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IMPROVED METHOD OF ISOLATING THE SUPRAEPITHELIAL LAYER OF MUCUS FROM THE SMALL INTESTINE OF EXPERIMENTAL ANIMALS

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Key Words: small intestine; intestinal mucus; method of obtaining; digestive enzymes

Many recent investigations have shown that the supraepithelial layer of mucus of the small intestine not only performs a protective function but also has a very important role in the final stage of digestion, by participating in the degradation of products of intraluminal hydrolysis and their subsequent transport [3, 6, 13, 14]. The mucous layer has been shown to contain active pancreatic and intestinal enzymes proper, but the role of contact (taking place in the mucous layer) digestion has been interpreted differently [6, 10, 11]. A solution to this problem is possible only by the use of a standard method of obtaining mucous deposits, so that the juxtamural layer can be separated sufficiently completely without damage to the epithelial cells.

The method suggested previously [3], namely removing mucus with a spatula under a binocular microscope is applicable only to the intestine of large animals and cannot be used for quantitative assessment of the role of the enzymes of this layer, because in this case it is possible to obtain only the intraluminal part of the mucus, and there is great probability of injuring the epithelium.

Some studies of the radial distribution of enzymes of the final stages of hydrolysis of food substances in the small intestine have been undertaken by Pitran's method of isolating mucous deposits [6, 11]. In this method the supraepithelial layer of mucus is obtained from the everted intestine, fitted on to a glass rod into a test tube by manual forward and rotary shaking. It will be evident that the inadequate standardization of this procedure and the possibility of damaging the preparation on the wall of the tube, i.e., the fact that the efficacy of separation of the mucus depends on the experimenter's skill, has led to the obtaining of contradictory results.

The aim of this investigation was to develop a standard method of isolation of the supraepithelial layer of mucus with the aid of a special apparatus, eliminating the above deficiencies, and allowing the supraepithelial mucus to be separated more completely, while preventing the possibility of damaged villi and their fragments from the traumatized ends of the intestinal preparation from contaminating the fraction of mucous deposits.

EXPERIMENTAL METHOD

Experiments were carried out on male Wistar rats weighing 400-450 g. The rats were deprived of food for 12 h before sacrifice. The rats were killed by decapitation. In all experiments segments of small intestine 6 cm long, taken 10 cm distally to the stomach, were used. A ligature was applied to one end of the intestinal segment, after which the segment was everted and fitted on the working rod of the apparatus for shaking the juxtamural layer of mucus, which consisted of a system of a rigidly fixed rod, in contact with a toothed disk, mounted on the axle of an electric motor (Fig. 1). When the disk rotates, it induces vi-

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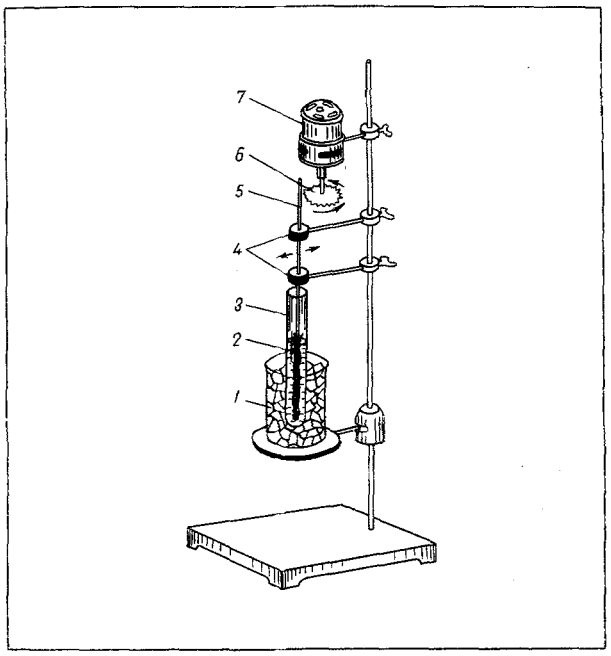

Fig. 1. Vibrating apparatus for obtaining supraepithelial mucous layer of small intestine from experimental animals. 1) Receptacle containing ice, 2) everted segment of small intestine, 3) test tube with isolation medium, 4) rubber bushes; 5) working rod (vibration 30-35 vibrations/sec); 6) toothed disk; 7) electric motor (60 rpm).

