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ENTEROKINASE AND DISACCHARIDASE ACTIVITIES OF THE SMALL INTESTINAL MUCOSA IN INFANTS WITH PROTEIN - ENERGY MALNUTRITION AND IN NORMAL INFANTS*

*Semra Sökücü***

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Protein-energy malnutrition (PEM) in infants leads to histological changes in the intestinal mucosa[1-4]. The functional disturbances accompanying these changes have been well documented for disaccharide absorption. It is known that there is a fermentative diarrhea in some cases of kwashiorkor and of marasmus secondary to a failure of lactase synthesis. This failure results from atrophy of mucosal epithelial cells[5-9]. This type of diarrhea can be corrected by the elimination of lactose from the diet[10,11].

Reports on alterations in protein digestion and related enzymes are limited, and to our knowledge do not include protein-energy malnutrition cases. This study was designed to investigate intestinal enterokinase activity in infants with protein-energy malnutrition and to relate this activity to histologic changes as well as to the activity of the disaccharidase enzymes.

Material and Methods

This study was carried out on 18 PEM cases and 9 control infants admitted to the Department of Pediatrics of Istanbul University Faculty of Medicine between August and November 1982. The mean ages of the PEM cases and of the control infants were 14.3 ± 5.64 months and 11.44 ± 3.62 months respectively. Assessment of PEM was based on anthropometric and clinical indices, in accordance with the Wellcome and McLaren criteria[12,13]. Height and weight values of normal Turkish infants were used as the standards of reference[14]. Clinical and laboratory data for the PEM and control groups are given in Table I. Fasting blood sugar, BUN, serum cholesterol, serum electrolyte levels including calcium and phosphate levels were within normal ranges in all subjects.

* From the Department of Pediatrics, İstanbul University Faculty of Medicine, Istanbul.

** Associate Professor of Pediatrics, Istanbul University Faculty of Medicine.

Parental consent to perform an intestinal biopsy was obtained for all cases. Following a fasting period of 4 to 6 hours, a pediatric size Crosby - Kugler capsule was inserted orally under direct fluoroscopic vision. When the capsule was located in the stomach, the patient was positioned on his right side and fluoroscopy was repeated once or twice within the next two hours to check the passage. Two biopsy specimens were obtained in each patient from the mucosa at the level of the duodeno-jejunal junction. One sample was placed in 10% buffered formaldehyde for histology and the second was put on dry ice immediately, then washed in normal saline, blotted gently, weighed and kept at −20 °C. All samples were assayed for enzyme activity within a period of two months.

For histologic examination by light microscopy, sections were cut at a right angle to the mucosal surface and stained with hematoxylin and eosin. The mucosa was examined with particular attention to a) the appearance and height of the villi, b) the depth of the crypts and c) the appearance of the villous epithelium and cellularity and/or edema of the lamina propria. Using a modification of the description advanced by McNicholl and Egan[15], the histologic findings were graded as: a) normal jejunal mucosa b) third degree alteration = total villous atrophy or flattened mucosa c) first and second degree alteration (intermediate forms) = flat or short villi with atrophy in the epithelium and an increase in the cellular infiltration of the lamina propria.

For the enzymatic assays, the biopsy specimen was homogenized by hand in 100 ml of distilled water at 4 °C, using a glass tissue homogenizer. Each sample was then treated with 600 µl of sodium maleate buffer until a homogenous solution was obtained.

Enterokinase activity was determined in two steps as described by Kunitz[16] as a micromodification of the method used by Hadorn et al[17]. The procedure used was as follows: Step 1 : Trypsinogen was incubated with the enterokinase containing solution. The activation mixture consisted of 50 µl of homogenate (dilution with 600 µl sodium maleate), 50 µl of 0.1 M sodium maleate at pH 6.0, and 500 µl of distilled water. The mixture was preheated to 25 °C incubation temperature for 10 minutes. The activation step of 60 minutes was initiated following the addition of 50 µl of trypsinogen solution at 25 °C. (2 mg of trypsinogen per milliliter of 0.005 M HCl). Step 2 : Analysis for determination of amount of trypsin activity produced. These solutions are used.

