Glucose- and Phlorrhizin-Protected Thiol Groups in Pig Intestinal Brush-Border Membranes

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Purified brush borders, prepared from newborn pig intestine, were incubated in the presence of ^{203}Hg -labelled *p*-chloromercuribenzenesulphonic acid and the membrane proteins later separated by polyacrylamide-gel electrophoresis. The presence of either D-glucose or phlorrhizin, during a preliminary incubation in non-radioactive *p*-chloromercuribenzenesulphonic acid, increased the subsequent binding of the ^{203}Hg -labelled compound to a protein of molecular weight 31 500. This increase appeared to be specific for the low-molecular-weight protein, provided that the concentration of protecting agent used corresponded to that used to produce a biological response in the intact tissue. These results are discussed in relation to the known properties of other presumptive sugar carriers isolated from different membranes.

Thiol-reacting compounds have long been known to inhibit the facilitated diffusion of sugars across a wide variety of biological membranes. This inhibition can be antagonized by the presence of transportable sugars. It was the exploitation of this finding which led originally to the isolation of a β -galactoside-binding protein from a particulate fraction of *Escherichia coli* (Fox & Kennedy, 1965). The molecular weight of the protein, determined by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, was about 30 000 (Jones & Kennedy, 1969). A similar *N*-ethylmaleimide-labelled protein has since been isolated from rat kidney brush-border membranes (Thomas, 1973). In each case it has been suggested that the low-molecular-weight protein is identical with, or is a part of, the sugar-transport system in membranes.

Another thiol-reacting compound, *p*-chloromercuribenzenesulphonic acid, has been shown to inhibit the ability of galactose to interact with the intestinal mucosa of rabbits (Schaeffer *et al.*, 1973). A similar effect of *p*-chloromercuribenzenesulphonic acid occurs when glucose interacts with newborn pig intestine (Henriques de Jesus & Smith, 1974). The present work uses radioactively labelled *p*-chloromercuribenzenesulphonic acid in an attempt to identify the glucose-transport system in pig intestinal brush borders. The properties of a *p*-chloromercuribenzenesulphonic acid-labelled protein are then compared with those of other putative sugar carriers isolated previously from different sources.

Materials and methods

Pigs obtained at birth from the Babraham herd were killed and the small intestine was dissected

into ice-cold bicarbonate saline (Krebs & Henseleit, 1932). The mid-portion of the tract was then either mounted in an Ussing chamber for the measurement of short-circuit current as described previously (Henriques de Jesus & Smith, 1974), or used for the preparation of brush-border membranes by the method of Porteous (1968). Homogeneity of brush-border preparations was assessed by phase-contrast microscopy. Contamination from nuclei was negligible. Alkaline phosphatase activity, measured with 1 mM-*p*-nitrophenyl phosphate as substrate in the presence of 20 mM-MgCl₂ at pH 9.6, was high and comparable with values reported previously ($3.0 \pm 0.6 \mu\text{mol}$ of *p*-nitrophenyl phosphate hydrolysed/min per mg of pig brush-border protein; 4.9 for the rat; Forstner *et al.*, 1968).

Purified brush borders were finally suspended in 2 ml of bicarbonate saline (2–5 mg of protein/ml). Half the suspension was incubated for 15 min at room temperature in the presence of 5 mM-*p*-chloromercuribenzenesulphonic acid. The other half was treated identically but with 2.5 mM-glucose or 0.2 mM-phlorrhizin present. Both suspensions were centrifuged at 30 000g for 10 min and the pellets washed in fresh bicarbonate saline. Brush borders, recovered from this suspension by a second centrifugation at 30 000g for 10 min, were then mixed with 1 ml of bicarbonate saline containing 5 mM-*p*-chloro[^{203}Hg]-mercuribenzenesulphonic acid. These suspensions were shaken occasionally at room temperature for 60 min. They were then centrifuged and washed as above. Samples were taken for protein determination (Lowry *et al.*, 1951) and the remaining pellets were used for preparative polyacrylamide-gel electrophoresis by the method of Ferguson & Twite (1974). Membranes were dissolved in 3% (w/v) sodium

dodecyl sulphate in 10mM-sodium phosphate buffer, pH 7.2, containing 4M-urea but no 2-mercaptoethanol, since this might conceivably displace ^{203}Hg from membrane proteins. A total of 2mg of protein was placed on each gel for electrophoresis. After electrophoresis protein bands were stained with Coomassie Blue. The gels were then cut up into 3mm slices and ^{203}Hg radioactivity was counted directly on a Packard Autogamma spectrometer.

p-Chloro[^{203}Hg]mercuribenzenesulphonic acid came from The Radiochemical Centre, Amersham, Bucks., U.K., as a gift from Dr. P. J. Bentley and Dr. J. S. Charnock. It was stated to contain less than 3% ionic Hg. Non-radioactive *p*-chloromercuribenzenesulphonic acid came from Sigma Chemical Co., St. Louis, Mo., U.S.A. Phlorrhizin came from Phase Separation Ltd., Queensferry, Flints., U.K. All other chemicals were of A.R. grade.

Results and discussion

The interaction of glucose with intestinal brush borders can be studied indirectly in the intact tissue by measuring changes in short-circuit current. The primary action of glucose is to increase the permeability of the brush-border membrane to Na^+ .

