Developmental Pattern of Rat Intestinal Brush-Border Enzymic Proteins along the Villus–Crypt Axis

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At various postnatal stages, intestinal epithelial cells were isolated sequentially from villus tip to crypt base by successive EDTA treatments. According to the localization of marker enzymic activities, isolated cells were pooled into three cell compartments: villus (V), lower villus and upper crypt (VC) and crypt (C). Purified brush-border-membrane proteins were separated by 7.5%-polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Enzymic activities could be assigned to some protein bands: maltase/glucoamylase (protein band 3), sucrase–isomaltase (protein bands 3 and 6), lactase (protein band 5) and alkaline phosphatase (region of protein bands 8 and 9). The findings suggest the following. (1) Sucrase–isomaltase activities appeared in compartment C at 17 days with a simultaneous increase of the pre-existing protein band 3 and appearance of a well-defined protein band in position 6; the enzymic complex remained still present in the crypt cells until adulthood. From the day 21 onwards, sucrase–isomaltase was detected in compartments VC and V. (2) Lactase was only present in the three cell compartments until day 21; at this developmental stage its activity completely disappeared from compartment C, in spite of the persistence of a weak protein band. (3) Alkaline phosphatase activity could be detected as a single peak corresponding to protein band 9 in all three cell compartments until day 21; thereafter it was replaced by two peaks of activity showing a less precise correlation with the well-defined protein bands 8 and 9. In the crypt cells of the adult rat, however, the preweaning situation, which was regularly observed, is an unexpected phenomenon. (4) Maltase and glucoamylase did not display any marked qualitative or quantitative modifications either along the villus–crypt axis or during the period of postnatal development studied. Evidence is given from the present data that each brush-border enzyme investigated has a specific developmental pattern.

During postnatal development the digestive enzymes of the intestine undergo substantial changes. In particular, the evolution pattern of the two main disaccharidases (lactase and sucrase) has been extensively studied in rodents on tissue homogenates (for a review see Eloy *et al.*, 1977). The localization of most digestive enzymes along the villus–crypt axis of the adult rat has already been established either on sequentially isolated cells (Webster & Harrison, 1969; Weiser, 1973) or on successive horizontal sections (Nordström *et al.*, 1968). Moreover, the use of such techniques revealed that, in suckling rats, sucrase–isomaltase activities first appeared in intestinal crypt cells (Herbst & Koldovsky, 1972; Henning *et al.*, 1975; Raul *et al.*, 1977). More recently, gel-electrophoretic systems of purified brush-border membranes allowing the separation and characterization of enzymic proteins have been developed. Such techniques were successfully applied to the following studies: (1) biosynthesis of human brush-border enzymic glycoproteins (Hauri *et al.*, 1977); (2) induction of precocious appearance of sucrase–

isomaltase molecules by cortisol administration (Galand & Forstner, 1974*b*; Raul *et al.*, 1978*a*) or sucrose feeding (Raul *et al.*, 1978*b*) in the rat; (3) comparative postnatal development of rat brush-border enzymes located in proximal and distal small intestine (Seetharam *et al.*, 1977; Green & Hauri, 1977).

Isolated intestinal cells as a means of improving the homogeneity of the starting material used in brush-border-membrane isolation has never been reported. The present paper shows that sequentially isolated cells represent an appropriate model for studying the developmental pattern along the villus–crypt axis of enzymic brush-border proteins separated by gel electrophoresis.

Materials and Methods

Animals

Non-starved Wistar rats were studied from day 13 after birth through to the adult stage. In the postnatal series the young rats remained with their mother

until the following developmental ages were reached: 13, 16, 17, 18, 21 and 24 days. From each litter nine animals were divided into three groups. The intestinal segments of the three animals of each group were pooled and submitted in parallel to a sequential cell-isolation procedure. The first group served as control for the establishment of an enzymic gradient from villus tip to crypt base. The second and third groups were used in duplicate for the study of purified brush-border proteins of the sequentially isolated cells. In the adult series the intestines of three different rats (250 g body weight) were similarly treated and studied in parallel. In all cases the experiments were repeated at least three times.

