BBA 75049

STRUCTURAL AND FUNCTIONAL ORGANIZATION OF THE BRUSH BORDER OF INTESTINAL EPITHELIAL CELLS

III. ENZYMIC ACTIVITIES AND CHEMICAL COMPOSITION OF VARIOUS FRACTIONS OF TRIS-DISRUPTED BRUSH BORDERS

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(Received December 12th, 1966)
(Revised manuscript received March 16th, 1967)

SUMMARY

The composition of subunits of the intestinal brush border has been studied. Fractions obtained after Tris disruption and subsequent density-gradient centrifugation were analyzed for their enzymic and chemical content. These fractions include a major membrane component, a minor membrane component, microvillus cores and two that are unidentified. The major membrane fraction contains all of the alkaline phosphatase, maltase, invertase, lactase, isomaltase, trehalase and leucyl naphthylamide hydrolase activities of the brush border. It also contains ATPase. A different ATPase activity is found in one of the morphologically identified fractions. It appears to be associated with a fibrillar material which can be formed from this fraction by appropriate treatment. Lactase activity is occasionally found to be differently distributed from the other disaccharidases; *i.e.*, an appreciable amount appears in the minor membrane component. No enzymatic activities were found in the fraction composed of microvillus cores.

All fractions were analyzed for protein, carbohydrate, cholesterol, phospholipid and DNA. Cholesterol to phospholipid ratio of 1.1 was found in the major membrane fraction.

INTRODUCTION

The brush border of the intestinal epithelial cell has recently been described as a specialized cellular subunit containing specific elements concerned with the processes of digestion and absorption[1,2]. It is, thus, a functional organelle and it should be of interest to investigate the relationships of the various elements contained within it, not only from the specific physiological point of view of how each contributes to absorption and transport, but also from the more general biological point

of view of how specific organization may contribute to function and control in membrane systems. A useful step in this direction is our recent discovery of a means to disrupt isolated brush border in a specific way by the use of Tris, and to recover morphological identifiable subfractions. One of these subfractions has been shown to be composed entirely of microvillus membranes, and another substantially of microvillus cores[3,4].

From electron-microscopic and histochemical studies the brush border is known to contain alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) activity associated with the membrane[5]. Thus, it is possible by reference to alkaline phosphatase to decide the probable localization of other enzymes associated with the brush border. Preliminary investigation of enzyme distribution among fractions obtained by disruption previously indicated that alkaline phosphatase and maltase (maltose glucohydrolase) activity were predominantly to be found in the fraction containing microvillus membranes[3]. In the present report these studies are extended to include four additional disaccharidase activities, leucine naphthylamide hydrolase (EC 3.4.1.1) and ATPase. Studies have also been undertaken to determine the gross chemical composition of the brush border membrane and the fractions obtained after Tris disruption. A qualitative analysis of brush border lipids from thin-layer chromatograms was previously reported by FINEAN and his colleagues[6,7].

METHODS

Preparation of fractions

Brush borders from the intestinal epithelial cell of the hamster jejunum were isolated by a previous described modification of the original method of MILLER AND CRANE[3]. As enzyme analysis of these preparations for cytochrome oxidase indicated little contamination with mitochondrial material, that is, less than 0.003% of the total activity of this enzyme present in the original homogenate was found in brush border preparations, they were considered suitably purified for further fractionation. Full details of the subsequent procedures were provided in a previous paper[3]. In brief, we proceeded as follows: Brush borders, usually from the intestine of six hamsters, were collected and disrupted by the use of 1 M Tris. If undisrupted brush borders or other particulate material are found to contaminate the fraction after Tris disruption, such material can be removed by a 10-min centrifugation at 1000 $\times g$. The fragments were then separated by centrifugation on stepwise gradients prepared by successive introduction of equal volumes of 20, 30, 40, 50 and 60% glycerol solutions containing 0.05 M $MgCl_2$ to the bottom of the centrifuge tube. The tubes were centrifuged for 10 min at 63 000 $\times g$ in the bucket rotor of a Spinco Model L ultracentrifuge and the contents were removed in small aliquots beginning from the top by means of a device similar to that previously used by KAHLER AND LLOYD[10]. All fractions were dialyzed against distilled water in the cold room overnight and then against 5 mM EDTA (disodium salt) at pH 7.0 for a few hours.

For chemical assays, Tris-disrupted fractions were prepared and collected as described above except that larger numbers of hamsters (about 40) were used in order to provide adequate amounts of material for analysis. The collected fractions were subsequently dialyzed at 4° for at least 96 h in a dialystat against 4 l of distilled

water which was changed every 12 h. After dialysis, the fractions were analyzed for remaining glycerol by the procedure of LAMBERT AND NEISH[11] and the dialysis was continued until no glycerol could be detected (less than 1 μg glycerol/ml). An alternate procedure for the removal of glycerol from fractions was to subject them to centrifugation at 48 000 $\times$ g for 40 min in the Servall refrigerated centrifuge. The precipitated fractions were then lyophilized and their weight was recorded. The yields of lyophilized material from each of the fractions were in microgram quantities. Upon further drying over P_2O_5 at 56° for 24 h additional water loss in the brush border fraction was about 1 %.

