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AN ENZYME HISTOCHEMICAL INVESTIGATION OF THE INTESTINAL MUCOSA IN DIARRHEIC CALVES*

By

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LANDSVERK, T.: *An enzyme histochemical investigation of the intestinal mucosa in diarrheic calves.* Acta vet. scand. 1981, 22, 449—458. — Selected enzymes were examined in the small intestine of twelve 2—5 week-old calves, 8 with diarrhea and 4 convalescents. The diarrheic calves showed a reduction of enzyme reactions mainly in the duodenum and middle small intestine, and the crypt reactions appeared most severely affected. In the duodenum, villous alkaline phosphatase, adenosine triphosphate-(ATP)-splitting enzyme, and β-D-galactosidase were reduced in 3 calves; the reaction in the corresponding crypts was decreased in 6 calves for the ATP-splitting enzyme and in 4 calves for the β-D-galactosidase. Six calves showed decrease of villous brush border acid phosphatase, and 3 of villous non-specific esterase. In the middle jejunum, villous ATP-splitting enzyme was reduced in 3 calves, while 5 showed decrease of the corresponding crypt reaction. Convalescents had no enzyme reduction in the duodenum, whereas 1 showed marked reduction of the ATP-splitting enzyme and aminopeptidase in the middle and posterior jejunum. The decreased enzyme reactions in the present material may be caused by immaturity of epithelial cells associated with regenerative crypt hyperplasia and/or microbial destruction of enzymes.

enzyme histochemistry; small intestine; villous atrophy; diarrhea; calves.

Diarrhea has been associated with a range of structural and functional changes in the intestinal mucosa. Enzyme studies have given important insights into the pathogenetic mechanisms operating during different forms of diarrhea (*Lojda* 1974). In calves with certain diarrheic conditions biochemical studies have revealed a decrease in the activity of lactase and phosphatases (*Bywater & Penhale* 1969, *Abel et al.* 1972, *Benz & Ernst* 1976,

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Table 1. Average regional enzyme reaction detected histochemically in the small intestinal epithelial cells, average ratios of villous length to crypt depth (v:c), and clinical status of the calves.

Diet		B1					
Calf No.		1	2	3	4	5	6
Duodenum (d2)							
ALP	Villus	+*	+++	++	++	+++	+++
ATP-	Villus	++	+++	+++	+	+++	+++
s.e.	Crypt	0/+	0/++	+/+++	0/+	+/+++	+/+++
ACP	Villus BB	+	++	+	+	+++	+++
	Villus Cy	+++	++	+++	+++	++	++
β-D-gal	Villus	++	+++	+++	++	+++	+++
	Crypt	0/+	0/++	0/+	0/+	0/++	0/++
NSE	Villus	+	+++	+++	+	+++	+++
	v:c	1.1	2.8	1.1	0.7	1.7	1.8
Middle jejunum							
ATP-	Villus	+++	++	+++	+++	+++	+++
s.e.	Crypt	+/++	0/+	+/++	0/+	0/++	+/++
	v:c	1.1	1.5	2.0	1.3	1.3	2.4
Posterior jejunum (pj3)/ileum							
ATP- s.e.	Villus	+++	+	+++/++	+++	+++	+++
Am	Villus	+++	+++/+	+++/+	+++	+++	+++
	v:c (pj3)	1.4	1.0	0.6	0.8	1.5	1.3
Clinical status		D	D	D	D	C	C

ALP = alkaline phosphatase
ATP s.e. = adenosine triphosphate splitting enzyme
ACP = acid phosphatase
β-D-gal = β-D-galactosidase
Am = aminopeptidase
NSE = non-specific esterase
BB = epithelial brush border
Cy = epithelial supranuclear cytoplasm
D = diarrhea
C = convalescent
N = normal, no diarrhea

Table 1 (continued).

