Acta vet. scand. 1980, *21*, 402—414.

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HISTOCHEMICAL DISTRIBUTION OF ENZYMES IN THE SMALL INTESTINE OF YOUNG MILK-FED CALVES*

By

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LANDSVERK, THOR: *Histochemical distribution of enzymes in the small intestine of young milk-fed calves.* Acta vet. scand. 1980, *21*, 402—414. — The histochemical distribution of selected enzymes were examined in the small intestine of 5 about 3-week-old normal calves fed on whole cow's milk. Alkaline phosphatase and β-D-galactosidase (= lactase) in the epithelial brush border, and non-specific esterase in the cytoplasm showed a strong reaction in the villi of the anterior small intestine and a marked decrease in the posterior regions. Aminopeptidase in the brush border of the villi showed a reverse distribution, with the strongest reaction in the posterior small intestine. Adenosine-triphosphate-splitting enzyme in the epithelial brush border, acid phosphatase and succinate dehydrogenase in the cytoplasm of the epithelial cells gave a relatively uniform reaction in the villi throughout the small intestine. A fluoride-resistant acid phosphatase was demonstrated in the brush border of the villi in the anterior small intestine. The distribution of enzymes demonstrated in this study was generally compatible with the known absorptive functions of the various parts of the small intestine.

enzyme histochemistry; small intestine; epithelial cells; milk diet; calves.

Enzyme histochemical studies of the small intestine in domestic animals have revealed a differentiated enzyme pattern in the epithelial cells suited to their absorptive function (*Johnson & Kugler* 1953, *Fowler & Rohovsky* 1970, *Saloniemi & Rahko* 1972, *Hornich et al.* 1974). In the calf there have been some biochemical studies on the distribution of enzymes in the small intestine, but histochemical studies have been rare. *Abel et al.* (1972) studied some phosphatases in the ileum of the calf. The present work

* This study was supported by grants from the Agricultural Research Council of Norway.

originated in studies of diarrhoea in 2—4-week-old calves. As a basis for an enzyme histochemical approach to the problem it was necessary to define a normal state of the small intestine. The purpose of the present report is to describe the distribution of some enzymes throughout the small intestine in normal, about 3-week-old, calves fed on whole cow's milk.

MATERIALS AND METHODS

Tissue specimens were obtained from the small intestine of 5 healthy, 17-, 20-, 21-, 22- and 23-day-old calves fed on whole cow's milk, 110 ml/kg body weight/day. Four of these were included in a previous paper (*Landsverk* 1979). Details on diet and sampling procedures are given in the previous report. For enzyme histochemical studies, frozen sections of unfixed specimens cut at 8 μ in a cryostat were used. Fixation of sections, when used, was in 10 % neutral formol-calcium for 5 min at 4°C. The enzyme histochemical procedures always included heat-inactivated controls (80°C in aqua dest. for 10 min) besides the other control measures described under each method. The following enzymes were studied:

Alkaline phosphatase (EC 3.1.3.1) was demonstrated by Gomori's calcium-cobalt method (*Pearse* 1968). Mostly unfixed sections were used. Incubation was usually for 20 min at 37°C. Controls included inhibition of activity with 10 mM L-cystein and 50 mM L-phenyl-alanin.

Adenosine triphosphate (ATP) splitting enzyme was demonstrated by a modification of the *Wachstein & Meisel* (1957) technique (*Jacobsen & Jørgensen* 1969). The concentrations of the reagents were: 3 mM ATP, 80 mM Tris [tris- (hydroxymethyl-) aminomethan] (pH 7.2), 3.6 mM lead nitrate and 3 mM magnesium sulphate. Fixed sections were used, and incubation was mostly for 15 min at room temperature. The inhibitor tested was 100 mM sodium fluoride. Other control measures included omission of magnesium from the incubation medium and replacement of the substrate with equimolar concentration of adenosine diphosphate (ADP) or β-glycerophosphate. With the magnesium-deficient media, demineralized water was always used.

Acid phosphatase (EC 3.1.3.2) was demonstrated by Gomori's lead nitrate method (*Pearse* 1968). Fixed sections were used, and alternatively to fixation in formol-calcium, some sections were fixed in acetone at 4°C for 1 min. The procedure included

the following modification of the technique: 10 mM β-glycerophosphate was dissolved in 50 mM acetate buffer (pH 5.0) at 37°C. When the solution was clear, 4 mM lead nitrate was added to the medium, which was allowed to stand for 30 min at 37°C. The solution was then filtered, and incubation of sections mostly for 45 min at 37°C followed immediately. The inhibitor tested was 10 mM sodium fluoride.

β-D-galactosidase (EC 3.2.1.23) was demonstrated by the indigogenic method (*Lojda & Kraml* 1971), using 4-Cl-5-Br-3-indolyl-β-D-fucosid. As a rule unfixed sections were used. Incubation was usually for 4 h at 37°C.

Aminopeptidase (EC 3.4.11.2) was demonstrated by the azo-coupling method (*Lojda et al.* 1976). The substrates used were L-alanyl-β-naphtylamid and L-leucyl-β-naphtylamid, with hexazonium-p-rosaniline as the capturing agent. Unfixed sections were used, and incubation was mostly for 20 min at room temperature.

Non-specific esterase was demonstrated by the azo-coupling method (*Pearse* 1972), with α naphtyl acetate as substrate and hexazonium-p-rosaniline as the capturing agent. Unfixed sections were used, and incubation was for 15 min at room temperature.

Succinate dehydrogenase (EC 1.3.99.1) was demonstrated with nitro blue tetrazolium (*Pearse* 1972). Unfixed sections were used, and incubation was for 1 h at 37°C.