Enzyme assays

Disaccharidase activities were measured as described by DAHLQVIST[12]. Alkaline phosphatase was measured by the rate of splitting of *p*-nitrophenylphosphate in a glycine buffer (0.05 M) at pH 10.5 using 0.2 mM $MgCl_2$, 2 mM zinc acetate and 5 mM $CoCl_2$ as cofactors. ATPase was measured by the appearance of P_i, at pH 7.0, in presence of 5 mM ATP, 5 mM Mg^{2+}, 100 mM Na^+, 10 mM K^+ during a 30-min incubation at 37°. The reaction was stopped with trichloroacetic acid and P_i was determined by the method of FISKE AND SUBBAROW[13]. Leucyl naphthylamide hydrolase was determined by a slightly modified procedure of GOLDBARG AND RUTENBURG[14].

Activities are expressed in international units, *i.e.*, μM substrate reacted per min at 37°. Specific activity is expressed as units of enzyme activity per mg of protein. Protein was estimated by the procedure of LOWRY *et al.*[15].

Chemical assays

Carbohydrates were determined by the anthrone method of SCOTT AND MELVIN[16] as well as by reducing sugar assay[17] after hydrolysis in 0.5 M H_2SO_4 for 30 min at 100°. Cholesterol was assayed by the KENNY modification[18] of the Lieberman–Burchard reaction and esterified fatty acids by the procedure of STERN AND SHAPIRO[19]. Protein was estimated as above and P_i was estimated by the procedure of FISKE AND SUBBAROW[13]. DNA was estimated by the method of STUMPF[20]. Extraction of phospholipids was carried out in $CHCl_3$–methanol (2:1, v/v) at 4° overnight, using essentially the procedure of FOLCH, LEES AND SLOANE-STANLEY[21]. Prior to the determination of P_i the fractions were washed with water as recommended by FOLCH. Controls indicated this procedure to be as complete, with brush borders, as extraction with a boiling mixture of ethanol and ether and $CHCl_3$–methanol in a Soxhlet extractor. Phospholipids were measured as $CHCl_3$–methanol-extractable phosphate and calculated as lecithin. Similarly, esterified fatty acids were calculated as tripalmitin.

RESULTS AND DISCUSSION

Fig. 1 is a schematic diagram of the fractions of Tris-disrupted brush borders separated by centrifugation on the density gradient and visualized by the Tyndall effect. Fractions A and B have as yet not been morphologically identified. Fractions C and C′ contain microvillus membranes. Fraction D contains microvillus cores, occasionally contaminated by membranous material resulting from incomplete disruption of brush borders as shown below.

The various fractions when recovered from the density gradient, diluted five-

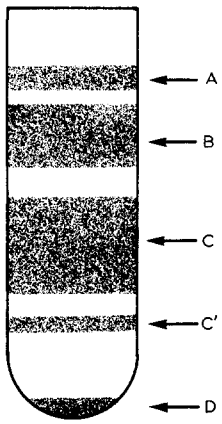
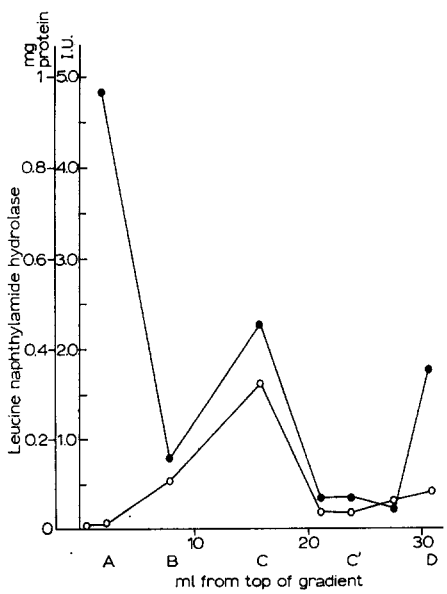


Fig. 1. Diagrammatic illustration of the separation of fractions of Tris-disrupted brush borders by density-gradient centrifugation.

fold with distilled water and centrifuged at 150 000 × *g* for 60 min, yield sediments containing the total protein and enzymic activities of the brush borders. Clearly, the enzyme activities remain associated with particulate material throughout Tris disruption and subsequent separation procedures. The C fraction can be similarly sedimented by centrifugation at 48 000 × *g* for 30 min.