Fig. 1. Effect of glucose and phlorrhizin on the short-circuit current of newborn pig intestine

The intestine was mounted in an Ussing chamber in Krebs bicarbonate saline gassed with $\text{O}_2 + \text{CO}_2$ (95:5), containing 5mM-glucose, at 37°C. This medium was replaced with one containing no glucose and the short-circuit current allowed to decay to a constant value. Different amounts of glucose were then added in small volumes to the mucosal side of the preparation; increments in short-circuit current were measured and represented as a percentage of the maximal response to glucose (○). In a second series of experiments different concentrations of phlorrhizin and 5mM-glucose were added simultaneously to the intestine bathed in glucose-free solution. The increment in short-circuit current was then compared with that found with 5mM-glucose. Results are expressed as percentage inhibition of a mean response to 5mM-glucose (●). Each point gives the mean $\pm$ S.E.M. of 9–12 observations.

Na^+ entering the mucosa is then pumped electrogenically to the blood side of the tissue, causing the short-circuit current to increase rapidly. The whole process appears to follow Michaelis–Menten kinetics with an apparent K_m for glucose of 1.9mM (Fig. 1). Phlorrhizin acts as a competitive inhibitor of this response. The glycosidic portion of the molecule possesses all the structural features necessary for binding to the sugar-transport mechanism (Alvarado & Crane, 1964). The kinetics of phlorrhizin inhibition of a standard glucose response are shown in Fig. 1. The response to glucose was halved in the presence of 0.2mM-phlorrhizin. These initial experiments on intact tissue allow one to define concentrations of glucose and phlorrhizin which could be expected to produce effects on purified preparations of intestinal brush borders.

The normal distribution of ^{203}Hg from *p*-chloro[^{203}Hg]mercuribenzenesulphonic acid among proteins separated from intestinal brush borders is shown in Plate 1. Significant amounts of ^{203}Hg bind to membrane proteins, even though these proteins have been subjected previously to a 15min contact with 5mM non-radioactive *p*-chloromercuribenzenesulphonic acid. All proteins show this secondary binding of *p*-chloro[^{203}Hg]mercuribenzenesulphonic acid, but it is most noticeable with a single protein of mol.wt. 31 500 (mean value; 10 determinations). This delayed binding of *p*-chloromercuribenzenesulphonic acid to thiol groups could reflect a restricted access of inhibitor to the reactive group. It is known that biological membranes are only slightly permeable to *p*-chloromercuribenzenesulphonic acid (Rothstein, 1970). These slow-reacting thiol groups could be partially protected from *p*-chloromercuribenzenesulphonic acid by their hydrophobic surroundings.

The lower half of Plate 1 compares the binding of *p*-chloro[^{203}Hg]mercuribenzenesulphonic acid to glucose- or phlorrhizin-treated membranes with that found under control conditions. An *R* value greater than 1 indicates that glucose or phlorrhizin has caused some additional protection of thiol groups during the initial incubation with non-radioactive *p*-chloromercuribenzenesulphonic acid, causing more *p*-chloro[^{203}Hg]mercuribenzenesulphonic acid to bind during the second period of incubation. There was no consistent protection of high-molecular-weight proteins with glucose or phlorrhizin, used at concentrations known to cause approximately 70% of the maximal effect on the intact tissue. Variation in *R* values over the initial part of the gel appeared random, but there was some protection of the 31 500-mol.wt. protein, the *R* value increasing to maximum values of 1.1 and 1.2 from a steady baseline of 0.9. Repeating these experiments with a higher concentration of phlorrhizin (5mM) caused protection of several proteins, including the one of mol.wt. 31 500. These results, showing non-specific

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of pig intestinal brush-border membranes

Purified brush borders, previously incubated in *p*-chloro[^{203}Hg]mercuribenzenesulphonic acid, were dissolved in 3% (w/v) sodium dodecyl sulphate as described previously (Ferguson & Twite, 1974). Bands are numbered according to their estimated mass in daltons calculated from their respective R_F values in relation to cytochrome *c* (C). The origin (O) is marked to the left of the gel. The upper trace shows the distribution of ^{203}Hg in a control experiment. The lower trace shows the ratio *R* of counts for glucose- or phlorrhizin-treated membranes to that for control membranes. Values of *R* represent, in each case, the mean of three experiments using either 2.5mM-glucose (—) or 0.2mM-phlorrhizin (----) to protect proteins against the initial binding of non-radioactive *p*-chloromercuribenzenesulphonic acid.

protection with a so-called specific ligand, highlight the importance of keeping the concentrations of suspected protecting agents as low as possible in this type of work.

Proteins with a molecular weight of about 30000, having specific binding affinities for sugars and phlorrhizin, have already been isolated from both bacterial and kidney brush-border membranes and it may be that all these proteins have structural features in common. Whether they are an integral part of the sugar-transport system has still, in our opinion, to be proved conclusively. The low-molecular-weight protein in pig intestinal brush borders is present in quite large amounts (as much as 15% of the total membrane protein). This seems an unusually large proportion of protein to devote to a single transport process in a membrane which is known to encompass many different transport functions. Binding of glucose and phlorrhizin to intestinal brush borders, although specific in itself, could conceivably alter the conformation of surrounding proteins, changing their binding affinities for thiol-reacting agents. Such an effect might well amplify an initial binding response into one where a large proportion of membrane protein shows changed binding characteristics for *p*-chloromercuribenzenesulphonic acid. These reser-

vations as to physiological importance of the glucose-protected protein will only be finally dispelled once a way has been found to isolate the protein without denaturation, so that its physiological transport properties might again be demonstrated in a reconstituted membrane.

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