1. 43.5 mg of Na-Benzoyl-DL-Arginine p Nitroanilide (pfs) HCl as substrate (dissolved in 0.2 ml of dimethyl sulfoxide).
2. Tris buffer 0.05 M, pH 8.2 ....605.2 mg
   HCl 0.1 M .... 8 ml
   $CaCl_2$ .... 222 mg

Three of them were dissolved in 50 ml distilled water (pH 8.2 at 25 °C). The volume was brought to 100 ml by a further addition of distilled water. Solutions 1 and 2 were combined (substrate solution) and a temperatüre of 25 °C was maintained throughout the procedure. After preincubation at 25 °C, 500 µl of substrate solution obtained in step 2 was mixed with / 100 µl of the activation mixture, and incubated at 25 °C for 60 minutes. The reaction was stopped by the addition of 0.3 ml of 30% (v/v) acetic acid, mixed to remove turbidity and read in a spectrophotometer at 409 nm.

The procedure to determine the amount of trypsin formed was immediately started by the addition of an aliquot of the activation mixture to a tube with the reagents for the assay of trypsin activity. Different solutions of each sample were usually analysed to check the proportionality and to obtain an optimum reading value. A blank tube was run with omission of trypsinogen. This tube would also reveal any trypsin accidentally present in the enzyme sample. The separate tubes were also checked to ascertain that the trypsinogen itself was free from trypsin and that no autoactivation of the trypsinogen occurred during the experimental procedure.

At the end of the assay, a standard curve was drawn from the readings of different dilutions and enterokinase activity was calculated. A unit of trypsin is the activity that hydrolyzes 1 µmol of Na-Benzoyl-DL-Arginine p-Nitroanilide HCl per minute and 1 unit of enterokinase is the activity that activates 1 unit of trypsin per minute. A unit of enterokinase activity is expressed in micromoles of substrate hydrolyzed per gram of protein per minute at 25 °C.

The activities of lactase, sucrase and maltase were measured by a modification of the Dahlquist method[18]. The activity was expressed in micromoles of hydrolyzed substrate per gram of protein per minute at 37 °C. The protein content of the homogenate was determined according to the method of Lowry et al[19].

Results

Mucosal atrophy of the first degree was established in 3, and atrophy of the second degree in the remaining 15 PEM cases (Table II, Figure 1).

Enterokinase, lactase, sucrase and maltase levels in PEM cases and in the control group are summarized in Table II.

As seen in Table II, enterokinase activity in the PEM group was significantly lower than in the controls ($p<0.05$). Similarly, lactase and sucrase activities of the PEM group were significantly less than those of the controls. The difference in maltase activity was not significant between the two groups (Fig. 2). A significant difference was not established in the enterokinase and disaccharidase activities between the mild to moderate (first and second degree) group and the severe PEM group when a statistical comparison was made by "rank sum analysis".

