STUDIES ON THE ORGANIZATION OF THE BRUSH BORDER IN INTESTINAL EPITHELIAL CELLS

I. Tris Disruption of Isolated Hamster Brush Borders and Density Gradient Separation of Fractions

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ABSTRACT

Brush borders isolated from the epithelial cells of hamster jejunum have been dissociated by treatment with 1 M Tris(hydroxymethyl)aminomethane into several subfractions which can be separated by means of centrifugation on glycerol density gradients. Investigation of the chemical specificity of disrupting agents suggests that the amino group of Tris, in its positively charged state, is involved. Five individual bands or fractions have been routinely recovered from density gradients. The distribution of alkaline phosphatase and maltase activities among these fractions has been studied and the results indicate that both enzymes are predominantly associated with one fraction which has been identified in a companion paper as being composed of the membranes of the brush border microvilli. A fibrillar material of unidentified origin has also been obtained from Tris-disrupted brush borders.

During the past few years, various investigations along several independent lines have revealed the brush border of the epithelial cells of the small intestine to be an exquisitely differentiated subcellular organelle in which certain of the digestive and absorptive capacities of the cell appear to be fixed, possibly in intimate molecular juxtaposition. The process of sugar absorption, which is a particularly clear example of sodium ion–dependent active transport (2, 3), occurs in the brush border (11), as do the disaccharidases (12) which are responsible for the terminal stages of carbohydrate digestion and provide the substrates for absorption. Alkaline phosphatase (5) and leucine aminopeptidase (9, 14) are localized there also, along with an ATPase (17) which has characteristics permitting the assumption that it represents an energy-dependent sodium ion pump. What is now needed is a knowledge of the relationship of these enzymic and functional activities to one another and their identification with the various morphological elements of the brush border.

Biochemical studies, particularly of the influence of sodium ions and phlorizin on the *in vitro* accumulation of sugars released by disaccharidase action (2, 15), have provided a limited amount of information on the relative localization of digestive and absorptive functions. The data permit the tentative conclusion that these two functions, at least with regard to sugars, are close to the free cell surface and are so positioned that the products of disaccharidase activity are formed near the site of absorption. Alkaline phosphatase has been localized histochemically in the plasma membrane of the microvilli (1), and the biochemical studies suggest a similar localization for the disacchari-

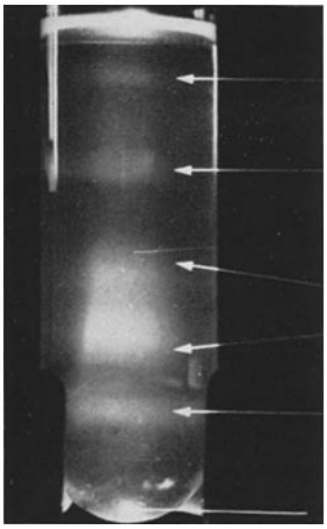

FIGURE 1 Photograph of a typical density gradient separation of Tris-disrupted brush borders.

dases. Such studies, taken in connection with modern concepts of membrane transport (19), have permitted the construction of hypotheses which have structural implications and are consistent with available information but for which there is as yet no evidence based upon an enzymic or functional analysis of microvillus fine structure.

The availability of a method for obtaining the brush border portion of the intestinal epithelial cell as a discrete entity substantially free of contamination by other subcellular organelles (13) provides a starting point for further fractionation. The present report describes a method of fractionating hamster brush borders into several distinct subfractions. A preliminary report of this work has been given (7).

METHODS

PREPARATION OF BRUSH BORDERS: Brush borders were prepared by a slight modification of the method of Miller and Crane (13). The modification involved the use of a Powerstat variable transformer at a setting of 90 to decrease the speed of blade rotation in the Waring Blendor, a decrease in time of homogenization to 15 seconds, and filtering of the homogenate through No. 25 bolting silk. Larger contaminants were removed by centrifugation in an International Clinical Centrifuge at its lowest setting for 1 minute. Hamster brush borders, prepared by this modified procedure, were washed by being suspended in 0.005 M ethylenediamine tetraacetate (EDTA), pH 7.0, and sedimented by centrifugation at 500 *g* for 10 minutes. The supernatant fluid was decanted. Washing was repeated a minimum of three times or until the supernatant fluid was clear. Brush borders prepared in this way were substantially free of other particles, as determined by light microscope and by electron microscope.