RESULTS

The average regional enzyme reaction in the epithelial cells is given in Table 1.

Alkaline phosphatase gave a strong reaction in the brush border of villous enterocytes in the duodenum, anterior and middle jejunum (Fig. 1). A weaker reaction was also found in the supranuclear cytoplasm, possibly corresponding to the Golgi region (Fig. 2). In the posterior 1/4 of the small intestine only scattered areas of the villous brush border were positive (Fig. 3). L-cystein and L-phenyl-alanine in the concentration used inhibited the activity.

ATP-splitting enzyme was strong in the brush border of epithelial cells along the villi throughout the small intestine (Figs. 4—8). A weak to moderate staining was present in the crypt cell brush border, with the strongest reaction in the apical crypt portion. A weak staining was seen also at the other sides

Table 1. Average regional enzyme reaction detected histochemically in the epithelial cells of the calf small intestine.

Enzymes	Middle duodenum (d2)		Anterior jejunum (aj2)		Middle jejunum (mj)		Posterior jejunum (pj1)		Posterior jejunum (pj3)	
	villus	crypt	villus	crypt	villus	crypt	villus	crypt	villus	crypt
ALP	+++[1]	0	+++	0	++	0	+	0	+/0	0
ATP s.e.	+++	+/+++	+++	+/+++	+++	+/++	++	0/++	+++	0/++
ACP	++	+	++	+	++	+	++	+	+++	0/+
β-D-gal	+++	0/++	+++	0/++	++	0	+	0	0/+	0
Am	0	0	0	0	++	0	++	0	+++	0
SDH	++	++	++	++	++	+	++	+	++	+
NSE	+++	+	+++	+	++	+	++	+	+	0

[1] Key: +++ = strong; ++ = moderate; + = weak; 0 = no reaction; / = variation of the reaction, see text for further explanation.

ALP = Alkaline phosphatase
ATP s.e. = adenosine triphosphate splitting enzyme
ACP = acid phosphatase
β-D-gal = β-D-galactosidase
Am = aminopeptidase
SDH = succinate dehydrogenase
NSE = non-specific esterase

of the epithelial cell membranes (Fig. 4). Replacement of substrate with equimolar concentration of ADP gave results similar to those obtained with ATP. Replacement by β-glycerophosphate or inhibition by 100 mM sodium fluoride gave a distribution of staining almost corresponding to that of alkaline phosphatase (Figs. 5—8). The results of the control study are summarized in Table 2.

Table 2. Control studies of the phosphatase reactions recorded by the modified Wachstein & Meisel procedure (*Jacobsen & Jørgensen* 1969). Incubation was for 15 min.

Substrate (3.0 mM)		Middle duodenum (d2)	Posterior jejunum (pj3)
ATP	Villus	+++[1]	+++
	Crypt	+/+++	0/++
ADP	Villus	+++	+++
	Crypt	+/+++	0/++
β-glyc	Villus	++	0/+
	Crypt	0	0
ATP without $MgSO_4$	Villus	++	+
	Crypt	0	0
ATP + 100 mM NaF	Villus	++	+
	Crypt	0	0

[1] Key: +++ = strong; ++ = moderate; + = weak; 0 = no reaction; / = variation of the reaction, see text for further explanation.

ATP = adenosine triphosphate
ADP = adenosine diphosphate
β-glyc = β-glycerophosphate

Acid phosphatase was shown as a granular deposit confined to an area in the supranuclear cytoplasm of the enterocytes (Figs. 9, 10). In the duodenum, where a closer interpretation of the localization of the reaction product was possible, owing to the greater cell height, the reaction was seen close to the cell nucleus, possibly corresponding to the Golgi region (Fig. 9). In the duodenum and anterior jejunum staining was also found in the villous brush border region (Fig. 9). The brush border reaction was weak at the tips of the villi. The staining in the supranuclear cytoplasm was inhibited by fluoride, while the brush border staining was resistant to fluoride. In the lamina

propria some cells showed a fluoride-sensitive acid phosphatase reaction (Fig. 10). They were interpreted as macrophages.

In general β-D-galactosidase was strong in the brush border of villous enterocytes in the anterior small intestine (Fig. 11). In the duodenum apical villi staining was weak, the intensity of staining reaching a maximum on the sides of the villi. A weak to moderate reaction was also seen in the crypts, with the strongest staining in the apical portion. In the anterior and middle jejunum the villi were stained evenly and a slight crypt staining was present, whereas the posterior jejunum had a progressively weaker reaction, the staining being present only at the tips of the villi (Fig. 12).

The aminopeptidase reaction was moderate to strong in the brush border of enterocytes in the villi of the middle and posterior small intestine (Fig. 13), while the anterior parts of the small intestine were negative. The staining was not seen in the crypts.

Succinate dehydrogenase showed a moderate reaction in the supra- and infranuclear cytoplasm of epithelial cells in the entire crypt-villous unit (Fig. 14). The reaction was relatively uniform throughout the small intestine.

Non-specific esterase was strong in the apical cytoplasm of the villous enterocytes in the anterior small intestine (Fig. 15). The crypt reaction was weak. In the posterior small intestine a progressively weaker reaction occurred, the staining being strongest towards the tips of the villi (Fig. 16). Non-specific esterase-positive leukocytes of the lamina propria were interpreted as macrophages.