Cell-isolation procedure and determination of enzymic activities

After ether anaesthesia of the animals, the duodenum and the proximal half of the jejunum were removed, rinsed thoroughly and everted. To obtain sequential cell release from villus tip to the crypt base, the intestines were submitted to successive incubations in phosphate-buffered saline (no Ca^{2+} or Mg^{2+}) containing 1.5 mM-EDTA + 0.5 mM-dithiothreitol as described previously (Raul *et al.*, 1977). The number of incubations necessary to obtain the last crypt-cell fraction varies between 9 (for the youngest animals) and 12 (for the adults). The final pellet of each cell fraction from the first group was homogenized and the following enzyme activities were determined: maltase and sucrase (by the method of Messer & Dahlqvist, 1966); lactase (by the method of Asp & Dahlqvist, 1972); alkaline phosphatase (by the method of Louvard *et al.*, 1973). Proteins were assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

According to the villus-crypt gradient of the marker digestive-enzyme activities established in the first group, sequentially isolated cells could be pooled into villus (V), lower-villus and upper-crypt (VC) and crypt-cell (C) fractions. Limits of the cell compartments were also checked in some experiments by using labelled thymidine as marker of the proliferative cells (results not shown).

Separation and characterization of brush-border proteins

Details of the techniques used have already been described elsewhere (Raul *et al.*, 1978a). Purified brush-border membranes from each cell fraction (V, VC, C) were submitted to polyacrylamide-gel electrophoresis (7.5%, w/v) in the presence of 2% (w/v) sodium dodecyl sulphate. Similar amounts of brush-border proteins (~150 µg) from fractions V, VC and C were layered on to the gels. After electrophoresis the gels were cut longitudinally in two equal halves. A half gel was stained with Coomassie

Brilliant Blue and scanned at 565 nm with a Vernon spectrophotometer. Brush-border enzyme activities were determined in a semi-quantitative manner on 0.3 mm slices of the corresponding half gel. The correspondence of the protein bands and their enzymic activities was established.

Results

Enzymic activities in cells isolated from the villus-crypt axis during the postnatal development

The evolution of maltase, lactase and alkaline phosphatase activities along the villus-crypt axis during postnatal development is illustrated in Fig. 1. That of sucrase activity has been described in a previous paper (Raul *et al.*, 1977). Between 13 and 24 days of postnatal life, maltase showed maximum activity in the intermediate cell fractions, the lowest values being present in the crypt zone. This enzymic pattern was no longer maintained in the adult stage, where maltase activity became the highest in the upper-villus cells and decreased progressively towards the base of the crypts. The activity of lactase was always maximum in the middle part of the villus and low in the crypts. From 17 days onwards, no more activity was detectable in the cells originating from the base of the crypts. A sharp decrease of alkaline phosphatase activity from villus tip to crypt base was observed at all stages studied.

Fig. 1 also illustrates the quantitative variations in specific enzymic activities occurring between 13 days and the adult stage. The highest specific activity of lactase decreased from 50 munits/mg of protein to 3 munits/mg of protein, whereas a progressive increase was noted for maltase (100 to 1200 munits/mg of protein), for alkaline phosphatase (200–2000 munits/mg of protein) and for sucrase (5–100 munits/mg of protein; results not shown). Moreover, the calculation of the total amount of the specific activities of each cell fraction confirmed the above-mentioned results for lactase and sucrase. In contrast, the highest total specific activities of maltase and alkaline phosphatase were attained between postnatal days 21 and 24 and were followed by a decline at the adult stage.

The establishment at each developmental stage of enzymic gradients was necessary firstly to define the three compartments (V, VC and C) along the villus-crypt axis and secondly to allow a direct comparison between the enzymic patterns of the cell homogenates and of the purified brush borders.

Developmental pattern along the villus-crypt axis of enzymic brush-border proteins separated by gel electrophoresis

The protein and enzymic profiles of the purified brush-border membranes were compared in the

Fig. 1. Representative gradients of maltase, lactase and alkaline phosphatase activities in intestinal cells isolated along the villus-crypt axis at different postnatal stages

Enzymic activities were determined in homogenates of successive cell fractions. The ordinate shows specific activities expressed as munits/mg of protein. (One unit of enzymic activity hydrolyses 1 μ mol of substrate/min.) The abscissa shows the percentage of cells isolated in each successive fraction determined by the proportion of cell protein isolated in a given fraction. '100%' of cells isolated corresponds to the sum of the fractions expressed as protein. According to the enzymic gradients obtained, isolated cells could be pooled into three cell compartments: V (villus cells) VC (lower-villus and upper-crypt cells) and C (crypt cells).

above-defined cell compartments as a function of age. The evolution of each enzymic activity and its corresponding protein band of the most characteristic developmental stages is illustrated in Fig. 2.

