Release of Intestinal Surface-Membrane Glycoproteins Associated with Enzyme Activity by Brief Digestion with Papain

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Rat intestinal surface-membrane glycoproteins were labelled by intraperitoneal injection of [$1\text{-}^{14}\text{C}$]glucosamine 4h before the animals were killed. At this time, density-gradient centrifugation of disrupted brush borders indicated that glycoprotein radioactivity was distributed identically with sucrase, a plasma-membrane marker. Labelled brush borders were digested by papain for brief time-intervals known to release surface-enzyme particles without disruption of the unit membrane. Digestion for 5min released 90% of the surface sucrase, and almost one-half of the brush-border glycoprotein and label. On Sepharose 4B column chromatography most of the glycoprotein and label emerged as a single peak. This peak contained the most actively labelled glycoprotein in the brush border and was closely associated with maltase, sucrase, β -naphthylamidase and alkaline phosphatase. The peak was partially resolved on polyacrylamide-gel electrophoresis into three bands. Each band contained a distinctive enzyme or enzyme pair, and was labelled by [$1\text{-}^{14}\text{C}$]glucosamine. No periodic acid-Schiff-negative protein was observed in the peak material. Glycoproteins susceptible to brief digestion with papain are therefore closely linked to released surface-enzyme particles. Intestinal surface glycoproteins are heterogeneous with respect to molecular weight, electrophoretic mobility and function.

Glycoproteins present in or on the intestinal brush-border plasma membrane can be labelled by [$1\text{-}^{14}\text{C}$]glucosamine *in vivo* (Forstner, 1968). The specific radioactivity of glycoprotein hexosamine reaches a maximum between 90 and 180min (Forstner, 1970). During this period until at least 12h after intraperitoneal injection of the precursor label the specific radioactivity of glycoprotein hexosamine is higher in brush-border glycoproteins than in glycoproteins of other subcellular fractions (Forstner, 1968, 1970). Since a significant portion of the total membrane glycoprotein may be attached to the external leaflet of the plasma membrane as a glycocalyx (Ito, 1965a), the following experiments were designed to determine whether mild conditions of proteolytic digestion (Eichholz, 1968), a procedure known to remove surface-enzyme particles without altering unit-membrane structure (Johnson, 1967), would also be effective in releasing glycoprotein. The release of several labelled glycoproteins by short exposure to papain is described. Evidence is also presented indicating that the most highly labelled of the released glycoproteins are closely related to, if not identical with, several similarly released surface enzymes.

EXPERIMENTAL

Preparation of labelled brush borders. Male Wistar rats weighing 100-200g and fed *ad libitum* were injected intraperitoneally with [$1\text{-}^{14}\text{C}$]glucosamine (New England Nuclear Corp., Boston, Mass., U.S.A.; $50\mu\text{Ci/mg}$) in 0.2ml of 150mM-NaCl. After a suitable time-interval, usually 4h unless otherwise specified, the rats were killed by a blow on the head. The small intestine was removed from the ligament of Treitz to a point 6cm proximal to the ileocaecal valve, rinsed with 50.0ml of ice-cold 150mM-NaCl and everted. Brush borders were prepared from scrapings of the everted mucosa as described by Forstner (1970), except that the initial homogenization was performed in 100 vol. of 5.0mM-EDTA, pH 7.4, rather than 50 vol.

Preparation of density-gradient fractions from disrupted brush borders. Brush-border pellets obtained from 3.0g of intestinal mucosa scrapings were drained of supernatant EDTA (pH 7.4) and suspended in 12.0ml of ice-cold 1.0M-tris-HCl buffer, pH 7.0 (Eichholz & Crane, 1965). The suspension was vigorously stirred on a vortex mixer for 3min. A portion (4.0ml) was then placed on a discontinuous sucrose density gradient containing 5.0ml each of 10, 20, 30, 40 and 50% (w/v) sucrose in 0.5M-MgCl₂. The gradient was centrifuged for 10min at 63000g. Fractions (4.0ml) were removed from the top of the gradient by Pasteur pipette, and a final fraction was

obtained by vigorously suspending material on the bottom of the gradient tube with a spatula. Fractions were dialysed against two changes of 5.0 mM-EDTA, pH 7.4, at 4°C and frozen at -10°C until analysed.