DISCUSSION

The histochemical distribution of enzymes in the calf small intestinal epithelium seemed to follow a certain pattern. The anterior and the posterior parts of the small intestine appeared to have the most uniform enzyme activities, while a portion of the middle small intestine showed steeply decreasing or increasing reactions of alkaline phosphatase, β-D-galactosidase, non-specific esterase, and aminopeptidase. The anterior and posterior areas are therefore to be preferred in the selection of sites for comparative investigations. The varying enzyme reactions in the different portions of the small intestine suggest

that the functions of these areas are different and may be correlated to the activity of each enzyme.

Alkaline phosphatase may catalyze hydrolysis of phosphorylated compounds which otherwise would not readily penetrate cell membranes (*Crane* 1968). *Moog & Glazier* (1972) showed that phosphatase activity was necessary for the utilization of phosphate from organic phosphate esters. It is possible that alkaline phosphatase may act as transferase in the cell membrane (*Morton* 1958). Alkaline phosphatase apparently plays a role in fat absorption (*Linscheer et al.* 1971), but the exact mechanism is unknown. At the ultrastructural level, alkaline phosphatase has been localized to the villous brush border and to the Golgi region in the epithelial cells (*Hugon & Borgers* 1966). This seems to be in accordance with the present results. With the technique used in the present study, however, no firm conclusion with regard to the subcellular localization of enzymes is justified. The anterior—posterior gradient for the alkaline phosphatase found in this study is in agreement with biochemical assays in the calf (*Benz & Ernst* 1976) and with histochemical studies in the cat, dog and rat (*Johnson & Kugler* 1953). On the other hand a strong ileal reaction has been reported in pigs (*Saloniemi & Rahko* 1972, *Hornich et al.* 1974). The anterior—posterior gradient found for alkaline phosphatase in the calf coincides with the known heavy absorption of nutrients in the calf anterior small intestine (*Mylrea* 1966).

The intestinal epithelium has a membrane Na-K-ATPase supposed to perform the coupled active transport of sodium and potassium (*Berg & Chapman* 1965). However, the relation of the Na-K-ATPase to the Mg-ATPase demonstrated by the Wachstein & Meisel technique is uncertain. Objections have been made to the Wachstein & Meisel technique because of non-enzymatic hydrolysis of ATP caused by the lead nitrate (*Rosenthal et al.* 1966) and because lead has been claimed to inhibit a large percentage of the ATPase activity (*Moses et al.* 1966). *Jacobsen & Jørgensen* (1969) showed that lead in the concentration used (3.6 mM) definitely inhibited the Na-K-ATPase, but only partially inhibited the Mg-ATPase that might be demonstrated in the cell membranes. The modification of the Wachstein & Meisel technique used in this study included an increase of the ATP concentration (3 mM versus 0.83 mM) and a decrease of magnesium concentration (3 mM versus 10 mM) as compared with

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Figure 1. Alkaline phosphatase, middle duodenum (d2). Strong reaction in the villous brush border. Note the abrupt disappearance of staining at the crypt-villus junction. Lightly counterstained with hematoxylin. × 150.

Figure 2. Alkaline phosphatase, middle duodenum (d2). Strong reaction in the villous brush border. Note the granular staining in the supranuclear cytoplasm of the enterocytes (arrows), probably corresponding to the Golgi region. Lightly counterstained with hematoxylin. × 150.

Figure 3. Alkaline phosphatase, posterior jejunum (pj2) outside Peyer's patches. The brush border staining is discontinuous and occurs only in the upper half of the villi. Positive leukocytes in the lamina propria. In the upper part of the micrograph the supporting liver specimen shows reaction in the bile canaliculi. Lightly counterstained with hematoxylin. × 150.

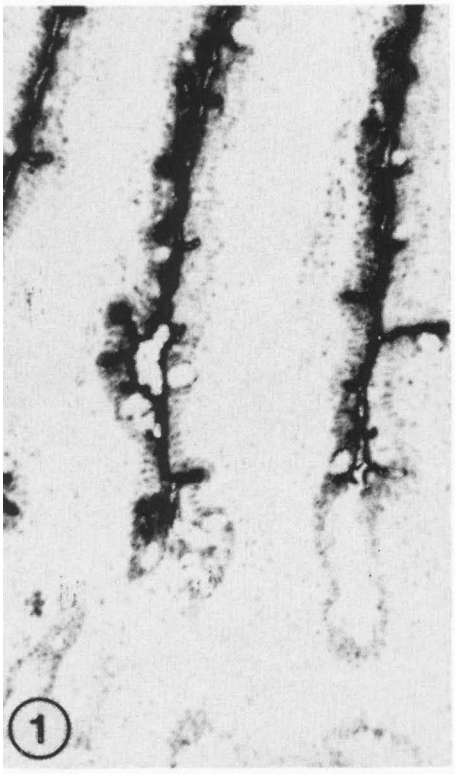
Figure 4. ATP-splitting enzyme, middle duodenum (d2). Strong reaction in the villous brush border. The lateral and basal sides of the enterocytes are stained weakly. The crypts show staining of the brush border. No counterstain. × 150.

Figure 5. Control incubation, complete ATP medium, middle duodenum (d2), incubation for 15 min. The section is cut obliquely. There is an increase in brush border staining from the bottom to the top of the crypts. The villous brush border reacts strongly. × 150.

Figure 6. Control incubation, complete ATP medium + 100 mM NaF, section from the same specimen as Fig. 5, incubation for 15 min. The brush border of the villi is moderately positive. No crypt staining. No counterstain. × 150.

Figure 7. Control incubation, complete ATP medium, posterior jejunum (pj3) over Peyer's patches, incubation for 15 min. Strong staining of the brush border and weak staining of the basolateral epithelial cell membranes. The supporting liver specimen above the villi shows staining of the bile canaliculi. No counterstain. × 150.