Lactase. As described previously, a good correspondence between lactase activity and protein band 5 has been regularly observed (Raul *et al.*, 1978a). Up to 17 days, a high lactase activity was present along the whole villus-crypt axis; fraction C showed, however, a lower activity than that found in fractions V and VC. From postnatal day 21 onwards, lactase activity progressively declined in fractions V and VC and completely disappeared in fraction C. It is noteworthy that protein band 5 showed a parallel decrease, but that a distinct protein band or at least a shoulder

persisted in the crypt patterns despite the absence of enzymic activity on and after day 21.

Sucrase-isomaltase. Previous results showed that, in the adult rat intestine, the sucrase-isomaltase complex migrates at two positions corresponding to protein bands 3 and 6 (Raul *et al.*, 1978a). Gels were completely devoid of those activities until day 17, the stage at which weak activities appeared in the crypt (C) compartment. At day 21, sucrase-isomaltase activities also appeared in the VC and V compartments. Afterwards they gradually increased in these two cell compartments, remaining always higher than in compartment C. As soon as the sucrase-isomaltase activities appeared in compartment C (day 17) and in compartments VC and V (day 21), an increase of the

Fig. 2. Evolution of representative protein patterns and enzymic activities (lactase, sucrase and alkaline phosphatase) as a function of age in the three cell fractions V, VC and C (defined in Fig. 1)

Polyacrylamide-gel electrophoresis of the brush-border membranes was performed in 7.5% (w/v) gels in the presence of 2% sodium dodecyl sulphate. Identical quantities of proteins ($\sim 150 \mu\text{g}$) were applied to each gel. Underneath each protein scan, lactase (L), sucrase (S) and alkaline phosphatase (AP) are illustrated. One half-gel was stained with Coomassie Brilliant Blue and scanned at 565 nm with a Vernon spectrophotometer. The ordinate shows the enzymic activities determined in a semi-quantitative manner on 0.3 mm slices of the corresponding half-gel and expressed as absorbance (A). Isomaltase, assayed with an artificial substrate (palatinose), is not illustrated, since it follows exactly the sucrase profiles. The abscissa shows the migration of the high-molecular-weight proteins from the left towards the right; alkaline phosphatase migrates to approximately the middle of the gel.

pre-existing protein band 3 and the transformation of the minor shoulder present in position 6 into a well-defined protein band occurred.

Enzymic shift between lactase and sucrase-isomaltase. Up to 17 days of postnatal life the major protein (band 5) corresponded to lactase activity. Thereafter, protein band 3 associated with sucrase-isomaltase, maltase and glucoamylase activities became the major protein band. This enzymic shift could be clearly shown by considering the protein patterns of the three cell compartments of 17-day-old animals (Fig. 2). Indeed, in compartment V, band-5 protein still remained in greater amount than band-3 protein; in compartment VC, these two proteins were present to nearly the same extent, whereas in compartment C band-3 protein was more prominent than band-5 protein. The modifications in the three protein patterns were correlated with the simultaneous appearance of sucrase-isomaltase activity and the decrease of lactase activity.

Maltase and glucoamylase. These enzymes did not display any marked qualitative or quantitative modifications either in the three compartments of the villus-crypt axis or during postnatal development, and results for them are therefore not shown. Maltase and glucoamylase activities were associated with band-3 protein, and a weak maltase activity could be detected in band-6 protein simultaneously with sucrase-isomaltase activities.

Alkaline phosphatase. It has been described previously, firstly that at 13 days, a single peak of activity migrated with the same relative mobility as protein band 9, and secondly that, in the adult, alkaline phosphatase activity was present in two distinct peaks migrating in a broad region comprising protein bands 8 and 9 without good correlations of the relative mobilities (Raul *et al.*, 1978a). The present study shows the persistence of a single peak of activity in fractions prepared from all three cell compartments of young rats until day 21. From this stage onwards two distinct peaks of activity could be detected in fractions taken along the villus-crypt axis. Moreover, the adult stage is characterized by the presence of two peaks of activity in fractions V and VC but surprisingly of only one peak in fraction C. During the postnatal development, maximal alkaline phosphatase activity roughly increased in all three cell compartments, with generally higher values in compartments V and VC than in compartment C.