DISRUPTION OF BRUSH BORDERS: Washed brush borders were disrupted in the cold, by addition of a volume of freshly prepared 1.0 M Tris (pH 7.0) equal to 4 times the weight of the original mucosa, and stirring on a Vortex mixer for 3 minutes.

DENSITY GRADIENT CENTRIFUGATION: Both continuous and discontinuous gradients were used. The stepwise gradients were prepared by successive introduction of 20, 30, 40, 50, and 60 per cent glycerol solutions containing 0.5 M $MgCl_2$ through a syringe to the bottom of the centrifuge tubes. The continuous glycerol gradient was formed by mixing a 60 and 20 per cent glycerol solution according to the method of Tung and Taylor (18). Centrifugation was carried out for 10 minutes at 63,000 *g* in a Spinco Model L Ultracentrifuge using either an SW 25.1 or an SW 39-L swinging bucket type rotor. The rotor was accelerated manually at a slow rate. Fractionation of the contents of the centrifuged tubes was carried out either by puncture through the bottom of the tube or by an especially designed syringe device (8) which permitted collection of small fractions from the top without disturbing the precipitate. The collected fractions were then dialyzed against distilled water overnight and then against 0.005 M EDTA for several hours. Dialysis is necessary since glycerol at high concentration has been found to inhibit several of the disaccharidases.

ENZYME ASSAYS: Disaccharidase activities were measured as described by Dahlqvist (4). Glucose was estimated with the glucose oxidase reagent on a Technicon autoanalyzer. Alkaline phosphatase was measured by the rate of splitting of *p*-nitrophenylphosphate (Sigma Chemical Co.) in a glycine buffer (0.05M) at pH 10.5 using 0.2 mM $MgCl_2$, 2 mM Zn acetate, and 5 mM $CoCl_2$ as cofactors.

PROTEIN DETERMINATION: Protein was estimated by the use of the Lowry method (10) using crystalline bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Separation of Components of Brush Borders

When hamster brush borders are treated with 1 M Tris as described above and the resulting suspension is examined under a phase contrast microscope, identifiable brush borders are found to have disappeared and to be replaced by a collection of smaller fragments having a variety of shapes and sizes. When such a suspension is layered on a density gradient and centrifuged as above, it sepa-

rates into several bands which can be localized by the Tyndall effect from a strong light passed vertically through the tube. Fig. 1 is a photograph of a representative gradient showing the position of the bands obtained. Bands C and D of the gradient have been studied with the electron microscope, and the morphology of their components is described in the accompanying paper (16).

Upon recovery and recentrifugation on another gradient, fraction A tends to agglomerate and precipitate, fractions B and C again yield single bands, and fraction D remains homogeneous if disruption is carried to completion. However, if fraction D is dialyzed against distilled water and shaken vigorously on the Vortex mixer, the large structures of which this fraction is composed break up into smaller fragments which have not been identified.

Formation of Fibrillar Material

In earlier studies of guinea pig brush borders, a gel-like material was obtained when the brush borders were treated with distilled water and

TABLE I

Disruption of Brush Borders by Compounds Other than Tris

All compounds were tested at 1 M concentration and at pH 7.4 unless otherwise specified. Meaning of symbols: +++, complete disruption; ++, nearly complete disruption; +, poor disruption; + −, distortion and slight disruption; −, no disruption.

Compound	Immediate effect
Tris analogues	
2-Amino, 2-methyl-1,3-propanediol	+++
2-Amino, 2-methyl-1-propanol	+++
tert-Butylamine	++
2,2-Dimethyl-1,3-propanediol	−
2-Hydroxymethyl, 2-methyl-1,3-propanediol	−
Amines other than Tris analogues	
Choline	−
Acetylcholine	−
Tetramethylammonium	− slight distortion
Triethanolamine	+++
Ethanolamine	+++
Histamine	+++
N-Butylamine	++
Urea	+++
Non-ionic organic compounds	
D-Glucose	+ −
D-Mannitol	+ −
D-Fructose	+ −
L-Sorbose	+ −
Glycerol	−
Ethanol	−
Organic anions	
Ethylenediamine tetraacetate 0.05 M	−
Inorganic ions	
Sodium phosphate	+ −
Ammonium chloride	+ −
Sodium chloride	+ −
Potassium chloride	+ −
Sodium hydroxide, pH 9.0	−
Magnesium chloride	+ −