Figure 8. Control incubation, magnesium-deficient ATP medium, section from the same specimen as Fig. 7, incubation for 15 min. Note the decrease in brush border staining as compared with Fig. 7. No staining of the basolateral cell membranes of the enterocytes. No counterstain. × 150.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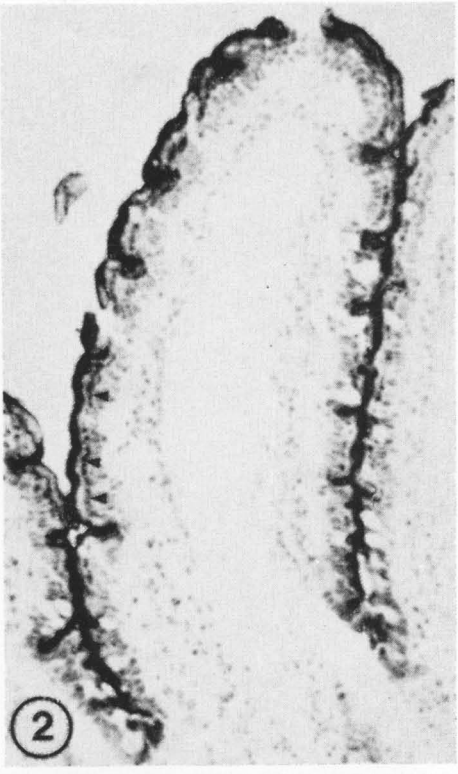
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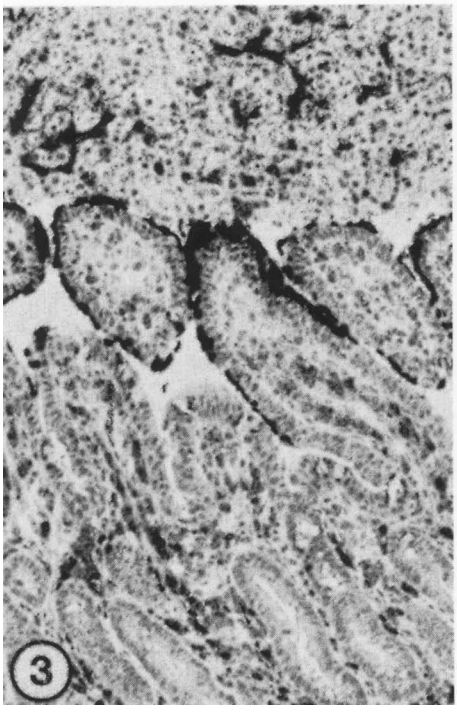
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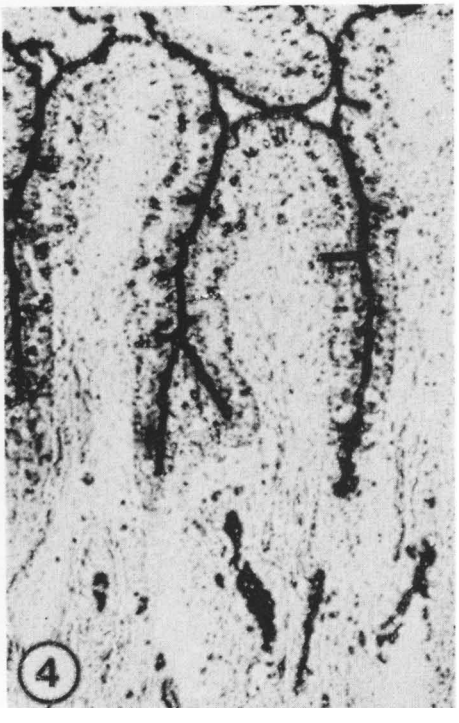
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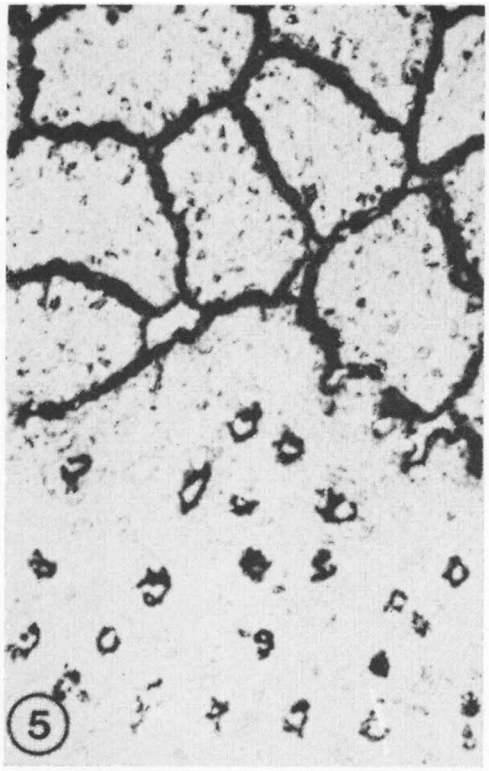
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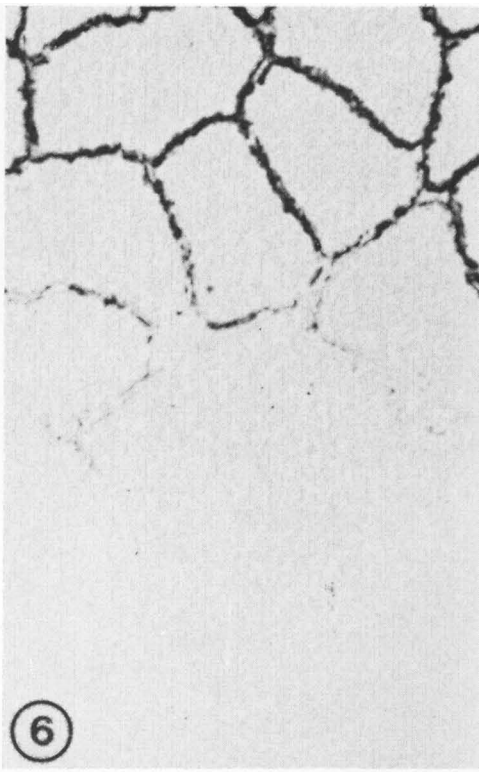
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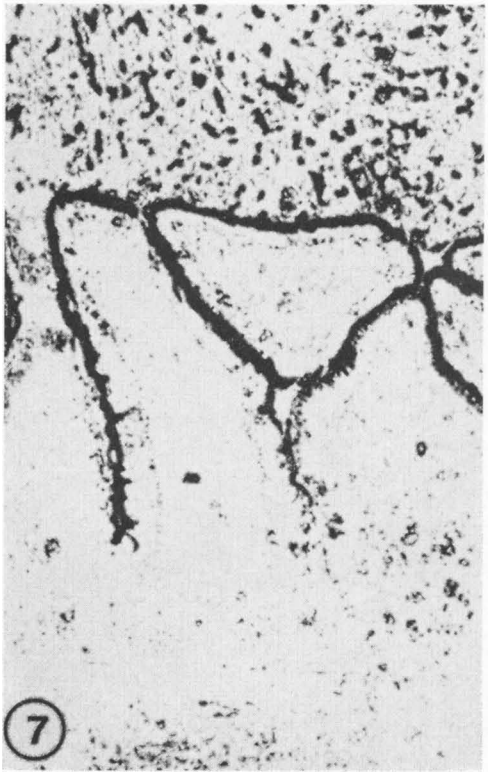
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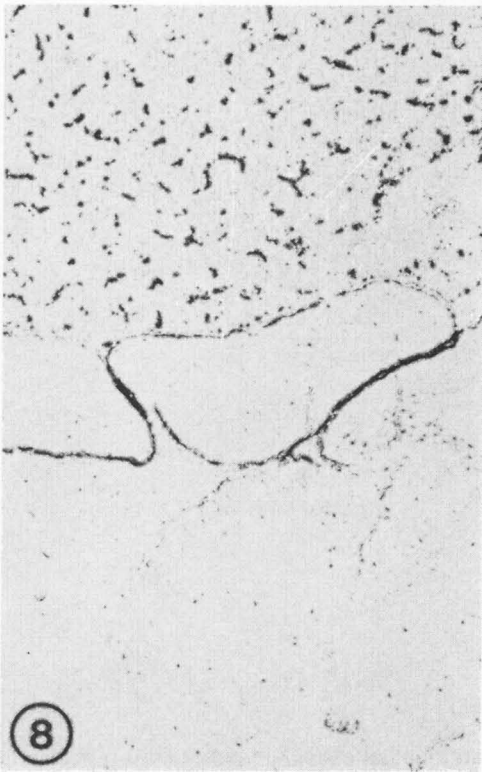
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Figure 9. Acid phosphatase, anterior duodenum (d1). A linear staining in the villous brush border and a granular staining in the supranuclear cytoplasm of the epithelial cells (arrows); the localization near the nuclei suggests a reaction in the Golgi region. The linear staining in the brush border is weak at the villous tips. Acid phosphatase positive leukocytes in the lamina propria are probably macrophages. Lightly counterstained with hematoxylin and eosin. × 150.