B2						Controls (WM)
7	8	9	10	11	12	17—21
+++	+++	+++	+++	+++	+++	+++
+++	++	ND	+++	+++	+++	+++
+/++	0/+	ND	0/++	+/+++	+/+++	+/+++
++	+	ND	+++	+++	+++	+++
+++	++	ND	++	++	+++	++
+++	++	+++	+++	+++	+++	+++
0/++	0/+	0/++	0/++	0/++	0/++	0/++
+++	++	ND	+++	+++	+++	+++
						$\bar{x}$ s
1.5	1.5	1.4	2.1	2.0	1.3	3.6±1.0
++	++	+++	+++	+++	++	+++
0/+	0	0/++	+/++	+/++	0/+	+/++
						**$\bar{x}$ s
1.5	2.6	1.5	3.0	2.1	1.6	3.6±0.5
+++	+++	+++	+++	+++	++/+	+++
+++	+++	+++	+++	+++	+++/++	+++
						$\bar{x}$ s
1.9	2.1	1.3	1.0	0.7	0.5	1.8±0.3
D	D	D	D	C	C	N

* +++ = strong; ++ = moderate; + = weak; 0 = no reaction; ND = not done; / = variation of the reaction between the basal and the apical portion of the crypts, or between two segments (pj3 and ileum).

** Measurements from posterior jejunum 1 (pj1) for calves 18, 19, 21 are considered to represent the lower normal limit for samples collected randomly in the middle small intestine (that was the case for the samples from middle jejunum of calves 1—12).

Halpin & Caple 1976). An enzyme histochemical investigation of calf viral enteritis revealed a decrease of the adenosine triphosphatase reaction in calf ileum (*Abel et al.*). In a previous paper pathomorphologic findings in spontaneous calf diarrhea were described (*Landsverk* 1981a). This study describes the histochemical distribution of enzymes in the small intestine of the diarrheic calves and discusses the significance of the findings with reference to the possible pathogenetic mechanisms implicated in the disease.

MATERIAL AND METHODS

The material comprises 8 diarrheic calves (1—4, 7—10) and 4 convalescent calves (5, 6, 11, 12) also used in other studies (*Landsverk* 1981a, b, *Landsverk et al.* in prep.). The calves were given milk replacers, with constituents including either normally treated (diet B1, calves 1—6) or heat-damaged (diet B2, calves 7—12) skim milk and whey powder. Five healthy calves (17—21) fed whole cow's milk served as controls. The calves with diarrhea were euthanized at different stages of the disease when they were 2—5 weeks of age. Further details on the experiments and procedures are given in the other reports (*Landsverk* 1981a, *Landsverk et al.* in prep.).

The enzymes studied, incubation times and temperatures were as follows: alkaline prosphatase (EC 3.1.3.1) — 10 and 20 min at 37°C; adenosine triphosphate-(ATP)-splitting enzyme — 10 min at room temperature; acid phosphatase (EC 3.1.3.2) — 45 min at 37°C; β-D-galactosidase (EC 3.2.1.23) — 4 h at 37°C; aminopeptidase (EC 3.4.11.2) (with L-alanyl-β-naphtylamid and L-leucyl-β-naphtylamid as substrate) — 20 min at room temperature, and non-specific esterase (with α-naphtyl acetate as substrate) — 15 min at room temperature. Further details on the histochemical methods are given in a previous report (*Landsverk* 1980). The small intestinal segments examined were: middle duodenum (d2), middle jejunum, and posterior jejunum (pj3). The localization has been described previously (*Landsverk* 1979, *Landsverk* 1981a).

RESULTS

A description of the outbreak and duration of diarrhea, the microbiologic examination, and the gross and microscopic examination of the gastrointestinal tract is given elsewhere

Figure 1. Alkaline phosphatase, middle duodenum (d2), control calf (21). Normal enzyme reaction with positive villous brush border. No counterstain. × 100.

Figure 2. Alkaline phosphatase, middle duodenum (d2), diarrheic calf (1). The staining of the villous brush border is weak and discontinuous. No counterstain. × 100.

Figure 3. ATP-splitting enzyme, posterior jejunum (pj3), control calf (21). Normal enzyme reaction with positive villous brush border. Note the weak staining at the base of the epithelial cells. No counterstain. × 100.