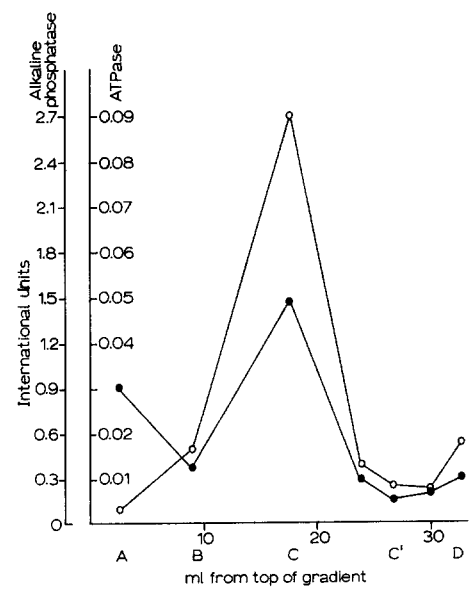


Fig. 2. Distribution of leucine naphthylamide hydrolase (○ — ○) and protein (● — ●). Letters designate center of visible bands.

Fig. 3. Distribution of alkaline phosphatase (○ — ○) and ATPase (● — ●). Letters designate center of visible bands.

Enzyme distribution

Figs. 2–4 show the distribution of the total enzymatic activity along the density gradient, compared to the distribution of the visible bands. It is apparent that all of the disaccharidase activities, alkaline phosphatase and the leucine naphthylamide-

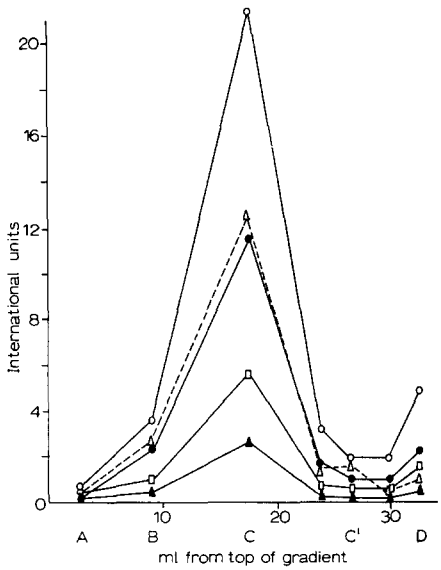


Fig. 4. Distribution of disaccharidases. ●, maltase; ○, sucrase × 10; △, lactase × 100; ▲, trehalase × 10; □, isomaltase × 2. Letters designate center of visible bands.

splitting activities are predominantly localized within the C fraction which is an almost pure preparation of microvillus membranes. ATPase activity appears there also, but some is found also within the B fraction. Due to a high amount of alkaline phosphatase and the nucleotide 5'-phosphatase (EC 3.1.3.5) activity present in brush borders, it was difficult to ascertain the nature of the activity of the ATPase in this fraction. Recoveries of all of the enzymatic activities are close to 100% except for maltase, isomaltase and trehalase which routinely showed greater recoveries of sometimes as much as 200%. This apparent "activation" has as yet not been explained. The specific activities of the enzymes in various fractions from a typical single run are shown in Table I. Fraction C shows the highest specific activities. The

TABLE I

SPECIFIC ACTIVITY OF FRACTIONS OBTAINED FROM THE GRADIENT

	BB	*A*	*B*	*C*	*C'*	*D*
Maltase	2.8	0.22	7.24	12.6	6.9	3.1
Invertase	1.2	0.05	2.3	4.7	2.6	1.3
Lactase	0.07	0.001	0.117	0.28	0.21	0.03
Isomaltase	1.6	0.11	3.1	6.2	3.4	2.1
Trehalase	0.037	0.009	0.15	0.28	0.15	0.06
Alkaline phosphatase	1.81	0.085	3.17	5.92	3.38	1.52
ATPase	0.037	0.030	0.076	0.100	0.074	0.03

increase in specific activity is about four-fold over that of the brush border preparation, which was in turn about ten-fold over the mucosa[22,23].

It is noteworthy, in comparing the specific activities of the various disaccharidases, that the specific activity of lactase, unlike that of other disaccharidases, remains occasionally high in the C′ fraction. The C′ fraction contains microvillus membranes electron-microscopically indistinguishable from those of the C fraction[24], and differs from it by the separation on the gradient, by its lack of all enzymatic activities except lactase and, as will be seen below, by its lower protein content. The anomalous behavior with respect to lactase is especially well shown by plots of enzyme activity per ml along the gradient, which reveal enzyme concentration in the gradient. This difference suggests that lactase activity is possibly concentrated within a different population of microvillus membranes, or that lactase is associated with a different kind of particle than are the other disaccharidases and may have a bearing on the clinical observation that lactase deficiency in comparison to other disaccharidase deficiencies, is relatively common[25]. Brush border lactase appears to be a specific enzyme as differentiated from a non-specific β-galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.23) present in the intestinal cell[26]. The small amount of enzymatic activity present in Fraction D can be removed by recycling through Tris and centrifugation. Upon such recycling all of the original fractions are again obtained concurrent with the removal of the contaminating enzymic activity. The enzymic activity of Fraction D is thus clearly the result of incomplete disruption of brush borders rather than a different localization of the enzymes.