Figure 10. Acid phosphatase, posterior jejunum (pj3), outside the Peyer's patches. A granular staining in the apical epithelial cell cytoplasm, the whole crypt-villus unit being positive. Positive leukocytes, probably macrophages, in the lamina propria. No counterstain. × 150.

Figure 11. β-D-galactosidase, anterior duodenum (d1). Strong reaction along the sides of the villi. Apical crypts (Cr) show less staining, whereas apices of villi are almost negative. The enzyme is probably located only in the brush border, the cytoplasmic staining probably being due to diffusion. Slightly counterstained with neutral red. × 150.

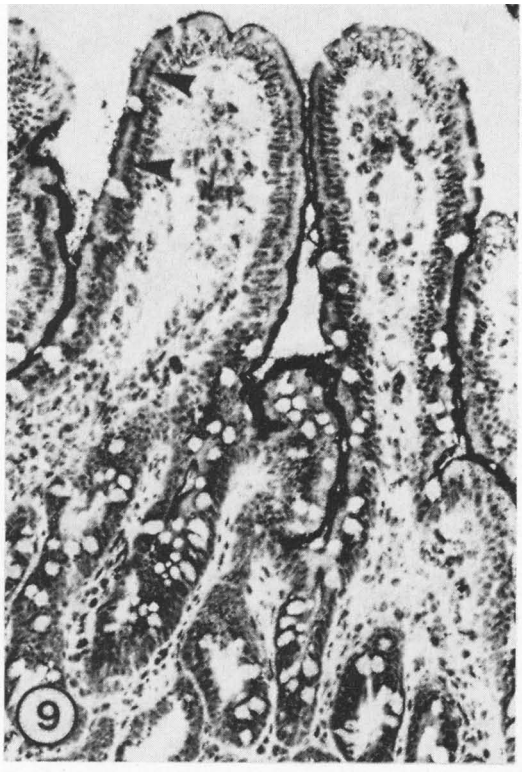
Figure 12. β-D-galactosidase, posterior jejunum (pj1). Positive staining only in the upper half of the villi. Counterstained with neutral red. × 130.