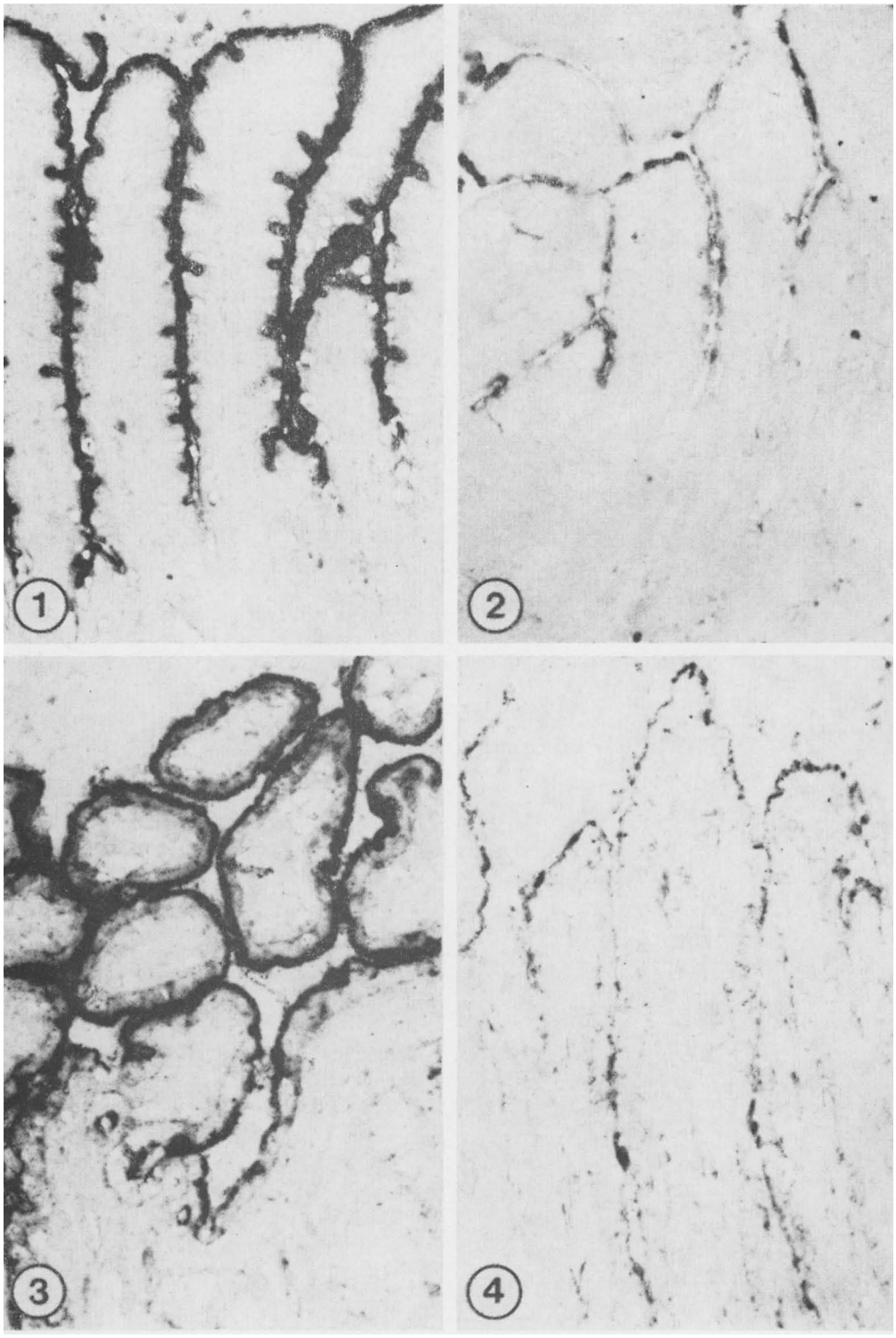
Figure 4. ATP-splitting enzyme, posterior jejunum (pj3), diarrheic calf (2). The villous brush border shows a weak staining. No counterstain. × 100.

Figure 5. Acid phosphatase, middle duodenum (d2), control calf (20). Normal enzyme reaction with strong staining of the villous brush border. Note the moderate staining of the supranuclear epithelial cytoplasm (arrows). Some positive cells in the lamina propria are probably macrophages. Weakly counterstained with hematoxylin. × 150.

Figure 6. Acid phosphatase, middle duodenum (d2), diarrheic calf (3). Note the weak reaction in the brush border of villous epithelial cells and the strong reaction in the supranuclear cytoplasm of these cells (arrows). There are many positive cells, probably macrophages, in the lamina propria. Weakly counterstained with hematoxylin. × 150.

Figure 7. Non-specific esterase, middle duodenum (d2), control calf (21). Normal enzyme reaction with strong staining in the supranuclear cytoplasm of the villous epithelial cells. No counterstain. × 100.