shaken on the Vortex mixer (6). Upon the addition of salt to the gel, fibrillar material was precipitated which was found to have an absorption spectrum in the ultraviolet and a content of organic phosphate suggestive of nucleic acid. In the present studies, dilution of Tris-disrupted hamster brush borders with distilled water or 0.05 M EDTA in a ratio of 1:2 or higher yields a fibrillar material apparently similar in composition to that described above. Judged from the ability to form fibers and from the ultraviolet absorption spectrum, this material appears to be concentrated within bands A and B of the density gradient.

Studies on the Nature of Disruption

Though the mechanism of disruption with Tris remains unknown, a certain degree of specificity is indicated by the fact that only a few of the many compounds tested acted like Tris, which disrupts brush border preparations almost immediately after their addition and a short mixing period. Most compounds either did not disrupt brush borders at all or caused visible deformation only after prolonged standing. Table I summarizes the behavior of the compounds tested. From the fact that analogues of Tris and other compounds having no amino group exhibit no activity, it is clear that disruption requires an amine.

In studying the pH dependence of the process, it was found that Tris disrupted in the pH range between 5 and 7.5, distorted brush borders at pH's 3 and 8, and had no effect at pH 9 and 10. This finding, taken together with the fact that quarternary amines do not appear to disrupt (see Table I), indicates that ionization of the amine is required but that a positive charge on the nitrogen alone is not enough: a replaceable hydrogen must be present. It would appear therefore that the following minimal structure is necessary for the disruption:

$$R\text{—}\overset{\overset{+}{|}}{\underset{|}{N}}\text{—}H$$

The role of the amine in disruption and the nature of the disrupted bond are not known. One possibility is that, in a lipid environment of the brush border, bonding involves an ionic or hydrogen bond which can be disrupted by a hydrogen exchange mechanism, and that the solubility characteristics of the disrupting compound account for the larger part of the specificity for disruption.

The concentration of the disrupting substance appears to be critical. All the active compounds tested showed little activity at or below 0.2 M. They deformed brush borders at 0.5 M, and disrupted at 0.6 M or above. Upon density gradient centrifugation of particles disrupted by amines other than Tris, bands very similar to those formed after disruption with Tris were obtained.

Distribution of Alkaline Phosphatase and Maltase after Density Gradient Centrifugation

Fig. 2 illustrates the distribution of alkaline phosphatase and maltase activities along the density gradient prepared as described above. The highest total and specific activities of alkaline phosphatase and maltase were found in fraction C. This finding strongly suggests that this fraction is

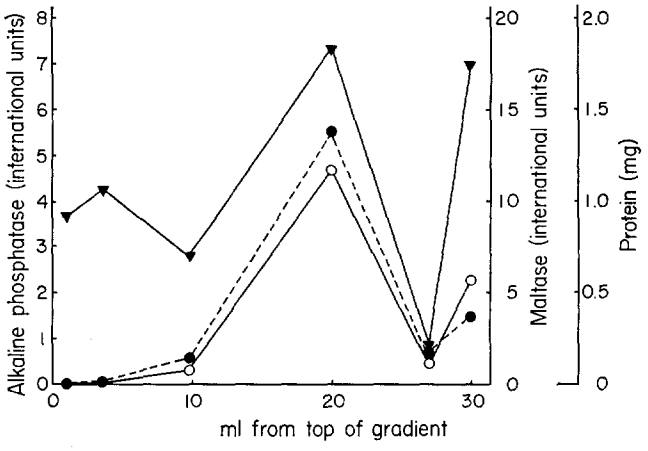

FIGURE 2 Distribution of maltase (solid circles), alkaline phosphatase (open circles), and protein (solid triangles) along the density gradient.

derived from the plasma membrane of the microvilli. High recoveries (80 to 90 per cent) of enzymatic activities can be obtained in fraction C if the brush borders are subjected to prolonged and vigorous mixing during disruption with tris. It is also of interest that alkaline phosphatase and maltase activities do not separate if fraction C is recovered, sonicated, and recentrifuged on a gradient, although the material separates into several distinct bands. Fraction A contains a large amount of protein but substantially none of the measured enzymatic activities.