Although the presence of DNA in brush borders is intriguing in view of the recent findings of DNA in mitochondria, chloroplasts and the red blood cell membrane, it is present here in such large amounts that much of it is probably an artifact of isolation and derived from the nucleus of the cell.

The membrane of the microvillus (Fraction C) contains approx. 50% protein by weight. Thus, in this respect it is more like the erythrocyte membrane than the lipid-rich myelin membranes. In several experiments we have calculated the ratio of cholesterol to phospholipid. The results on whole brush borders varied in values between 0.7 and 0.9. One should bear in mind, however, that the value for the phospholipid is based on organic solvent-extractable phosphate and the amount found may vary depending on the nature of the phospholipid. This value is similar to those reported by FINEAN and his co-workers[6] (0.5) and that by ASHWORTH AND GREEN[28] (0.46).

TABLE II

Values are expressed as percentages of dry weight of the fractions and represent averages from several experiments. The variations arise mainly from differences in sample preparation.

Fraction	*% protein*	*% carbohydrate*	*% cholesterol*	*% phospholipid*	*% esterified fatty acid*	*% DNA*
A	63 ± 7	10 ± 5	3 ± 1.6	7 ± 1	3	8
B	29 ± 3	7 ± 3	4 ± 1	7.5 ± 0.5	6	20
C	51 ± 6	4 ± 2	4 ± 1	7 ± 1	4	—
C′	26 ± 4	4	—	8	3	—
D	53 ± 4	5 ± 1	3 ± 1	7 ± 2	5	10
BB	24 ± 9	10 ± 2	5 ± 1	8 ± 1.4	10	24

FINEAN and his co-workers have reported a rising cholesterol to phospholipid ratio on purification of brush border membranes[7,8]; similarly FORSTNER, SABESIN AND ISSELBACHER[9] have recently reported in a preliminary communication, a high ratio similar to that found in red blood cells or myelin.

We have also observed a relatively high value in several experiments varying from 1.0 to 1.3. The values reported in Table II are averages and do not apply to single experiments.

Chemical composition

A rough estimation of the overall chemical composition of fractions obtained after Tris disruption and density-gradient separation is represented in Table II. These results are averages of four to six determinations on pooled fractions obtained as described in the METHODS section.

Fraction A is highest in percentage protein. It also contains the highest amount of P_i not extractable by organic solvents.

Fraction B contains low amounts of protein and phospholipid. The DNA previously reported to be present in guinea-pig and hamster brush border preparations[3,27] is found in this fraction. The identity of the DNA has been shown in both species by the usual reactions with diphenylamine, by the Schiff reaction, by treatment with periodate and subsequent determination of the malonaldehyde formed, as well as the hyperchromic shift after enzymatic reaction with deoxyribonuclease and acid hydrolysis and the chromatographic identification of DNA bases. From this fraction, as previously described[3] a fibrillar material can be formed which contains protein to DNA in a 1:1 ratio. This material carries also the ATPase activity of the B fraction. Additional evidence that the fibrillar material resides in the B fraction was obtained by removal of the fibrillar material after Tris disruption but prior to density-gradient centrifugation. The resulting gradient showed decreased amounts of protein mainly in B and D fractions, which contain substantially all the DNA of the brush border.

Fraction D consists of microvillus cores. Electron-microscopically this fraction has been found to vary in purity from preparation to preparation as would be expected since it is collected as a precipitate. In pooled samples from a large number of experiments this fraction is presumably not homogeneous and contains contaminating material. This fraction is relatively high in protein.

Recently we have attempted to investigate the nature of the lipids found in brush border fraction by use of thin-layer chromatography. No detectable amounts of cholesterol esters were found. The phospholipids consisted mainly of phosphatidyl inositol and phosphatidyl choline; also phosphatidyl serine, phosphatidyl inisitol and sphingomyelin were found. Among neutral lipids in addition to cholesterol, diglyceride, triglyceride and free fatty acids were detected.

ACKNOWLEDGEMENTS

The author is grateful to Dr. R. K. CRANE for his support, advice and interest during the course of this work. I would also like to express my appreciation to Mr. K. COLLYARD, Mr. S. BASS and Mrs. I. EDELSTEIN for their able assistance.

The research was supported by grant No. GM 09224 from the U.S. Public Health Service.

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