Figure 13. Aminopeptidase with L-alanyl-β-naphtylamid as substrate, posterior jejunum (pj1). Strong reaction in the villous brush border. No reaction in the crypts. No counterstain. × 150.

Figure 14. Succinate dehydrogenase, posterior jejunum (pj1). Reaction in the cytoplasm of the epithelial cells in the entire crypt-villus unit. No counterstain. × 150.

Figure 15. Non-specific esterase with α-naphtyl-acetate as substrate, middle duodenum (d2). Strong reaction in the apical epithelial cell cytoplasm in the villi. Only weak reaction in the crypts. No counterstain. × 150.

Figure 16. Non-specific esterase with α-naphtyl-acetate as substrate, posterior jejunum (pj1). Reaction only in the upper half of the villi. Positive leukocytes of the lamina propria are interpreted as macrophages. The supporting liver specimen in the upper part of the micrograph shows a strong staining of the hepatocytes. No counterstain. × 150.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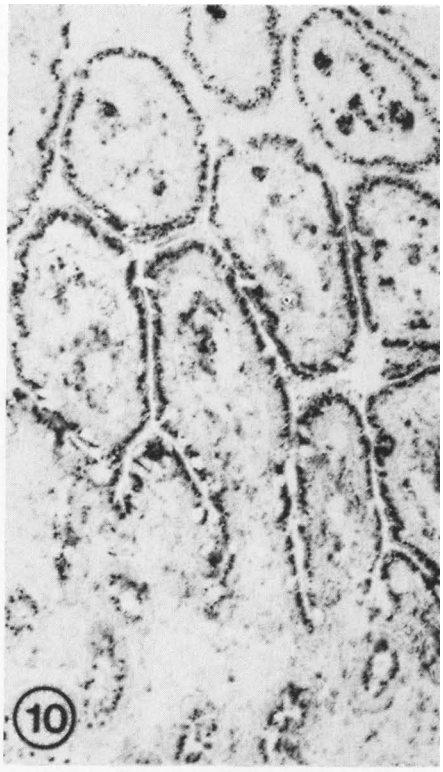
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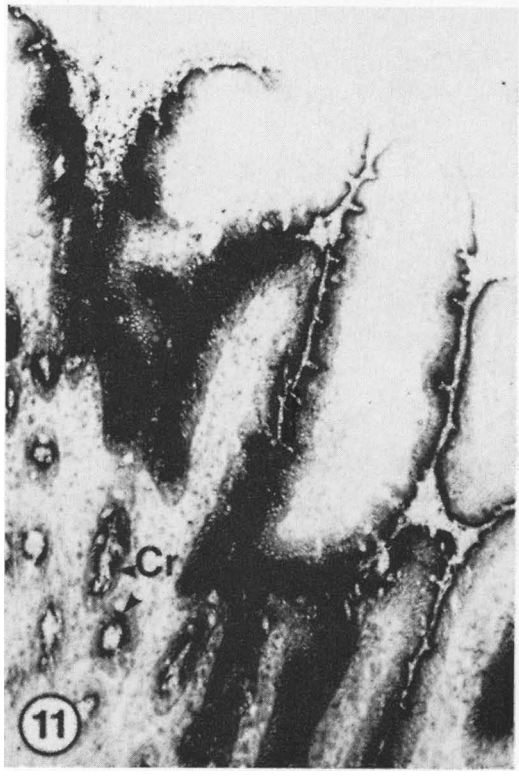
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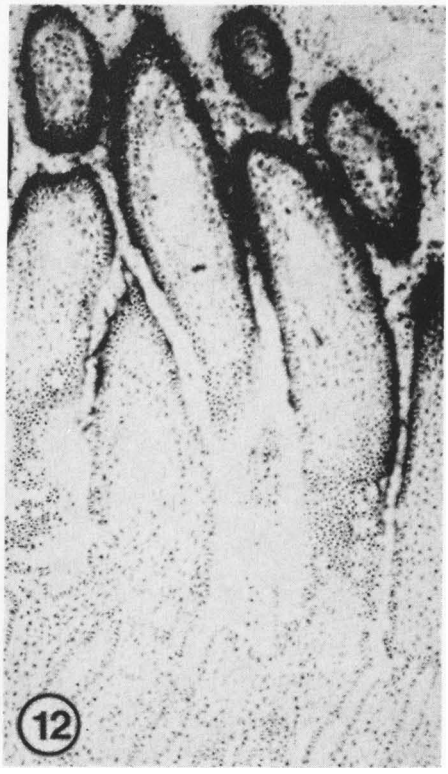
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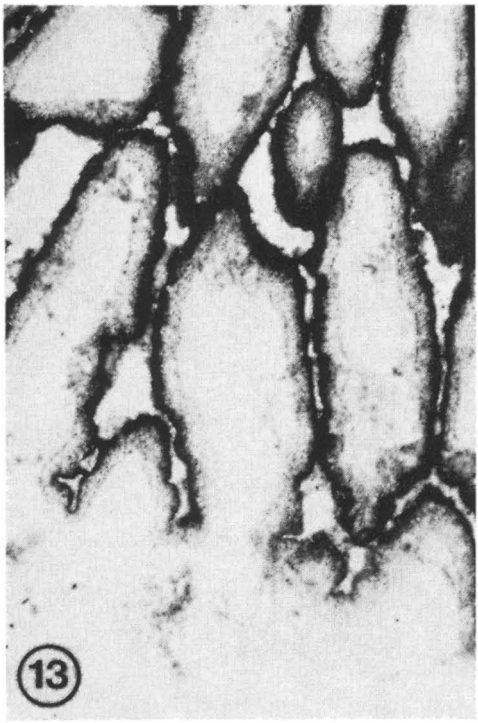
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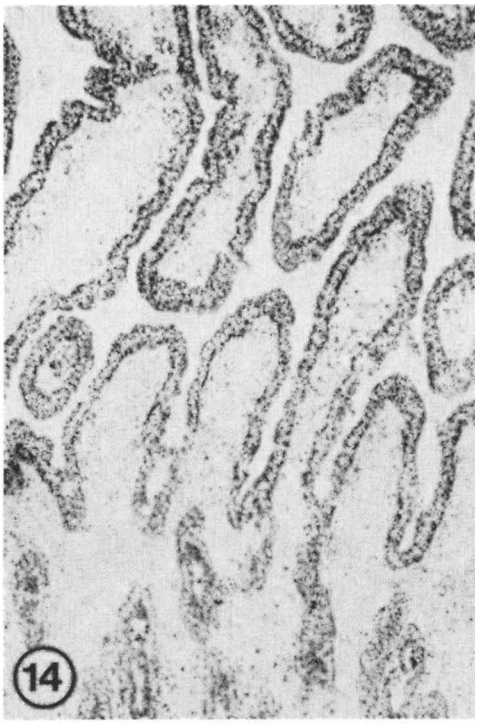
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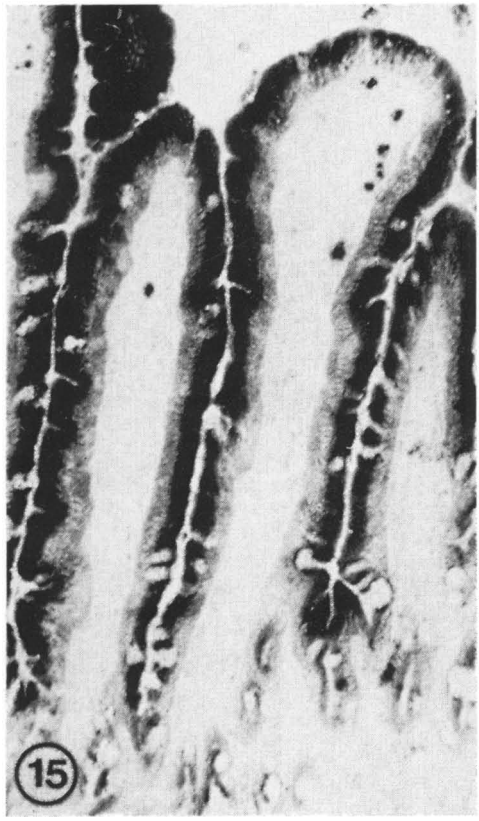
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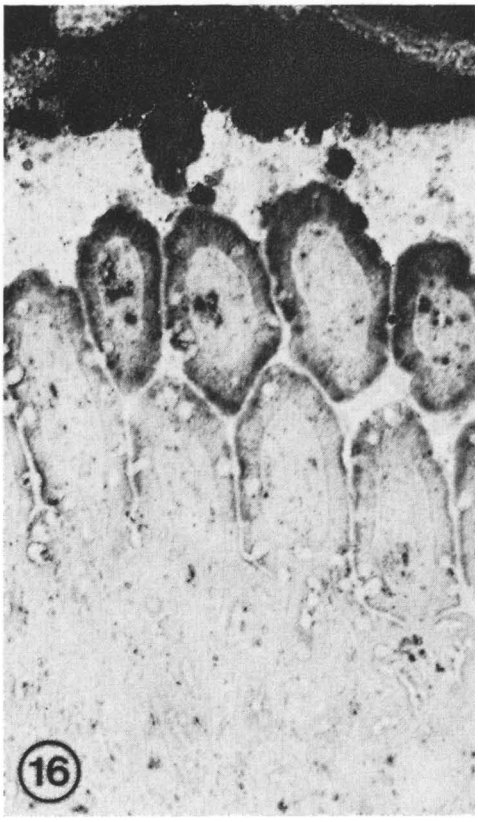