Hydrolysis of brush borders with papain. The method of Eichholz (1968) was used with slight modification. Brush-border preparations were incubated for 5 min and re-incubated subsequently for 60 min with papain at 37°C. The incubation mixture contained (per ml) 0.5 mg of brush-border protein, 10 µg of papain, 3.0 µmol of EDTA, 12.5 µmol of potassium phosphate buffer, pH 7.0, and 3.0 µmol of cysteine. At the end of each digestion period the mixture was cooled in ice-cold water and centrifuged at 13500g for 30 min. The supernatants were dialysed against three changes of ice-cold water for 20 h, then concentrated by freeze-drying to 0.5 ml or 1.0 ml.

Column chromatography. Sepharose 4B columns were prepared and used as described by Forstner (1970). The void volume was determined with Dextran Blue 2000 (Pharmacia, Uppsala, Sweden). The length of the gel column was 48 cm and width 0.9 cm. In most experiments the void volume was 10.0 ml and the total volume 34.4 ml. Molecular-weight determinations were derived from K_{av} values, with data provided by Pharmacia (Sepharose technical bulletin, Pharmacia), and are subject to the reservations discussed by Forstner (1970).

Sephadex G-200 was prepared by swelling in water for 48 h, then packed by gravity into a column 60 cm long × 0.9 cm diam. The length of the gel column was 53 cm. The void volume was 11.0 ml. The column was rinsed with 100 ml of 10 mM-potassium phosphate buffer, pH 7.0, before use, and the sample was eluted by gravity with the same buffer.

Polyacrylamide-gel electrophoresis. Polyacrylamide gels [4% (w/v) final total concentration] were cast in 9.0 cm columns in soft-glass tubing (inside diam. 5 mm). Besides acrylamide the gel solution contained 0.15 M-tris-borate buffer, pH 8.6, 0.056% ammonium persulphate and 0.05% *NNN'*-tetramethylenediamine. The acrylamide/bisacrylamide ratio was 19:1. Where indicated, sodium dodecyl sulphate was added to a final concentration of 0.1%. During the gelation the monomer was carefully overlaid with approx. 0.3 ml of water. The water was removed and replaced with 0.09 M-tris-borate buffer, pH 8.6, when the gels had hardened (10–15 min at room temperature). The tubes were then placed in an electrophoresis apparatus which was filled with 0.09 M-tris-borate buffer, pH 8.6, to cover the electrodes. Excess of ammonium persulphate was removed by preliminary electrophoresis (3.0 mA/tube for 1 h) and the buffer solutions were replaced. The sample (usually 10–40 µl) dissolved in 10% (w/v) sucrose solution containing Bromphenol Blue as a marker was layered on to the upper surface of the gel column by microsyringe. Electrophoresis was carried out at 2.0 mA/tube, either until the marker had traversed the tube or, when indicated, for an additional 30 min.

Staining of gels. Protein bands were stained with 0.5% Amido Black in 7% (v/v) acetic acid and washed and stored in 15% (v/v) acetic acid. Glycoproteins were stained with a periodic acid-Schiff technique as described by Zacharius, Zell, Morrison & Woodlock (1969).

Determination of radioactivity and enzymic activity in gels. Gels were sliced with a razor blade by hand at 2.0 mm

intervals. For determination of radioactivity gel slices were broken up in a tuberculin syringe with a 21 gauge needle in 0.4 ml of water and washed into counting vials, to which Bray's (1960) solution was added. For determination of enzyme activity gel slices were added to 0.5 ml of water, stirred at 4°C every 5 min for 1 h and left overnight at 4°C. The supernatant solution was then analysed for enzyme activity as described below.