TABLE 1. Enzyme Activity and Protein Concentration in Fraction of Mucous Deposits, with Different Times of Shaking Preparation of Rat Jejunum

Time, sec	Alkaline phosphate		Gly-DL-Leu DP		Sucrose		Trypsin		Protein, mg/ml
	I	II	I	II	III	IV	III	IV	
20	0,14±0,026	1,50±0,17	0,39±0,13	2,16±0,64	27,9±4,16	168,3±11,0	6,63±1,46	40,3±6,77	0,14±0,02
40	0,13±0,031	0,89±0,28	0,48±0,22	1,67±0,47	33,5±5,20	138,2±16,1	8,44±3,18	41,9±14,9	0,21±0,05
60	0,22±0,061	0,95±0,17	0,73±0,20	2,14±0,31	59,2±19,5	167,4±17,0	6,10±1,98	24,0±10,9	0,29±0,08

Legend. Activity of enzymes: I) in μmoles/min/cm intestine; II) in μmoles/min/mg protein; III) in nmoles/min/cm intestine; IV) in nmoles/min/mg protein. DP) Dipeptidase.

bration of the working rod with an amplitude of 1-2 mm. The frequency of vibration varied from 60 to 2250 oscillations/min depending on the number of teeth on the disk. When the fraction of mucous deposits is to be obtained the intestinal preparation is placed in a test tube containing the working solution, without immersing the proximal section of the intestinal segment or the site of its fixation to the rod. When the optimal duration of shaking was chosen, three intervals were tested: 20, 40, and 60 sec. During choice of the optimal medium for obtaining the fraction of mucous deposits, the duration of shaking was constant at 20 sec. Isolation was tested in three media: physiological saline + 100 mM mannitol, Ringer's solution + 100 mM mannitol, and 0.05 M Tris-HCl-buffer, pH 7.54, in physiological saline. In a preliminary experiment the optimal osmolarity of the solution was chosen. For this purpose the intestine was placed in fixative with different degrees of osmolarity (300, 350, 400, and 450 milliosmoles) as a result of addition of the corresponding quantity of D-mannitol, and later the degree of swelling or compression of the epitheliocytes was estimated under the light microscope. The fraction of intestinal contents was obtained by collecting the chyme during eversion of the segment in a test tube containing 6 ml of working medium, followed by shaking of the segment immersed in this solution twice (switching the apparatus on and off), or immersing the everted intestinal preparation in the isolation medium 3 or 4 times. The homogenate of the mucosa was prepared by the usual method from mucosa scraped from 3 cm of intestine, previously shaken, in the corresponding working solution in the ratio (*w/v*) of 1:20. In all the experiments to isolate the supraepithelial layer of mucus, a morphological control was set up to examine the fraction obtained for damaged epi-

TABLE 2. Distribution of Enzyme Activities (in μmoles/cm intestine/min) in Fractions of Rat Jejunum, Using Different Isolation Media

Enzyme	Fraction	Physiological saline	Ringer's solution	Tris in physiological saline
Gly-L-Phe dipeptidase*	Ch	0,20±0,09 (1,8)	0,08±0,03 (0,6)	0,24±0,06 (3,0)
	M.d.	1,89±0,37 (16,7)	2,13±0,09 (15,1)	1,99±0,23 (24,6)
	H	9,22±1,26 (81,5)	11,9±2,14 (84,3)	5,86±1,64 (72,4)
Gly-DL-Leu dipeptidase*	Ch.	0,49±0,18 (2,0)	0,21±0,05 (0,7)	0,59±0,14 (2,1)
	M.d.	4,45±0,87 (18,4)	4,74±0,26 (16,3)	4,43±0,49 (16,1)
	H	19,3±2,64 (79,6)	24,1±3,62 (83,0)	22,5±3,94 (81,8)
Ala-DL-Leu dipeptidase*	Ch	0,41±0,15 (2,3)	0,18±0,05 (0,8)	0,46±0,09 (2,3)
	M.d	3,16±0,6 (17,9)	3,35±0,18 (14,8)	3,05±0,36 (15,0)
	H	14,1±1,58 (79,8)	19,1±3,24 (84,4)	16,8±3,66 (82,7)
Alkaline phosphatase*	Ch. M.d.	0,06±0,03 (2,2)	0,05±0,03 (2,1)	0,06±0,04 (2,4)
	H	0,37±0,09 (13,3)	0,28±0,06 (11,6)	0,30±0,05 (12,0)
Monoglyceride lipase*	Ch	2,36±0,61 (84,6)	2,09±0,45 (86,4)	2,13±0,08 (85,5)
	M.d.	0,05±0,02 (7,1)	0,03±0,01 (4,5)	0,03±0,01 (4,0)
	H	0,15±0,08 (21,4)	0,12±0,05 (18,2)	0,16±0,03 (21,1)
Lipolytic activity*	Ch	0,50±0,08 (71,4)	0,51±0,09 (77,3)	0,57±0,09 (75,0)
	M.d.	93,5±47,0 (16,6)	77,5±51,5 (12,9)	68,5±42,1 (13,8)
	H	185,1±64,4 (32,9)	286,5±69,4 (47,5)	209,1±47,9 (42,1)
Saccharase*	Ch	283,3±43,8 (50,4)	239,1±88,7 (39,6)	219,3±31,9 (44,1)
	M.d.	11,8±3,58 (2,4)	7,37±2,25 (2,3)	2,96±1,47 (0,9)
	H	60,1±17,6 (12,1)	51,9±19,5 (13,4)	8,01±2,42 (2,5)
Trypsin**	Ch	426,6±79,0 (85,6)	326,7±39,9 (84,6)	309,4±64,6 (96,6)
	M.d.	11,6±3,27 (56,6)	20,4±7,99 (74,4)	19,7±6,24 (64,2)
	H	8,88±1,39 (43,4)	7,02±2,76 (25,6)	11,0±4,57 (35,8)
Amylolytic activity***	Ch	Trace	Trace	Trace
	M.d.	78,4±38,9 (34,1)	118,6±89,3 (30,3)	123,2±87,9 (49,5)
	H	128,2±43,4 (55,7)	234,9±135,6 (60,1)	100,4±46,8 (40,4)
		23,4±4,39 (10,2)	37,3±12,3 (9,5)	25,1±9,10 (10,1)