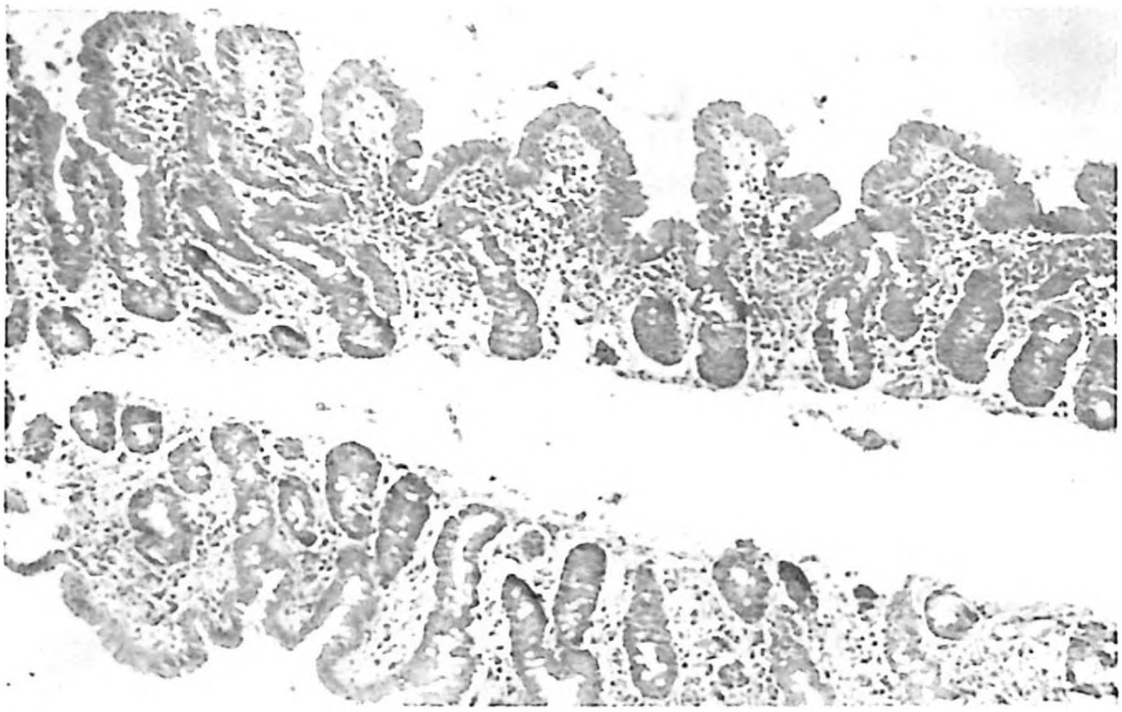

Fig. 1: Biopsy characteristics of the most severe architectual abnormalities found among this group of patients. The villi are shortened and blunted (HE x 75).