Enzyme assays. Disaccharides were determined as described by Dahlqvist (1964). Alkaline phosphatase was determined with *p*-nitrophenol as substrate under conditions described by Forstner, Sabesin & Isselbacher (1968). β -Naphthylamidase was determined by a modification of the procedure of Goldberg & Rutenberg (1958). The final incubation mixture contained (in 1.0 ml), in addition to enzyme, 0.342 µmol of L-leucine β -naphthylamide, 50 µmol of sodium phosphate buffer, pH 7.0, and 1.5 µmol of ZnCl₂. One unit of enzyme activity is defined as the amount required to hydrolyse 1 µmol of substrate in 1 min at 37°C.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Glycoprotein hexosamine was determined by methods described by Forstner (1970).

Determination of radioactivity. Samples in less than 0.5 ml of water were added directly to vials containing 15.0 ml of Bray's (1960) solution. The radioactivity in each vial was counted in a Packard scintillation counter. Quenching was determined and corrected for by a channels-ratio counting method (Forstner, 1970).

RESULTS

In all experiments unless otherwise indicated rat brush borders were labelled radioactively by intraperitoneal injection of 5–10 µCi of [¹⁴C]-glucosamine and isolated when the rat was killed 4 h later. Fig. 1 shows that virtually all of the radioactive label is confined to plasma-membrane glycoprotein at 4 h, although not necessarily at earlier time-periods. Brush-border fractions were examined by density-gradient centrifugation after treatment with 1.0 M-tris-HCl buffer, pH 7.0. Sucrase was used as a marker for the plasma membrane (Forstner *et al.* 1968). At 30 and 120 min radioactivity was found in acid-precipitable lipid-free protein in the least dense fractions from the top of the gradient as well as in the fractions rich in sucrase. At 4 h the distribution of radioactivity in acid-precipitable lipid-free glycoprotein coincided very closely with that of sucrase, as might be expected of a labelled glycoprotein on or in the plasma membrane.

Brush borders were digested by papain for 5 and 60 min. Solubilized material was separated from the remaining insoluble membrane by centrifugation as described in the Experimental section, dialysed against three changes of water at 4°C and concentrated for assay by freeze-drying. Initial results are shown in Table 1. As expected from the work of Eichholz (1968) most of the membrane-

Fig. 1. Density-gradient separation of labelled disrupted brush borders. Three rats were injected intraperitoneally with $5.0 \mu\text{Ci}$ of $[1\text{-}^{14}\text{C}]\text{glucosamine}$ and killed after (a) 30 min, (b) 120 min and (c) 240 min. Brush borders were disrupted with 1.0M -tris-HCl buffer, pH 7.0, and the fragments were separated by density-gradient centrifugation as described in the Experimental section. Three density-gradient centrifugations were performed for each rat. Corresponding fractions from each gradient were combined and analysed for sucrose (▲), acid-precipitable lipid-free protein (○) and acid-precipitable lipid-free glycoprotein radioactivity (Δ). Recovery is expressed as a percentage of the total activity on the gradients.

bound sucrose was released into the supernatant fraction after 5 min digestion with papain. At the same time 45% of the total radioactivity in the brush border was released. An additional 17% of the radioactive label was released into the supernatant by further digestion with papain for 60 min, and 21% of the label remained in the sediment from this digestion. Since radioautographic experiments have indicated that the surface glycocalyx is labelled in preference to the plasma membrane (Ito & Revel, 1966), it is noteworthy that the 5 min supernatant was the only fraction in which the specific radioactivity of glycoprotein hexosamine was greater than that of the undigested brush border.