Figure 8. Non-specific esterase, middle duodenum (d2), diarrheic calf (1). The cytoplasm of the villous epithelial cell shows a weak staining. No counterstain. × 100.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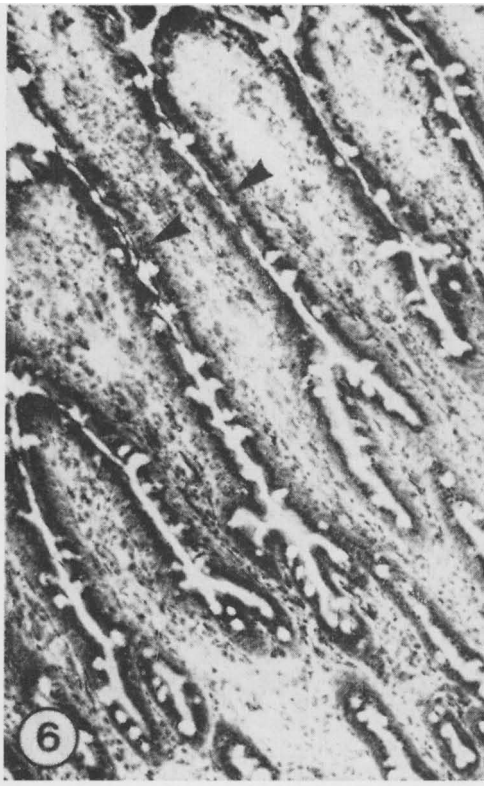
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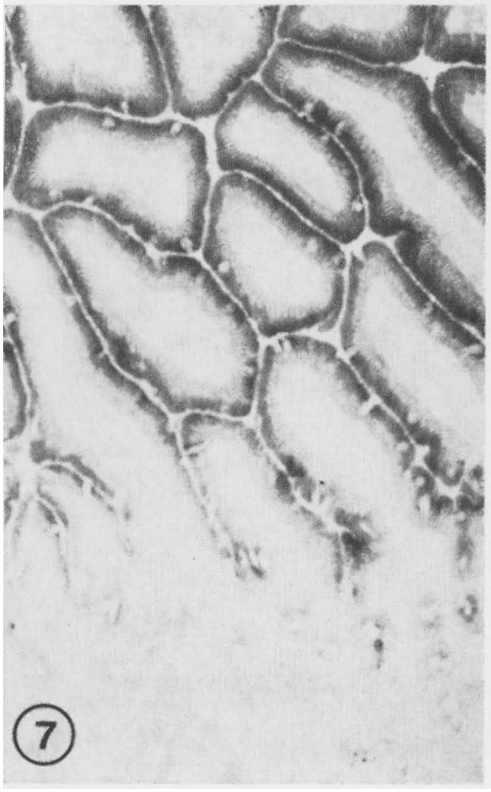
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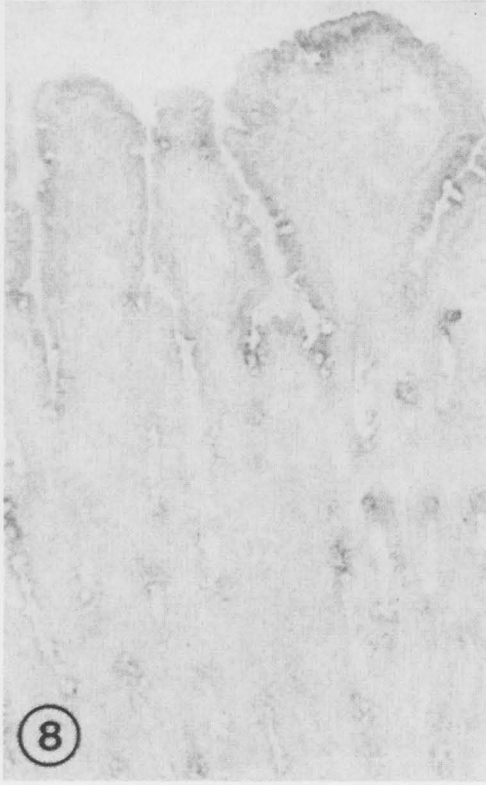
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(*Landsverk* 1981a, b, *Landsverk et al.* in prep.). For the purposes of the present report a brief statement of the disorder described in the other reports may be given: The diarrhea of calves 1—12 was mainly attributed to the occurrence of intestinal infections. Serologic examinations made a widespread rotavirus infection probable, and studies of frozen sections showed positive immunofluorescence for rotavirus in intestinal villi of calves 1 and 8. Calf 1 showed in addition occurrence of large numbers of Gram negative bacteria, probably Escherichia coli, on the intestinal villi. Calf 9 had large numbers of pseudomonas in the ruminal and intestinal contents. Calves 3, 5, 11 and 12 showed a chlamydial infection confined to the posterior small intestine. Some relevant clinical and morphometric data are included in Table 1.