Discussion

The intestinal-cell-separation procedure based on successive EDTA treatments has proved to be suitable for the study of postnatal evolution of the following brush-border enzymes: sucrase, alkaline phosphatase, aminopeptidase and enterokinase (Raul *et*

al., 1977). In the present paper, data from this previous study have been extended to two other enzymes involved in the digestion processes: lactase and maltase. Moreover, the developmental patterns of enzymic proteins have been investigated in three well-defined cell compartments determined after analysis of an enzymic gradient along the villus-crypt axis established systematically. The existence of clear-cut differences in enzymic content from one cell compartment to another supports the fact that the cell types of the different fractions were homogeneous, although a possible contamination especially of crypt cells by mature enterocytes cannot be completely excluded. This particular point will be considered later in the discussion. From an experimental point of view the analysis of purified brush-border-membrane proteins along the villus-crypt axis as a function of age has two main advantages over studies on intact cells: (1) in purified brush-border-membrane proteins the enzymic specific activities are approximately 10 times increased (Raul *et al.*, 1978a); (2) the separation by electrophoresis of purified brush-border-membrane proteins permitted observation with high sensitivity and at a molecular level of the age-related enzymic modifications occurring along the villus-crypt axis.

It is well established that lactase activity decreased from birth on through to adult age (Doell & Kretschmer, 1962), but it has never been demonstrated so far that in suckling rats this enzyme is present in crypt cells, although its activity is regularly lower than in villus cells. Furthermore, lactase activity progressively disappeared from the bottom of this cell compartment as soon as 17 days of life (cell homogenates) and at 21 days (purified brush-border proteins). Moreover, at 17 days, the decline of lactase activity in the crypt zone occurred simultaneously with the appearance of sucrase-isomaltase activities. It must be noted that the precise stage of intestinal development at which this enzymic shift occurs may vary from one litter to another. However, our systematic study carried out with 13-, 16-, 17-, 18-, 21- and 24-day-old rats revealed that the first appearance of sucrase-isomaltase activities was the most frequently detectable at the postnatal day 17. We were unable to establish any correlation between the stage of appearance of sucrase-isomaltase activities and the number of animals per litter, but the variability encountered by us could explain the age-related differences reported in the literature (Rubino *et al.*, 1964; Doell *et al.*, 1965; Raul *et al.*, 1977). The analysis of the relative mobilities of brush-border proteins separated by gel electrophoresis indicated a good correlation of protein band 5 with lactase activity as well as of protein bands 3 and 6 with sucrase-isomaltase activities in every cell fraction and at each developmental stage. Generally, the height of the protein peak well reflected the import-

ance of the corresponding enzymic activity. However, the existence of a shoulder or of a small protein band without any corresponding enzymic activity could be sometimes observed. In the case of protein band 6, this may be attributed either to a weak maltase activity or to the presence of an inactive sucrase-isomaltase precursor (Silverblatt *et al.*, 1974; Dubs *et al.*, 1975). Concerning protein band 5, the existence of a co-migrating undefined protein cannot be excluded. Such a remaining protein band deprived of enzymic activity has already been mentioned in human lactase deficiencies (Freiburghaus *et al.*, 1976; Crane *et al.*, 1976; Keding *et al.*, 1978).