The solubilized material obtained by digestion with papain for 5 min therefore contained a major fraction of the total radioactive label in the brush border, as well as virtually all of the surface-coat enzyme sucrose. Since the unit membrane is not altered under these conditions of digestion (Johnson, 1967), it seemed probable that the released label represented a solubilized surface-coat glycoprotein. The supernatant from the 5 min digestion was therefore examined in greater detail. Multiple preparations were studied because it was not possible often to obtain sufficient material on one experiment for all analyses. The reproducibility of the digestion procedure was reasonably good, however. In nine preparations the supernatant from the 5 min digestion contained $43 \pm 1.8\%$ (S.E.M.) of the label, and $90 \pm 2.1\%$ (S.E.M.) of the sucrose.

To determine the distribution of radioactivity, protein and protein-bound hexosamine, a single rat was injected with $5.0 \mu\text{Ci}$ of $[1\text{-}^{14}\text{C}]\text{glucosamine}$ and the radioactive homogenate was pooled with unlabelled homogenate obtained from two rats killed at the same time. Brush borders were prepared and digested with papain, as in Table 1. The supernatant from 5 min digestion was dialysed

Table 1. Digestion of $[1\text{-}^{14}\text{C}]\text{glucosamine}$ -labelled brush borders by papain

Three rats received $[1\text{-}^{14}\text{C}]\text{glucosamine}$ intraperitoneally 4 h before death. Brush borders were isolated and digested with papain as described in the Experimental section. Sucrose and radioactivity were determined in brush borders before digestion, material solubilized by 5 min digestion with papain, or by redigestion for 60 min, and in the sediment from the 60 min digestion. Glycoprotein hexosamine radioactivity was determined in acid-precipitable lipid-free material as described by Forstner (1970).

Fractions	Sucrose		Radioactivity		Sp. radioactivity of glycoprotein hexosamine (c.p.m./ μg)
	units	%	Total c.p.m.	%	
Brush border before digestion	9.9	100	94500	100	57
Supernatant from 5 min digestion	9.0	91	42300	45	69
Supernatant from 60 min digestion	0.14	1.4	16250	17	56
Sediment from 60 min digestion	0.29	2.9	19550	21	51
Recovery (%)		95.3		83

Fig. 2. Sepharose 4B column chromatography of '5 min supernatant' obtained by papain digestion of labelled brush borders. Samples (1.0 ml) of the column eluate were analysed for radioactivity (Δ) and protein ($\circ$). Total hexosamine (----) was determined on combined 4.0 ml samples.

Table 2. *Specific radioactivity and total recovery of glycoprotein hexosamine in brush-border fractions derived by papain digestion and Sepharose 4B chromatography of the '5 min supernatant'*

Three rats were used. One rat was injected with 5.0 μ Ci of [$1\text{-}^{14}\text{C}$]glucosamine to label glycoproteins. Homogenates were pooled and brush borders prepared. Radioactivity incorporated into glycoprotein was determined after acid precipitation and lipid extraction in all cases except in fractions obtained from the Sepharose 4B column, which were dialysed but not lipid extracted, to avoid loss of material.

Fractions	Sp. radioactivity of glycoprotein hexosamine (c.p.m./ μ g)	Glycoprotein hexosamine	
		μ g	% of total recovered
Homogenate	10.9		
Brush border	22.6	400	
5 min papain digestion column fractions			
15-18 ml	28.6	38.0	8.7
19-22 ml	33.1	98.0	22.4
23-26 ml	29.0	65.0	15.0
27-30 ml	21.8	38.3	8.8
Remainder	—	16.4	3.7
60 min papain digestion			
Supernatant	18.0	80	18.3
Precipitate	21.2	102	23.4
Total recovered		437	100

against water at 4°C, concentrated by freeze-drying to 0.5 ml and chromatographed on Sepharose 4B. Fractions (1.0 ml) of the eluate were analysed for radioactivity and protein. Fractions were also pooled at 4.0 ml intervals for determination of protein-bound hexosamine and hexosamine radioactivity. As shown in Fig. 2, two major protein peaks were obtained with peak elution volumes of 21 and 29 ml. The radioactive label was associated with peak 1. This peak was fairly symmetrical, with a rather delayed shoulder in its descending

limb beginning at approx. 25.0 ml. Total protein-bound hexosamine in the pooled fractions was greatest in the area of the radioactive peak. The hexosamine specific radioactivity and total hexosamine in the column fractions were compared with those found in protein-bound material obtained by acid precipitation and lipid extraction of the original brush-border preparation, the supernatant from the 60 min papain treatment and the residual precipitate (Table 2). The three fractions with highest hexosamine specific radioactivity corres-