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the original technique. These changes are claimed to enhance enzymatic hydrolysis of ATP and lower lead-catalyzed hydrolysis of ATP (*Jacobsen & Jørgensen*). It is known that several phosphatases, including alkaline phosphatase, may participate in the splitting of ATP (*Abel* 1969). Such a participation by alkaline phosphatase probably was the case in the anterior and middle jejunum in the present investigation. Replacement of the substrate by β-glycerophosphate revealed a distribution of staining almost similar to that of alkaline phosphatase. A similar distribution was found in magnesium-deficient ATP media, indicating the participation of an enzyme less dependent on magnesium than the Mg-ATPase, presumably the alkaline phosphatase. However, the ATP-splitting reaction in the posterior jejunum was probably due to the activity of a nucleoside phosphatase since alkaline phosphatase activity was very low in this region. Lead-catalyzed hydrolysis of ATP was controlled in magnesium-deficient media. With these media no crypt staining was demonstrated, indicating the absence of any significant lead-catalyzed hydrolysis under these conditions and the presence of a real magnesium-dependent nucleoside phosphatase in this region. The enzyme histochemical localization of an ATP-splitting enzyme to the brush border is in accordance with other reports (*Fric & Lojda* 1964, *Fowler & Rohovsky* 1970, *Hornich et al.*). Localization of the enzyme to the plasma membrane by the modified or unmodified Wachstein & Meisel procedure has likewise been demonstrated in several other types of epithelia (*Goldfischer et al.* 1964, *Abel* 1969, *Jacobsen & Jørgensen, Russo & Wells* 1977, *Gossrau* 1978). The even distribution of the ATP-splitting enzyme reaction between the various sites of the small intestine described in the present report is in agreement with that described in piglets (*Hornich et al.*).