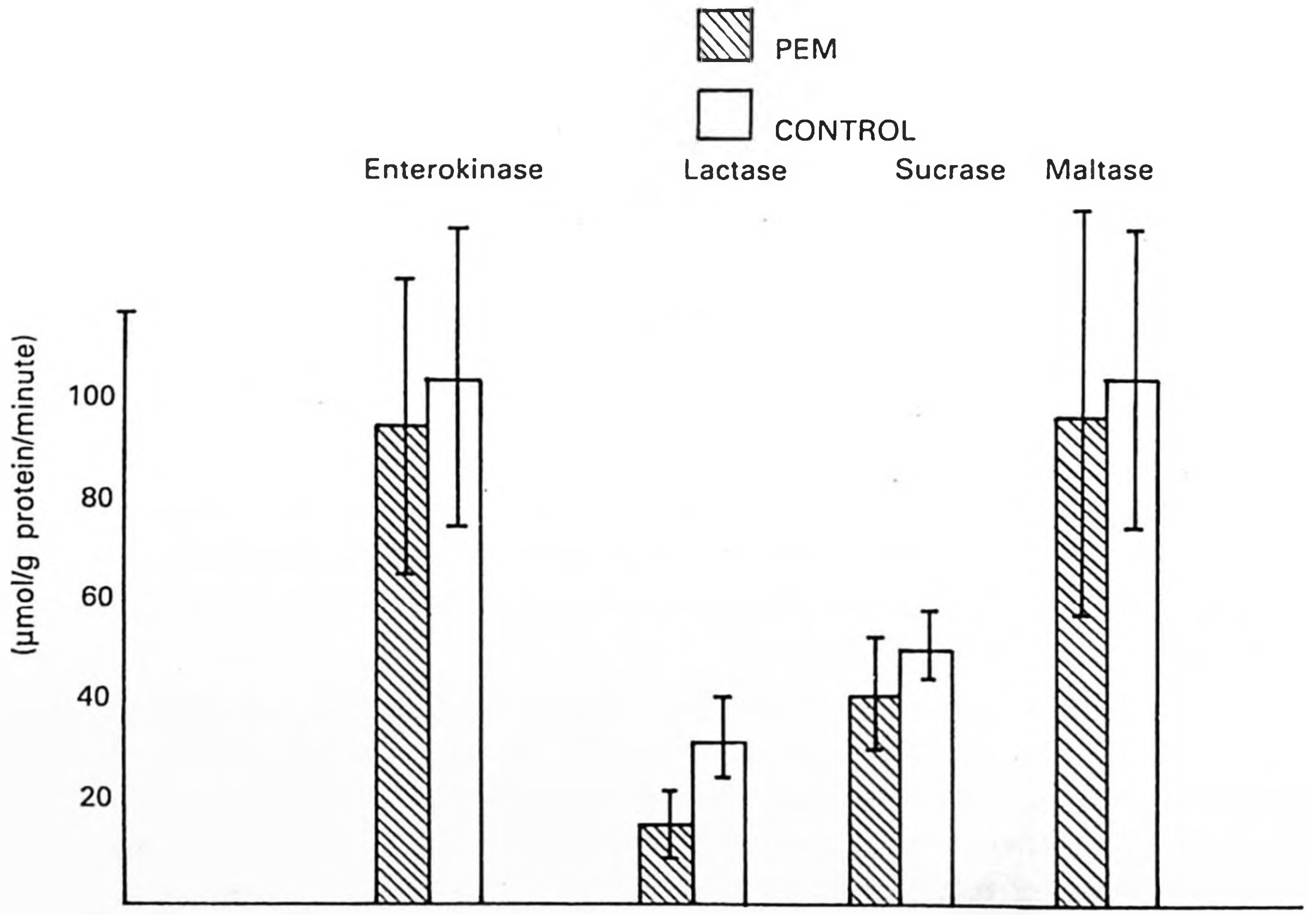

Fig. 2: Mucosal enterokinase and disaccharidase activities in PEM and control infants.

TABLE I: Clinical and Laboratory Characteristics of Infants with PEM and Control Infants

Case no. PEM	Age (month)	Weight (kg)	Height (cm)	W/A %	W/H %	Edema	T. Protein (g/dl)	Clinical Diagnosis
1	12	7.08	70.0	70.8	78.6	–	5.9	M (2nd degree)
2	12	7.60	73.0	76.0	84.4	–	6.3	M (2nd degree)
3	11	6.70	67.0	70.5	78.8	+	3.7	K
4	12	7.60	73.0	76.0	80.0	–	5.2	M (2nd degree)
5	18	6.65	69.0	58.0	78.2	+	3.0	MK
6	24	9.30	77.0	76.8	86.5	–	6.5	M (1st degree)
7	14	7.45	78.0	65.0	69.3	–	4.7	M (3rd degree)
8	15	5.90	67.0	51.9	67.5	+	6.3	MK
9	24	6.80	73.0	55.7	70.8	–	5.9	M (3rd degree)
10	18	7.20	75.0	64.4	72.0	–	6.6	M (3rd degree)
11	15	5.60	71.0	48.9	62.2	–	6.2	M (3rd degree)
12	14	6.18	74.0	59.4	70.8	–	5.8	M (3rd degree)
13	12	6.50	73.0	65.0	68.4	–	5.6	M (3rd degree)
14	18	4.95	64.0	45.0	66.8	–	5.9	M (3rd degree)
15	11	5.00	67.0	52.6	64.1	–	6.6	M (3rd degree)
16	9	5.30	63.0	58.8	76.8	–	5.6	M (3rd degree)
17	20	7.80	70.0	64.4	86.6	–	6.4	M (1st degree)
18	14	5.80	65.0	50.6	76.3	–	4.9	M (2nd degree)
(n) : 18								
Mean values	14.3	6.63	70.51	61.60	74.33	–	5.66	
SD (±)	5.46	1.08	4.24	9.60	7.23	–	0.96	
Control cases								
1	15	10.50	77.0	91.7	98.1		7.2	bronchopneumonia (recovered)
2	9	8.50	71.0	98.8	93.4		* 4.9	bronchopneumonia (recovered)
3	8	8.20	66.0	103.2	102.5		6.3	bronchopneumonia (recovered)
4	10	9.10	72.5	94.7	94.6		6.3	allergic disorders
5	9	8.20	73.0	86.3	86.3		5.3	inguinal hernia (post op.)
6	20	10.60	79.0	84.1	95.4		4.5	inguinal hernia (post op.)
7	11	10.00	76.0	105.0	90.9		6.1	inguinal hernia (post op.)
8	9	9.50	72.0	109.0	100.0		5.8	bronchopneumonia (recovered)
9	12	10.10	75.0	101.0	100.0		5.8	bronchopneumonia (recovered)
n : 9								
Mean values	11.44	9.41	73.5	97.08	95.6		5.8	
SD (±)	3.62	0.89	3.62	8.02	4.79		0.76	