Fig. 3. Sepharose 4B column chromatography of '5 min supernatant' obtained by papain digestion of labelled brush borders. Samples (1.0 ml) of the column eluate were assayed for radioactivity (Δ), maltase ($\bullet$), sucrase ($\blacktriangle$), β -naphthylamidase ($\square$) and alkaline phosphatase ($\blacksquare$).

ponded to the major radioactive peak obtained from the column. These fractions contained 46% of the recovered protein-bound hexosamine. Thus, as well as releasing a major portion of the radioactive label, brief digestion with papain also released a major portion of the total glycoprotein in the brush borders. Most of this glycoprotein hexosamine was found in the labelled chromatographic peaks and the specific radioactivity of the glycoprotein hexosamine in this peak was greater than that of any other fraction derived from the membrane.

In similar experiments column eluates were analysed for maltase, sucrase, β -naphthylamidase and alkaline phosphatase, since these enzymes are released by papain and were most likely to represent independent surface molecules or complexes (Eichholz, 1968). Representative results are shown in Fig. 3. All of the enzymes assayed had elution profiles that were partially superimposed on the radioactive peak. Maltase consistently appeared earlier than the peak and alkaline phosphatase consistently followed it. Sucrase and β -naphthylamidase always appeared in the vicinity of the radioactive peak, although their elution curves were rarely identical with that of the radioactive material. These results suggest that the labelled glycoprotein released by papain within 5 min was a macromolecule corresponding to solubilized surface hydrolytic enzymes in molecular weight and being possibly closely associated with, if not identical with, them. From a consideration of elution volumes, and determination of K_{av} values, the radioactive glycoprotein and sucrase behaved as molecules with molecular weights of approx. 200 000. This value for sucrase agrees well with reported estimations by independent methods, of 202 000 and 211 000 for sucrase-isomaltase (Kolinska & Semenza, 1967).

However, the apparent molecular-weight range of the four enzymes was rather large, varying from approx. 70 000 for alkaline phosphatase to 400 000 for maltase, indicating that this particular column had a limited ability to separate molecules of the above indicated molecular-weight range. Further resolution of the enzyme-rich peak was therefore attempted by electrophoresis on polyacrylamide disc gels.

The major glycoprotein peak and the bulk of the enzymic activity, usually from 16 to 25 ml on this column, was combined, dialysed against cold water, concentrated by freeze-drying and subjected to polyacrylamide-gel electrophoresis. The results of a typical experiment are shown in Fig. 4(a). Two major protein bands were invariably obtained. Band A was sharp and clearly demarcated from band B. Band B on the other hand was wide and variably resolved into two bands, B_1 and B_2 . Instead of a single band staining positively for carbohydrate, as expected from the chromatographic experiments, all three bands stained strongly with the periodic acid-Schiff reagent. The relative mobilities of these bands were unaltered by the addition of 1 or 0.25% sodium dodecyl sulphate, or by the use of a variety of buffer systems.

Several experiments were performed to establish the relationship of these bands to enzymes eluted from Sepharose 4B. In the first experiment sequential fractions corresponding to the area of the maltase peak (16–18 ml), the radioactive peak (19–23 ml) and the radioactive 'tail' (24–31 ml) were taken from the Sepharose 4B column eluate, dialysed and concentrated. When examined by polyacrylamide-gel electrophoresis the maltase peak (Fig. 4b) appeared as a single band in the A region and stained positively with the periodic acid-Schiff stain. The material from the radioactive peak (Fig. 4c) produced all three bands, A, B_1 and B_2 .