The average regional enzyme reactions in the small intestinal epithelial cells are given in Table 1.

Duodenum

Convalescents showed no reduction of enzyme reactions. Diarrheic calves showed decrease of several enzymes: alkaline phosphatase (Figs. 1, 2) and the ATP-splitting enzyme of the villous brush border were reduced in 3 calves. The corresponding ATP-splitting enzyme of the crypt was reduced in 6 calves. Acid phosphatase of the villous brush border was reduced in 6 calves (Figs. 5, 6), whereas the cytoplasmic acid phosphatase reaction was either similar or increased as compared with the controls. Three calves showed reduction of the villous brush border β-D-galactosidase, while 4 had decrease of the corresponding crypt reaction. Non-specific esterase in the cytoplasm of the villous epithelial cells was reduced in 3 calves (Figs. 7, 8).

Middle jejunum

Owing to the random sampling of this area, only the ATP-splitting enzyme was examined. This enzyme had been found to be evenly distributed between the various small intestinal sites in healthy calves (*Landsverk* 1980). One of the convalescents had a decrease of both the villous and the crypt reaction, and another had a slight decrease of the crypt reaction. Three diarrheic calves displayed decrease of the villous brush border reaction (Figs. 3, 4), whereas these and 2 additional diarrheic calves showed decrease of the corresponding crypt reaction.

Posterior jejunum and ileum

ATP-splitting enzyme and aminopeptidase were reduced in 3 calves, 2 of these were diarrheic and one convalescent. The cytoplasmic acid phosphatase reactions were similar to the controls.

DISCUSSION

The diarrheic condition of the present calves was associated with a decrease in the reaction of several enzymes in the intestinal mucosa. In healthy individuals epithelial cells populating the villi are highly differentiated and equipped with membrane bound enzymes of decisive importance to the absorption of nutrients. The process of epithelial cell proliferation and differentiation occurs in the crypts, the epithelial cells migrating continuously from the crypts to the villi (*Lipkin* 1973, *Sassier & Bergeron* 1978). Enzyme changes during diarrhea may therefore reflect disturbances in the crypt as well as the villous compartment and may be associated with marked structural alterations of the mucosa.

The pathomorphologic changes found in the present calves included villous atrophy and crypt hyperplasia probably resulting from enhanced epithelial cell loss (*Landsverk* 1981a). Alterations of this kind characterize a wide range of intestinal disorders, in particular viral infections affecting mature villous epithelial cells (*Kent & Moon* 1973). One of the best studied of these conditions is transmissible gastroenteritis of pigs (TGE). Prominent enzyme reduction in the villous epithelial cells has been found in TGE (*Thake* 1968, *Hornich et al.* 1977). Kinetic studies have revealed a marked acceleration of the migration rate of epithelial cells from the crypt to the villous tips (*Thake et al.* 1973, *Shepherd et al.* 1979). It may be that the enhanced migration rate during regenerative hyperplasia does not give the epithelial cells sufficient time to acquire the proper enzyme eqipment. Decrease of membrane bound enzymes in the villi has been found during regenerative hyperplasia after X-ray irradiation (*de Both et al.* 1974) and in a number of enteric disorders in man characterized by villous atrophy and crypt hyperplasia (*Lojda* 1974). Interference with epithelial cell maturation may also be a possible cause of the enzyme reduction in the present material; however, not all the results are explained satisfactorily this way.

In some calves, and particularly among the convalescents, villus to crypt ratios were reduced without any apparent decrease of enzyme reactions. Furthermore, enzyme decrease due to cell immaturity would be expected to affect all enzymes associated with cell maturation to the same degree. In the present material there seemed to be some imbalance between the reduction of the various enzymes. It is probable that a direct destructive effect of microbial agents is an additional cause of decreased enzyme reactions. Rather selective enzyme reduction, particularly of disaccharidases, has been demonstrated in experimental intestinal stasis with bacterial overgrowth and in the absence of apparent changes in the epithelial cell kinetics (*Jonas et al.* 1977). It is not possible to assess the actual contribution from the microflora precisely with respect to such direct effects in the present material, but it may be suggested that the rotavirus and chlamydial agents demonstrated in some of the calves were of significance. In this material, however, the disaccharidase examined (β-D-galactosidase) did not seem particularly vulnerable.