The developmental pattern of lactase and sucrase-isomaltase along the villus-crypt axis raises some problems about the regulatory processes involved in the synthesis of these two enzymes. Concerning lactase, one can admit that in the suckling rat this enzyme is synthesized along the whole villus-crypt axis, whereas in the adult its remaining synthesis is restricted to cells located along the villi. Concerning sucrase-isomaltase, the present study not only demonstrated that in the suckling rat an active sucrase-isomaltase complex first appears in crypt cells, but also that some active sucrase-isomaltase can still be observed in this cell compartment through the adult stage. Two questions arise in the interpretation of these results: (1) are the crypt cells able to synthesize active sucrase-isomaltase throughout life of the rat? or (2) are the observed sucrase-isomaltase activities in crypt cells due to a contamination by mature enterocytes? To answer the first question, the following conflicting results from the literature have to be considered. In the suckling rat the precocious induction of sucrase-isomaltase activities by cortisol is mediated via the cells of the crypts (Herbst & Koldovsky, 1972; Henning *et al.*, 1975). In the adult rat, studies on the cellular distribution of brush-border enzymes along the villus-crypt axis by sequential isolation of cells have shown that non-negligible amounts of active sucrase are present in the crypt-cell fraction (Webster & Harrison, 1969; Weiser, 1973; Schneider *et al.*, 1975; Raul *et al.*, 1977). In similar studies using cryostat transversal sections of fresh frozen intestine, results are more variable: either a weak sucrase activity (Fortin-Magana *et al.*, 1970) or a complete absence of this enzyme activity (Dahlqvist & Nordström, 1966; Nordström *et al.*, 1968; De Both & Plaisier, 1974) was found in crypt cells. Furthermore, it has been suggested that in the human (Silverblatt *et al.*, 1974) as well as in the rabbit intestine (Dubs *et al.*, 1975) sucrase protein appeared to be synthesized in crypts and was firmly bound to cell surface as an inactive precursor of the villus enzyme. Our experiments, which show the persistence of sucrase-isomaltase activities in crypt cells at all stages studied, indicate that these cells are able to synthesize active sucrase-isomaltase throughout life.

Moreover, the existence of higher protein amounts in villus than in crypt cells not only gives evidence that there is an activation of a pre-existing protein during migration of cells on to the villus, but argues in favour of continuous enzyme synthesis as cells mature; this has already been suggested after sucrase stimulation by cortisone (Herbst & Koldovsky, 1972). It must also be mentioned that increasing synthesis of brush-border enzymic glycoproteins occurred along the crypt-villus axis as demonstrated by James *et al.* (1971) and Weiser (1973). Concerning the second question, contamination of the crypt-cell compartment by mature enterocytes seems unlikely on the basis of morphological observations by phase-contrast and light-microscopy (results not shown) and of previous results that have clearly shown that crypt cells were completely devoid of enterokinase, a marker enzyme of the villus cells (Raul *et al.*, 1977). Moreover, if contamination exists, lactase activity would be detectable in crypt cells as long as it is present in the villus cells and would not disappear from day 17 onwards.

The study of the variations of maltase activity in the cell homogenates during the postnatal development revealed that maximal activities were reached in the 21–24-day interval (already shown by Rubino *et al.*, 1964, and by Moog *et al.*, 1973). The absence of any marked differences in maltase-activity patterns after gel electrophoresis, especially from one cell compartment to another, contrasting with the existence of an enzymic gradient in the cell homogenates, could be due to the presence of a solubilized brush-border maltase and/or of a lysosomal acid maltase (Galand & Forstner, 1974a; Seetharam *et al.*, 1977) quantitatively higher in fractions V and VC than in fraction C.

Concerning the developmental pattern of alkaline phosphatase, the present data obtained with intact cells and with *p*-nitrophenyl phosphate as substrate fully agree with findings of others (Moog & Yeh, 1973; Raul *et al.*, 1977). Gel electrophoresis allowed us to observe two peaks of activity in fractions taken along the whole villus-crypt axis as soon as at 21 days, only one peak being detectable before this stage. The good correlation between the single peak and protein band 9 and the loss of correlation between the two peaks of activity and well-defined protein bands 8 and 9 suggest that these age-related modifications could be attributed to the transformation of a primitive molecule into two different molecular forms. Moog & Yeh (1973) have already shown that a first class of isoenzymes was present at all stages of development from birth to adulthood, whereas a second class of isoenzymes manifested itself in the third postnatal week, suggesting that alkaline phosphatase differentiation reflects the post-translational modification of a core molecule. The presence of a preweaning situation in the crypts of the adult remains unexplained, but

constitutes a further argument against villus-cell contamination of this compartment. Such a situation sets the problem about the factors controlling the synthesis of alkaline phosphatase. Elucidation of the nature of the two molecular forms and their relation to each other at different postnatal stages needs further investigation.

The methodology developed in the present study yielded new information about the differentiation of brush-border enzymes along the villus-crypt axis during normal development of the rat. It will help to further characterize the molecular events involved in brush-border-enzyme synthesis under the influence of regulatory factors such as hormones or/and nutritional substrates.

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