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W/A weight for age
W/H weight for height
PEM protein energy * malnutrition
M marasmus
K kwashiorkor
MK marasmic-kwashiorkor

TABLE II: Enterokinase and Disaccharidase Activities in Intestinal Biopsies from Infants with PEM and Control Infants

Case no. PEM	Activities (µmol / g protein / min) Enterokinase	Lactase	Sucrase	Maltase	Protein g prot / g tissue	Morphological findings (degree of atrophy)
1	33.3	4	26.1	101.6	0.13	2nd
2	91.3	5	29.7	44.8	0.20	2nd
3	37.9	11.4	50.0	59.3	0.09	2nd
4	71.2	5.4	55.5	74 3	0.17	1st
5	86.6	25.0	56.7	181.7	0.10	2nd
6	69.3	18.3	54.2	81.6	0.08	1st
7	128.6	13.3	36.6	111.1	0.04	2nd
8	90.2	6 6	25.8	55.5	0.09	2nd
9	112.2	25.3	42.5	71.2	0.08	2nd
10	119.1	16.5	50.0	168.0	0.05	2nd
11	62.3	28.7	57.5	93.7	0.07	2nd
12	24.9	14.2	28.4	163.8	0.13	2nd
13	56.7	10.6	25.2	55.1	0.09	2nd
14	98.3	9.3	27.3	60.5	0.10	2nd
15	108.2	11.2	20.5	58.9	0.09	2nd
16	105.3	21.1	40.0	58.2	0.09	2nd
17	33.8	23.1	46.2	92.5	0.08	1st
18	78.1	19.1	53.9	62.8	0.11	2nd
n : 18						
Mean values	78.2	14.89	40.34	88.59	0.099	
SD (±)	30.81	7.42	12.62	40.93	0.03	
Control infants						
1	68.7	42.4	52.8	88.9	0.08	N
2	92.5	39.5	47.3	99.8	0.09	N
3	115.2	22 9	41.6	142.9	0.11	N
4	138.0	46.5	60.0	160.0	0.05	N
5	76.5	26.7	44.1	122.3	0.08	N
6	120.7	31.7	60.9	67.2	0.13	N
7	127.4	25.0	50.0	90.0	0.10	N
8	152.1	37.0	57.4	106.4	0.10	N
9	75.8	33.7	40.5	71.2	0.10	N
n : 9						
Mean values	107.43	33.93	50.51	105.41	0.093	
SD (±)	28.37	7.66	7.31	29.52	0.02	
Comparison of mucosal enzyme activities (t)	2.295	5.978	2.152	1.057	0.57	
	$p<0.05$	$p<0.001$	$p<0.05$	$p>0.05$	$p>0.05$	

Discussion

Enterokinase is the most important enzyme of the intestinal mucosa because of its cardinal role in protein digestion. The method of assessment of enterokinase activity is a meticulous and difficult process. One of the problems encountered is in the assessment results from trypsin present in the duodenal fluid or formed by the autocatalytic activation of trypsinogen. Autocatalytic activation may also occur with the crystalline bovine trypsinogen used in the procedure[16]. Another source of error in the estimations is the production of inert protein from trypsinogen under certain conditions. Kunitz[16], has suggested that if the trypsinogen concentration of the medium is kept low, both these problems, namely autocatalysis of trypsinogen and formation of inert protein, might to a great extent be eliminated. Further this author showed that inert protein production could be inhibited by keeping the pH lower than 6 in the medium of activation. Others have also suggested methods to eliminate inert protein production[20,21]. Hadorn et al[17], by passing the enterokinase containing solution on Sephadex G-100, were able to remove the interfering amounts of trypsin in the samples. This procedure is lengthy and its application is difficult. Bett[22] was able to show that trypsin could be completely eliminated from the medium by washing the mucosal specimens with an isotonic saline solution.