Apparently, brush border acid phosphatase was the enzyme mostly affected in the present material. This acid phosphatase reaction is fluoride-resistant (*Landsverk* 1980), and it is possible that the enzyme represents the "tail" of an alkaline phosphatase reaction that may persist at this low pH (5.0) (*Connock & Sturdee* 1975). If this is the case, the results for brush border acid phosphatase may reflect general inadequacies of enzyme histochemical methods, i.e. the limitations with regard to quantitative estimates. Thus the applied technique for acid phosphatase may have disclosed minor differences between the calves in the fraction of alkaline phosphatase activity, which are not detectable at pH optimum.

The decreased enzyme reactions and the villous atrophy with reduction of the intestinal surface area may have contributed to a state of malabsorption in the present calves. Such a state may include insufficient digestion of proteins, carbohydrates, and fats, resulting in excess of available substrate for bacterial fermentation. A malabsorption state may also include disturbances in the transport of electrolytes and water; it is mainly this disorder which is responsible for the clinically recognizable diarrhea (*Rowland* 1978). The absorption of sodium is especially important in this connection, since transport of this solute

appears to be closely linked to the absorption of water (*Parsons* 1967). Sodium absorption in the intestinal epithelium is mediated by the Na-K-ATPase (*Charney & Donowitz* 1978), and the Na-K-ATPase activity is reported to be greater in the villi than in the crypts (*Gall et al.* 1976). Immaturity of epithelial cells along the villi probably contributes to the decreased ATPase activity and the defective sodium transport in TGE (*Kelly et al.* 1972, *Kerzner et al.* 1977). A similar mechanism may have been operating in the present case, though, probably not reflected in the results for the ATP-splitting enzyme, since the Na-K-ATPase is inhibited by the lead in the incubation medium (*Jacobsen & Jørgensen* 1969). It is probable that the villous portion of the ATP-splitting enzyme includes the activity of alkaline phosphatase as well as nucleoside phosphatase (*Landsverk* 1980). It is of interest that the crypt reaction, which is distinguished from the villous reaction by its magnesium dependency (*Landsverk* 1980), was markedly affected in the present calves, indicating an influence of the disorder on nucleoside phosphatase systems.

In conclusion, the intestinal enzyme reduction in the present calves indicates a decreased absorptive capacity of the intestine. It may be of significance that the alterations were mainly confined to the anterior and middle small intestine, where digestive and absorptive processes are most intense. It seems that the posterior regions were not able to compensate this defect.

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SAMMENDRAG

En enzym-histokjemisk undersøkelse av tarmmukosa hos kalver med diaré.

Et utvalg av enzymer i tynntarmsmukosa ble studert hos tolv 2—5 uker gamle kalver, 8 med diaré og 4 rekonvalesenter. Diarékalvene viste reduksjon av enzymene spesielt i duodenum og midtre jejunum; krypt-reaksjonene syntes å være sterkest affisert. I duodenum var alkalisk fosfatase, adenosintrifosfat (ATP)-spaltende enzym og β-D-galaktosidase langs villi redusert hos 3 kalver; reaksjonen i de tilsvarende kryptene var nedsatt hos henholdsvis 6 kalver når det gjaldt ATP-spaltende enzym og 4 når det gjaldt β-D-galaktosidase. Seks kalver viste reduksjon av sur fasfatase i „børstesømmen" av villi og 3 hadde nedgang i reaksjonen av uspesifikk esterase i villi. I midtre jejunum var ATP-spaltende enzym langs villi redusert hos 3 kalver, mens 5 viste nedgang i den tilsvarende krypt-reaksjonen. Rekonvalesentene viste ingen nedgang av enzym-reaksjoner i duodenum, men i midtre og bakre jejunum var det en markert reduksjon av ATP-spaltende enzym og aminopeptidase hos én kalv. De nedsatte enzym-reaksjoner i dette materialet kan skyldes en mangelfull differensiering av epitelcellene forbundet med regenerativ hyperplasi i kryptene og/eller mikrobiell destruksjon av enzymene.

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