Acid phosphatase has been localized to the lysosomes of the intestinal epithelial cells by means of electron microscopic cytochemistry (*Barka* 1964). In the present study the duodenum, somewhat unexpectedly, showed reaction in the supposed Golgi region and not in the most apical cell portion. This may be explained by the heavy fat absorption taking place in these epithelial cells (*Landsverk* 1979), since oil administration has been shown to cause a redistribution of the enzyme in rat (*Barka*). However, acid phosphatase activity has also been described in the Golgi region (*Hugon & Borgers* 1968, *Ono* 1979). The fluoride-

resistant acid phosphatase found in the brush border of the duodenum in the present study parallels similar observations in mice (*Burt et al.* 1957) and rats (*Hugon & Borgers* 1968, *Ono*). *Burt et al.* suggested that the fluoride-resistant acid phosphatase of the brush border is due to a "tail" of the alkaline phosphatase rather than the acid variety. The distribution of acid phosphatase is otherwise largely in accordance with reports of studies in man (*Dawson & Pryse-Davies* 1963), germ-free cats (*Fowler & Rohovsky*) and piglets (*Hornich et al.*).

In biochemical subcellular fractionation studies β-D-galactosidase has been localized to the epithelial brush border (*Eichholz* 1969), its activity being to hydrolyse lactose. The distribution between different intestinal portions described in the present paper is in agreement with biochemical findings in the calf (*Toofonian et al.* 1974), and histochemical analysis in rats, monkeys and man (*Lojda & Kraml* 1971). The anterior-posterior gradient seems to be in agreement with a study of absorption (*Mylrea*), which established a similar gradient for the absorption of reducing substances. The decline of the reaction towards the tips of the villi found in the present report has also been described in rats (*Nordström et al.* 1968) and piglets (*Hornich et al.*). Reliable information of enzyme activities at the tips of the villi has been difficult to obtain, owing to inherent discrepancies of the techniques, e.g. damage of the membranes (*Nordström et al.*). In the present study the apical portions of the villi were protected by a supporting liver specimen, and it is presumed that damage to the cell membranes was prevented that way. Accordingly, the weak reaction at the tips of the villi may be interpreted as real.

Leucine aminopeptidase has also been found in the brush border fraction in biochemical studies (*Eichholz*), its activity probably being hydrolysis of smaller peptides. In the present study aminopeptidase showed a conspicuous anterior-posterior gradient, in contrast to the even distribution reported in man (*Dawson & Pryse-Davies*), pigs (*Hornich et al.*) and hamsters (*Andrews* 1973). The distribution of the aminopeptidase is difficult to explain in terms of absorption, even if the absorption of proteins seems to be more uniform throughout the small intestine than the absorption of reducing substances and lipids (*Mylrea*).

Non-specific esterase and succinate dehydrogenase have been associated with the endoplasmic reticulum and mitochondria,

respectively (*Roodyn* 1965). The distribution of these enzymes found in the present material is in accordance with findings in piglets (*Hornich et al.*). In man and hamsters, however, an even distribution of non-specific esterase has been found (*Dawson & Pryse-Davies, Andrews*). The endoplasmic reticulum is the most important subcellular structure for glyceride biosynthesis (*Hübscher et al.* 1962), and the regional distribution of non-specific esterase described in the present report is in agreement with the known pattern for fat absorption (*Mylrea, Landsverk*).

In conclusion, the calf small intestine shows a definite segmental organization for most of the enzymes examined. The pattern coincides with the major functional aspects of the various segments; i.e. absorption of the largest part of nutrients in the anterior parts of the small intestine and absorption of salt and water in the posterior parts (*Mylrea*).

ACKNOWLEDGEMENTS

I want to thank the Department of Pathology, Ullevål Hospital, and particularly E. Jaregg, for helping me with the β-D-galactosidase procedure. I am indebted to dosent Dr. med. H. A. Dahl, the Anatomical Institute, University of Oslo for constructive criticism of the manuscript.

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SAMMENDRAG

Histokjemisk fordeling av enzymer i tynntarmen hos unge melkefôrede kalver.

Den histokjemiske fordeling av visse epiteliale enzymer ble studert i tynntarmen hos 5 omkring 3 uker gamle kalver. Kalvene var friske og ble fôret med helmelk. Alkalisk fosfatase og β-D-galaktosidase (= laktase) i „børstesømmen" av epitelcellene langs villi samt uspesifikk esterase i epitelcellenes cytoplasma ga en sterk reaksjon i fremre tynntarmsavsnitt med en markert nedgang mot bakre avsnitt. Aminopeptidase i „børstesømmen" langs villi viste en motsatt fordeling og sterkest reaksjon i bakre avsnitt. Adenosintrifosfat spaltende enzym i „børstesømmen" langs villi samt sur fosfatase og oksalsyredehydrogenase i epitelcellenes cytoplasma viste en relativt jevn fordeling mellom tarmavsnittene. En fluoridresistent sur fosfatase ble registrert i „børstesømmen" av villi i fremre tynntarmsavsnitt. Fordelingen av enzymer som er beskrevet i denne studien synes generelt å være i samsvar med de kjente forhold når det gjelder den absorptive funksjon av de forskjellige tynntarmsavsnitt.

(Received May 27, 1980).

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