Legend. Ch) chyme, M.d.) mucous deposits, H) homogenate. *) Enzyme activity expressed in μmoles/min/cm intestine; **) in μmoles/min/cm intestine; ***) in mg starch/min/cm intestine; numbers in parentheses denote percentages.

theliocytes and epithelial sheets, and also to determine the integrity of the epithelium of the intestinal villi of the preparation subjected to the shaking procedure. Activity of the following enzymes was determined in each fraction: dipeptidases by the L-oxidase method [9], using glycyl-L-phenylalanine, glycyl-DL-leucine, and alanyl-DL-leucine; sucrose was determined by the glucose oxidase method in the modification in [2], alkaline phosphatase by the use of 0.6 M *p*-nitrophenyl phosphate as the substrate; monoglyceryide lipase by the method in [5]; pancreatic lipase activity by the use of olive oil as the substrate [4]; amylolytic activity by the iodine and starch test in the modification in [8]; trypsin activity by Erlanger's method in the modification in [12], and the protein concentration also was determined by Lowry's method in the modification in [7].

EXPERIMENTAL RESULTS

The experiments showed that the amplitude must not exceed 2 mm but the frequency of vibration can be varied within quite wide limits. In the working time interval (20 sec) even a very high frequency (2250 vibrations/min) does not damage the epithelial sheet, but enables the more complete separation of the mucous layer.

Choosing the optimal time of shaking the intestinal preparation showed (Table 1) that activity of the intestinal enzymes proper, when calculated per centimeter of intestine, in the fraction of mucous deposits obtained with a 20- and 40-sec interval, was virtually identical, whereas with a 60-sec interval, activity of the hydrolases was almost doubled. The morphological control showed that only single desquamated epithelial cells could be found in fractions of mucous deposits obtained after shaking for 20 and 40 sec, whereas after 60 sec, single epithelial sheets were found, and the integrity of the epithelium of the intestinal preraration also was disturbed. It can accordingly be postulated that increased activity of intestinal enzymes in the fraction after shaking for 60 sec was due to the fact that the preparation contained enzymes as well as damaged epitheliocytes, and not to the more complete separation of the layer of mucus. Evidence in support of this view also is given by the trypsin level which was the same in the fraction whatever the conditions of isolation. It is thus probable that, under the given conditions, the bulk of the mucous deposits separates in the course of 20 sec.