Fig. 4. Polyacrylamide disc-gel electrophoresis of selected fractions of the '5 min supernatant' obtained by column chromatography. Fractions obtained from Sepharose 4B columns: (a) enzyme-rich fraction (18–25 ml); (b) maltase-rich fraction (16–18 ml); (c) radioactive peak (19–23 ml); (d) radioactive 'tail' (24–31 ml); (e) 16–25 ml fraction before rechromatography on Sephadex G-200. Fractions obtained from Sephadex G-200 column: (f) fraction II, containing sucrose-isomaltase; (g) fraction I, containing maltase, β -naphthylamidase and alkaline phosphatase. In all cases electrophoresis was continued for 30 min after the marker had traversed the gel. Each gel was stained with Amido Black to demonstrate protein. In all gels except (d) bands stained by Amido Black also reacted with the periodic acid-Schiff reagent. A quantitative assessment of the amount of protein in each fraction is not possible since the amount of each fraction applied to the gel was varied. Thus the amount of protein in bands A, B₁ and B₂ shown in (d) reflect an approximately four-fold greater application than in (c), in order to demonstrate the more mobile proteins.

The radioactive 'tail' contained these plus a large number of faster-travelling proteins, some of which were also periodic acid-Schiff-positive (Fig. 4d). Thus the high-molecular-weight maltase, which appeared in the column eluate before the radioactive glycoprotein, accounted for at least part of band A, and also reacted as a glycoprotein with the periodic acid-Schiff stain. The radioactive 'tail', which appeared after the bulk of enzymic activity on Sepharose 4B, contained, on the other hand, multiple protein bands of faster mobility than band B₂, of which several appeared to be glycoproteins.

In the second experiment an unlabelled 5 min papain supernatant from nine rats was chromatographed on Sepharose 4B, and the enzyme-rich area (16–25 ml) was subsequently chromato-

Fig. 5. Sephadex G-200 chromatography of the 16–25 ml fraction from the Sepharose 4B column; ●, maltase; □, β -naphthylamidase; ■, alkaline phosphatase; ▲, sucrose; ○, isomaltase. Fractions I and II were each combined and concentrated by freeze-drying for polyacrylamide-gel electrophoresis shown in Fig. 4(f) and 4(g).

graphed on Sephadex G-200. As shown in Fig. 5 sucrose-isomaltase, which is probably retarded through enzyme-substrate interaction with Sephadex (Kolinska & Semenza, 1967), was separated partially from β -naphthylamidase, maltase and alkaline phosphatase, which appeared at or shortly after the void volume. Fractions I and II were combined as shown in Fig. 5 and examined with a sample of the 16–25 ml fraction on polyacrylamide gel (Figs. 4e, 4f and 4g). Fraction II, containing sucrose-isomaltase, appeared as a single periodic acid-Schiff positive band principally in the B₁ area (Fig. 4f). Fraction I, on the other hand, which contained the void-volume enzymes maltase, β -naphthylamidase and alkaline phosphatase, although showing considerable streaking, produced two major bands corresponding to bands A and B₂, both of which were periodic acid-Schiff-positive. These two experiments therefore indicated that band A contained a maltase distinct from sucrose-isomaltase and band B₁ contained sucrose-isomaltase, and suggested that band B₂ contained enzymes appearing close to the void volume on Sephadex G-200, possibly β -naphthylamidase and alkaline phosphatase.

To define the location of radioactive label as well as to confirm the position of the enzymes, a third experiment was performed. Three rats were given 10 μ Ci of [1-¹⁴C]glucosamine, and the '5 min supernatant' was prepared at 4 h after injection of label as before and placed on a Sepharose 4B column. Eluates containing the enzyme-rich area (18–26 ml) were then examined on three polyacrylamide gels. In two gels sequential 2.0 mm segments were removed and analysed for either radioactivity or

enzymes. The third gel was stained with the periodic acid-Schiff reagent. The results are summarized in Fig. 6. Band A (slices 13–16) contained maltase activity alone. A second maltase peak appeared in the B₁ area (slices 18–21) in association with the only sucrose peak. Although isomaltase was not determined because of lack of material it seems reasonable to conclude that the band B₁ maltase and sucrose represent the sucrose-isomaltase complex, since both components of this complex probably have maltase activity (Kolinska & Semenza, 1967; Dahlqvist & Telenius, 1969).