The fractions from the density gradient are being subjected to a more detailed chemical analysis and a complete determination of enzymes known to be present in brush borders. This work will be reported elsewhere.

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BIBLIOGRAPHY

1. CLARK, S., The localization of alkaline phosphatase in tissues of mice, using the electron microscope, *Am. J. Anat.*, 1961, **109,** 57.
2. CRANE, R. K., Hypothesis for mechanism of intestinal active transport of sugars, *Fed. Proc.*, 1962, **21,** 891.
3. CSAKY, T. Z., A possible link between active transport of electrolytes and monoelectrolytes, *Fed. Proc.*, 1963, **22,** 3.
4. DAHLQVIST, A., Method of assay of intestinal disaccharidases, *Anal. Biochem.*, 1964, **7,** 18.
5. DEMPSEY, E. W., and DEANE, H. W., The cytological localization, substrate specificity, and pH optima of phosphatases in the duodenum of the mouse, *J. Cell. and Comp. Physiol.*, 1946, **27,** 159.
6. EICHHOLZ, A. and CRANE, R. K., Subfractions of brush border membranes isolated from intestinal epithelial cells, *Fed. Proc.*, 1963, **22,** 416.
7. EICHHOLZ, A., Specific disruption of isolated brush borders from intestinal epithelial cells by the use of Tris(hydroxymethyl)aminomethane, *J. Cell Biol.*, 1964, **23,** No. 2, 27A.
8. EICHHOLZ, A., and CRANE, R. K., unpublished data.
9. HOLT, J. H., and MILLER, D., The localization of phosphomonoesterase and aminopeptidase in brush borders isolated from intestinal epithelial cells, *Biochim. et Biophysica Acta*, 1962, **58,** 239.
10. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 1951, **193,** 265.
11. MCDOUGAL, D. B., LITTLE, K. D., and CRANE, R. K., Studies on the mechanism of intestinal absorption. IV. Localization of galactose concentrations within the intestinal wall during active transport, *in vitro, Biochim. et Biophysica Acta*, 1960, **45,** 483.
12. MILLER, D., and CRANE, R. K., The digestive function of the epithelium of the small intestine. II. Localization of disaccharidase hydrolysis in the isolated brush border portion of the intestinal epithelial cells, *Biochim. et Biophysica Acta*, 1961, **52,** 293.
13. MILLER, D., and CRANE, R. K., A procedure for the isolation of the epithelial brush border membrane of hamster small intestine, *Anal. Biochem.*, 1961, **2,** 284.
14. NACHLAS, M. N., MONIS, B., ROSENBLATT, D., and SELIGMAN, A. M., Improvement in the histochemical localization of leucine aminopeptidase with a new substrate, *L*-leucyl-4-methoxy-2-naphthylamide, *J. Biophysic. and Biochem. Cytol.*, 1960, **7,** 261.
15. NEWEY, H., SANFORD, P. A., and SMYTH, D. H., Location of function in the intestinal epithelial cell in relation to carbohydrate absorption, *J. Physiol.*, 1963, **168,** 423.
16. OVERTON, J., EICHHOLZ, A., and CRANE, R. K., Studies on the organization of the brush border in intestinal epithelial cells. II. Fine structure of fractions of Trisdisrupted hamster brush borders, *J. Cell Biol.*, 1965, **26,** 693.
17. TAYLOR, C. B., Cation-stimulation of an ATPase system from the intestinal mucosa of the guinea pig, *Biochim. et Biophysica Acta*, 1962, **60,** 437.
18. TUNG, L. H., and TAYLOR, W. C., An improved method for preparing density gradient tubes, *J. Polymer Sc.*, 1956, **21,** 144.
19. WILBRANDT, W., and ROSENBERG, T., The concept of carrier transport and its corollaries in pharmacology, *Pharmacol. Rev.*, 1961, **13,** 109.