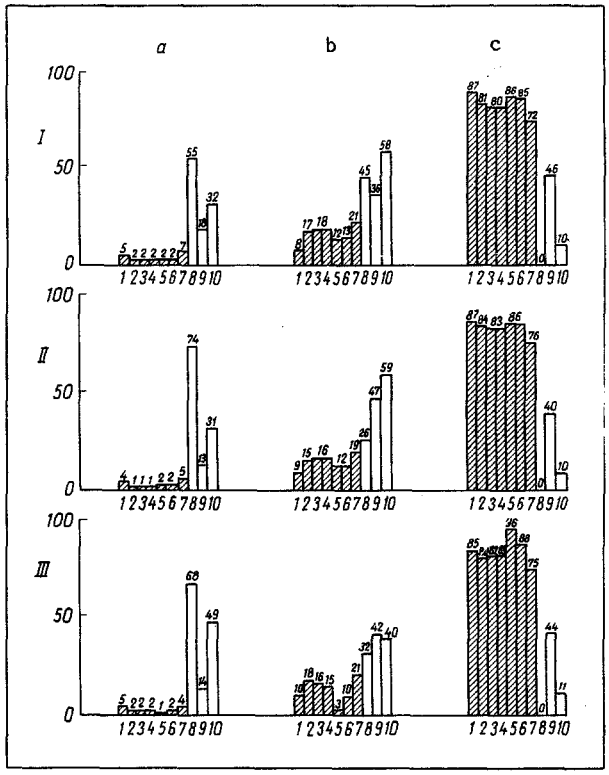

Fig. 2. Effect of isolation medium on radial distribution of intestinal and pancreatic enzymes in small intestine (calculated per centimeter length). I) Physiological saline, II) Ringer's solution, III) Tris-HCl buffer. a) Chyme, b) supraepithelial mucous layer, c) homogenate of mucosa. Abscissa: 1) protein, 2) Gly-Phe DF, 3) Gly-Leu DP, 4) Ala-Leu DP, 5) saccharase, 6) alkaline phosphatase, 7) Monoglyceride lipase, 8) trypsin, 9) lipolytic activity, 10) amylolytic activity; ordinate, distribution of enzymes (in %).

When the optimal ionic composition of the isolation medium of the mucous deposits was chosen, the possible effect of certain factors on the ability of the intestinal enzymes to pass into the juxtamural mucus was taken into account. for instance, Timofeeva and co-workers [10] have attempted to explain the high content of intrinsic intestinal hydrolases in the mucous fraction, observed in several investigations, by the absence of calcium ions in the solution and by exposure to Tris.

Our experiments showed that the different composition of the media used while keeping the conditions of isolation of the fractions otherwise the same does not affect the release of enzymes and their activity (Table 2, Fig. 2). The exception is saccharase, activity of which was reduced when Tris, made up in physiological saline, was used as the working medium, and this can evidently be explained by the inhibitory effect of the Tris itself [15]. The distribution and activity of the remaining enzymes studied in all fractions were constant; the relative activity of the enzymes in the fraction of mucous deposits, moreover, was very high, namely 6-21% of the total activity of all fractions for enterocytic hydrolases, and 25-45% for trypsin; the amylolytic (40-59%) and lipolytic (36-47%) activity in this fraction also was high.

Thus the vibration method of separating the supraepithelial mucous layer from an everted intestinal preparation has many advantages over the manual method, but in particular, it is standardized, so that it is possible to obtain comparable results in different experiments when studying enzyme activity and the radial distribution of hydrolases in the small intestine of experimental animals.

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USING FLUORESCENT CARBOCYANINE DYES TO STUDY PATHWAY ORGANIZATION IN AUTOPSY MATERIAL OF THE HUMAN BRAIN

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Key Words: neuromorphology; fluorescent dyes; retrograde and anterograde axonal transport; terminal ramifications.

One of the main trends in modern neuromorphology is the study of the human brain with its highly complex structural organization. Methods of axonal transport of substances (enzymes, salts of metals, fluorochromes) in association with operations, which are widely used to study pathways of the animal brain, naturally are not fully applicable for investigations of this kind of the human brain. The few methods available to study pathways of the human brain by teasing nerve fibers [3], by retrograde fiber degeneration and anterograde chromatolysis of cells, using clinical material [2], axonal transport of horseradish peroxidase in autopsy material [6]; and axonal iontophoresis of cobalt salts in weakly fixed autopsy material [1], also have a number of important limitations. The main problems which have to be solved for work on autopsy material from the human brain when the last two methods (both giving the best results) are used to ensure adequate preservation of the tissue throughout the experiment and to choose a transported material and the appropriate mechanisms of transport of that material along axons for use with it. Much progress in the study of human brain pathways may be expected by the use of prefixed material. However, axonal transport of materials is impaired by complete fixation of brain tissue, and this problem can be overcome only by the use of weakly fixed material and using an iontophoretic current to transport the materials [1]. When axonal iontophoresis of cobalt salts is used, when studying central projections of cranial nerves, afferent fibers are revealed much more clearly than efferent cells and their processes. This is because of the decrease in concentration of cobalt ions during their passage through the axon and cell body, which have a considerable volume [1].

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