Fig. 6. Polyacrylamide-gel electrophoresis of enzyme-rich fraction from Sepharose 4B column (18–26 ml). Three identical samples were applied to these gels. One gel was stained with the periodic acid-Schiff stain. The second gel was sliced at 2.0 mm intervals, and the radioactivity (Δ) determined in each slice. The third gel was sliced at 2.0 mm intervals, and in each slice maltase ($\bullet$), sucrose ($\blacktriangle$), β -naphthylamidase ($\square$) and alkaline phosphatase ($\blacksquare$) activities were determined. The enzymes are distributed in three areas related to bands A, B₁ and B₂, and each of these areas has a fairly well-defined radioactive peak. A small amount of radioactivity of unknown significance was found in a slightly periodic acid-Schiff-positive area preceding the enzymic bands.

As shown in Table 3, the B₁ maltase was also heat-sensitive, and thus resembled the maltase 1a and 1b activity of sucrose-isomaltase (Dahlqvist, 1962). By comparison (Table 3) the band A maltase is relatively heat-resistant, which together with its lack of sucrose and isomaltase activity suggests a resemblance to the maltases II and III described by Dahlqvist (1962). The B₂ area contained both β -naphthylamidase and alkaline phosphatase. All three bands were radioactively labelled, with the largest radioactive peak appearing in the B₁ area. All three glycoprotein bands in the enzyme-rich fraction therefore incorporate [1^{14}C]glucosamine, and each is represented by a characteristic enzyme or enzyme group, namely a heat-resistant maltase in band A, sucrose-isomaltase in band B₁ and β -naphthylamidase and alkaline phosphatase in band B₂.

DISCUSSION

Most of the membrane-bound saccharide in the brush border of the small intestine is localized on the external surface of the plasma membrane (Ito, 1964, 1965a). This surface possesses two important structural features. The first is a filamentous surface layer, or fuzz, which is stained by techniques specific for polysaccharide (Ito, 1965a; Rambourg & Leblond, 1967), and which appears to be the site of incorporation of a number of simple polysaccharide precursors (Ito, 1965b; Ito & Revel, 1966; Neutra & Leblond, 1966; Bennett, 1967, 1970). The second is a layer of surface knobs or particles, 40–60 Å in diameter, which is tightly apposed to the external leaflet of the plasma membrane (Oda & Seki, 1966; Johnson, 1966, 1967). These particles are thought to represent surface enzymes, since they are removed from the membrane by papain, along with the enzymes, and can be identified in column eluates containing concentrated disaccharidase fractions (Johnson, 1967). Unfortunately surface filaments and particles have not been demonstrated by the same technique, and consequently it is unclear whether these structures are actually different. As Johnson (1969) has

Table 3. Maltase activity in polyacrylamide-gel slices before and after heating at 50°C for 60 min

Slices 14–16 correspond to band A and slices 19–21 correspond to band B₁ as shown in Fig. 6.

Slice no.	Activity before heating (unit/slice)	Activity after heating (unit/slice)	Activity remaining (%)
14	0.455	0.234	51
15	0.605	0.296	49
16	0.146	0.069	47
19	0.182	0.014	7.7
20	0.203	0.021	10.0
21	0.127	0.003	2.0

pointed out, the filaments are also lost from the membrane during papain digestion. He has suggested that the apparent differences could be largely a matter of preparative technique.