In our study small amounts of trypsinogen were used in the activation medium in order to eliminate errors due to the production of inert protein and to autocatalytic activation. The biopsy specimens were washed with an isotonic saline solution at +4 °C in order to eliminate trypsin from the intestinal fluid. Also a control sample, with no added trypsinogen, was incubated at 25 °C to check the presence of trypsin in the homogenate. The medium which stained yellow during incubation, showing production of trypsin by enterokinase effect on the homogenate in the tubes to which trypsinogen was added did not show the color transformation in the control samples. Nordström[21] has shown that human enterokinase can be affected by the ionic power of the activation medium and that the use of malleate buffer provides an optimum pH with constant molarity. In our study malleate buffer at pH 6 was used.

A mean value of 109 ± 34.2 µmol/g protein/min is reported for mucosal enterokinase activity in normal infants[23]; comparable values of 107 ± 43 µmol/g protein/min were obtained in our control group conforming with those reported for normal infants[23].

Lebenthal and Lee[24] have studied disaccharidase activity in infants presenting with intestinal mucosal atrophy and have reported that lactase and sucrase activity show a decrease parallel to the degree of villous atrophy but that maltase activity does not reflect this relationship to the same extent. Lactase and sucrase activities in our series of 18 PEM cases, 15 of which showed 2nd degree mucosal atrophy, agreed with the results reported in cases with mucosal atrophy and the values were also significantly lower in our group of control cases. Maltase activity in our cases was low compared to results obtained by Lebenthal et al[23] but again was not significantly different from those in our control cases.

Secondary enterokinase deficiency is most frequently encountered in infants with intractable diarrhea. Lebenthal et al[25] also studied enterokinase and disaccharidase activities in infants with intractable diarrhea. They found both the disaccharidase levels and the enterokinase activity to be much lower than in the controls and found a correlation between these values and the degree of mucosal atrophy.

In our study, a significant difference did not exist between cases showing first degree mucosal atrophy and those with a more severe histopathology. This result may be due to the fact that there were only a few cases with first degree atrophy among the patients. Although the mean value of enterokinase activity in infants in the PEM group was not as low as those reported for infants with intractable diarrhea, these values were significantly lower than those reported for normal healthy infants and also lower than the values obtained in our control cases. Disaccharidase values in the PEM cases also agreed with values reported for children with intractable diarrhea[23].

Although severe mucosal atrophy also occurs in infants with untreated celiac disease, enterokinase activity in these patients has been reported to be normal[25-27]. One possible explanation for this finding is the difference in cell turnover rates in intractable diarrhea of infancy and celiac disease. According to Wooldley and Keane[27] and Eggermont et al[26], there is a high turnover of mucosal epithelial cells and an enhanced desquamation of the cells in untreated celiac disease. In contrast, Lebenthal et al[25] have shown that the turnover of mucosal epithelial cells is slight in intractable diarrhea of infancy. These authors also observed a diminished mitotic index in these patients, comparable to that seen in PEM.

Regenerative ability will also be decreased in mucosal cells in cases where the mitotic index is low. It has been speculated that a low turnover rate of the mucosal epithelial cells might be a causative factor in the relatively low enterokinase activity established in the PEM cases in our series.

Summary

With a view to assessing the effect of chronic malnutrition on the small intestinal mucosa, enterokinase and disaccharidase activities were measured in the biopsy specimens obtained by jejunal biopsy. Eighteen cases of protein-energy malnutrition aged between 9 and 24 months were studied and 9 normal infants of the same age were used as the control group. Mucosal atrophy of the small intestine was established in all protein-energy malnutrition cases. Enterokinase, lactase

and sucrase enzymes were found to be significantly lower in the protein-energy malnutrition group of infants, but the maltase enzyme levels were not found to be significantly affected by malnutrition. There was no significant difference in any of the enzyme levels between protein-energy malnutrition cases of second and third degree.

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