ratio form appears first, in the crypts and at the bases of the villi, and the high-ratio form appears later, when the cells have arrived at the outer zone of the villus. The intermediate values of the midvillus region probably are due to mixture of the two forms. This is a situation which at present appears to have no parallel in the literature on isozymes. The forms of malic dehydrogenase (27) and of glucuronidase (24) are spatially separate, and lactic dehydrogenase isozymes may succeed each other in time (4) but are not known to show the kind of structurally ordered progression revealed in this study.

The two isozymes in question are separable by chromatography on DEAE-cellulose and by electrophoresis in starch gel (21). They cross-react in immunological tests, but are not identical (9). They are too close in molecular size to comprise different numbers of subunits, but they may differ in their poly(oligo?)saccharide components. This possibility is an attractive one, for carbohydrate is an integral part of the intestinal phosphatase molecule (25), and the electrophoretic mobility of human kidney phosphatase at least is altered by treatment with neuraminidase (3). Though both isozymes are presumably membrane-bound, the difference between the two is not dependent on their orientation within the intact membrane, for it persists after the lipoprotein bonds are ruptured by extraction with butanol.

The nature of the difference between the two isozymic forms is a problem directly related to that of the nature of the differentiating mechanism that places the lower ratio form at the villi bases and the high ratio form at the tips. The possibility that the high ratio form is synthesized as the cells glide up the villi apparently is ruled out by the fact that the application of actinomycin D or puromycin elevates both phosphatase activity and PhP/bGP ratio in adult mice (20). This leaves two alternatives: (*a*) the lower ratio isozyme is converted to the higher as the cells move from base to tip; or, (*b*) the high ratio isozyme originally is synthesized in an inactive form that is activated gradually as the cells travel upward. The former alternative is favored by the apparent disappearance of the lower ratio type at the villi tips, where the average ratio rises above 6.0 (Table I); but it also is possible that the lower ratio form is degraded and lost as the other is activated. The second alternative is supported by the discovery that in young mice the duodenal crypts contain an enzymatically inactive material that cross-reacts with antihigh-ratio serum (10). Although there is little to choose between these two alternatives at present, it is clear that both are in agreement with the hypothesis that the production of a functional membrane is a multistep operation (7).

In mice of the low-activity strain, the highest and lowest PhP/bGP ratios (presumably corresponding to two isozymic forms) are quite different from those in the high-activity strain (Table I). This may indicate that in the low strain a structural gene has been modified to produce a slightly altered polypeptide, the difference being perhaps comparable to the variation that alters the mobility of glucose-6-phosphate dehydrogenase isozymes in deer mice of different genetic strains (26). Whether as a consequence of such a fundamental difference, or because of related factors in a situation known to be under polygenic control (22), the steps by which the manifestation of phosphatase activity is altered, as the cells move up the villus, do not seem to be identical in the two strains. Although the degree of change of PhP/bGP ratio from base to tip is approximately equal in the two, the specific activity at the tips in the high strain is 3.92 times that of the bases (C level) with PhP and 1.58 times with bGP, whereas in the low strain the comparable figures are 6.27 and 3.0. To analyze these changes adequately in terms of architectural and temporal determinants (24) will require further information not only about the structure of the phosphatase isozymes, their stability, and their relation to the expanding microvillar membrane, but also about such factors as the form of the villi and the microvilli, and the rate of movement of epithelial cells from crypt to extrusion zone.

SUMMARY

By sectioning intestinal villi at right angles to their length, it has been shown that alkaline phosphatase activity is highest at the tips and declines steadily toward the crypts in mice of both high- and low-phosphatase-activity strains, whether PhP or bGP is used as substrate. However, the rate of decline is not equal with the two substrates, so that the PhP/bGP ratio is significantly greater at the tips of the villi than at their bases. This phenomenon is most striking in the duodenum, in which this evidence indicates that the two major isozymes are spatially separate.

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