The time-course of [$1\text{-}^{14}\text{C}$]glucosamine incorporation into isolated rat brush-border membrane glycoprotein (Forstner, 1970) compares favourably with that seen radioautographically when monosaccharide incorporation into the surface coat is studied (Ito, 1965b; Ito & Revel, 1966; Ito, 1969; Bennett, 1970). This observation, together with the magnitude and specificity of the membrane-labelling process (Forstner, 1968, 1970) suggests that much of the radioactive membrane glycoprotein must reside in the surface coat. The present experiments provide strong additional support for this point of view. Brief digestion with papain released a major fraction of the total membrane glycoprotein, accounting for almost half of the entire protein-bound hexosamine as well as a similar fraction of the total radioactive label. Most of this material was recovered in a single peak from Sepharose 4B columns. This peak was not only highly labelled but was also rich in released surface enzymes, indicating that the glycoprotein was closely associated with a recognizable surface component.

This association with the surface enzymes persisted after more complete resolution of the material on polyacrylamide gel. Indeed, although the labelled glycoprotein peak was separated into three major bands, all of the bands contained enzymic activity, and each was labelled by [$1\text{-}^{14}\text{C}$]glucosamine. It therefore appears very likely that the enzymes are glycoproteins capable of incorporating [$1\text{-}^{14}\text{C}$]glucosamine. This conclusion is supported by the fact that no protein which appeared to be free from carbohydrate could be demonstrated in the enzyme-rich fraction when examined by polyacrylamide-gel electrophoresis. In addition sucrose-isomaltase, purified on Sephadex G-200, produced a single protein band on gel electrophoresis which was uniformly periodic acid-Schiff-positive, and the early eluates from Sepharose 4B, consisting chiefly of maltase activity, also accounted for a single periodic acid-Schiff-positive protein band. If these enzymes are glycoproteins and if the surface enzymes and knobs in the membrane coat are identical, it follows that the knobs are probably glycoproteins.

Since the enzyme-rich fraction is a major component of the total membrane glycoprotein, these knobs presumably contribute substantially to the glycoprotein of the surface coat, and probably account for a significant portion of the total polysaccharide-staining reaction of the intestinal surface. Since previous radioautographic work, which suggested labelling of the surface fuzz (Ito, 1965b; Ito & Revel, 1966; Bennett, 1970), employed tech-

niques that could not demonstrate the surface particles it is equally probable that part of the apparent labelling of the fuzz was due to incorporation of label into surface enzymes. This possibility is supported by our evidence that the solubilized enzyme-rich fraction contains the most actively labelled glycoprotein in the brush border, and that it accounts for a major portion of the labelled glycoprotein. Das & Gray (1970) have suggested that surface disaccharidases may turn over much more rapidly than other intestinal proteins. Such a rapid turnover could account for the very active labelling of the surface glycoproteins, as well as the apparent short half-life of the surface label described in radioautographic experiments (Ito, 1969).

My results are compatible with the suggestion that the surface filaments and knobs are identical (Johnson, 1969), since a second highly labelled glycoprotein fraction of quantitative significance was not released by papain. The relative contribution of the glycoprotein in the filaments to the total glycoprotein of the surface coat may depend on the species studied, however, and this contribution may be particularly small in the rat (Ito, 1965a). Filamentous glycoprotein may be lost also during brush-border preparation (Ito, 1969). Thus there is the possibility that the filaments represent a small fraction of the total isolated surface glycoprotein in this species. If so, non-enzymic filamentous glycoprotein might be represented by a low molecular-weight glycoprotein in the radioactive 'tail' area or quite possibly by a glycoprotein hidden within the enzyme-rich fraction itself. This fraction probably contains a number of additional enzymes and possibly other glycoproteins. Preliminary experiments indicate, for example, that sucrose-isomaltase accounts for no more than 50% of the label in the band B_1 region, and also suggest that lactase may be present in band B_1 .

Perhaps the most important contribution of these experiments is therefore to illustrate that the intestinal surface is covered not by a single type of glycoprotein but by a relatively complex mixture of glycoproteins with different functions. Further progress in understanding the role of the surface coat will depend on the speed with which additional functions are defined, as well as on improved methods of glycoprotein separation.

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