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# BIOSYNTHESIS OF BRUSH BORDER GLYCOPROTEINS BY HUMAN SMALL INTESTINAL MUCOSA IN ORGAN CULTURE *

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## Summary

The incorporation of [$^{14}$C]glucosamine into brush border glycoproteins by human small intestinal mucosa in organ culture has been investigated. The experiments were based on the observations that (1) isolated brush border membrane fragments from cultured explants showed an unchanged pattern of protein bands and brush border enzyme activities on sodium dodecyl sulfate/polyacrylamide gels after electrophoresis and (2) the rate of overall [$^{14}$C]glucosamine incorporation measured in the tissue homogenate remained constant up to 48 h.

After 24 h of culture, the radioactivity peaks on gels due to incorporation of [$^{14}$C]glucosamine were found exclusively in the high molecular weight region and corresponded to protein bands identified as maltase-glucoamylase, lactase, sucrase-isomaltase, enterokinase and alkaline phosphatase. Enzymatic activity could not be assigned to the three remaining labelled bands. Most of these glycoproteins were already labelled after 5 h. Newly glycosylated brush border enzymes remained predominantly associated with the brush border membrane of intact cells with little release into the medium up to 24 h.

## Introduction

Brush border enzymes of human [1—3] and animal [1,4—7] small intestinal mucosa are known to be glycoproteins. The sequence of events involved in the

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biosynthesis of these glycoproteins has been established in animal enterocytes. According to Bennett et al. [8] and Forstner [9] these proteins are glycosylated in the Golgi complex, migrate to the periphery of the cell and are incorporated into the brush border membrane. Little is known however of these mechanisms in man, since in vivo studies are hardly possible and in vitro investigations were limited by the short survival of intestinal tissue and by the small amount of tissue obtained by peroral biopsy. These difficulties have been overcome recently by the development of an organ culture method suited for intestinal mucosa [10] and by using specimens of mucosa prepared from operation material in addition to peroral biopsies [11]. In the present study we applied newly developed methods for the isolation [12] and chemical analysis of brush border membranes [2,13] to cultured explants and give evidence for the in vitro synthesis and release of brush border membrane glycoproteins, some of which could be identified as brush border enzymes.

## Material and Methods

*1. Patients*

Jejunal mucosa was obtained from patients undergoing bypass operations for morbid obesity. Suction biopsies were taken with a paediatric Watson capsule.

*2. Organ culture*

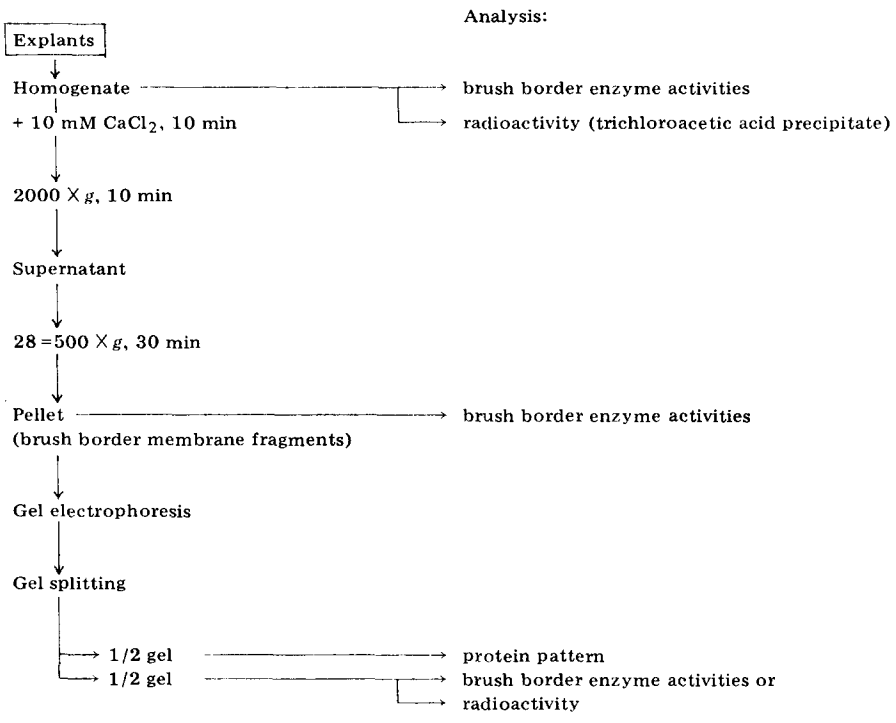
Small explants (2 × 5 mm) of dissected intestinal mucosa were cultured in Falcon dishes as described earlier [11]. The culture medium used was the synthetic solution RPMI 1640 (Gibco No. 240 52) supplemented with heat inactivated fetal calf serum (1 h, 56°C), insulin (0.5 mg/100 ml), glucose 366 mg/100 ml), and glutamine (30 mg/100 ml). Penicillin (100 units/ml) and streptomycin (100 μg/ml) were generally added. Each experiment was repeated 3 times using mucosa from three different subjects. An experiment was usually performed with three culture dishes in parallel each containing 6 explants. The explants were washed 3 times in fresh medium after culture and the 6 explants out of one culture were pooled for one determination. The three washings together with the mucus containing debris of extruded cells were added to the culture medium prior to analysis.

*3. Purification and separation of the brush border membranes*

Brush border membranes were isolated by a micromodification of the Schmitz technique [12] described in Table I. 5—70 mg of mucosa were used. Purified brush border membranes were resuspended in a small amount of distilled water by sonication and separated on 7.5% polyacrylamide gels in the presence of sodium dodecyl sulfate according to the methods of Laemmli [14]. 20—100 μg of protein were incubated with the sample buffer (containing 2% w/v sodium dodecyl sulfate, but no mercaptoethanol) for 15 min at room temperature. Non solubilized particles were removed by centrifugation 10 000 × *g*, 3 min) prior to layering the samples onto the gels. Immediately after the run the gels were split longitudinally into two equal halves. One half-gel was fixed with 12.5% trichloroacetic acid and protein bands were stained with Coomassie Brilliant Blue. The other half was frozen with solid $CO_2$ and cut in 0.3 mm or

TABLE I

Table showing the sequence of manipulations for the isolation and analysis of brush border membrane fragments.




0.5 mm slices with a Mickle Gel-Slicer for subsequent enzyme or radioactivity determinations. The stained half-gels were scanned with a Zeiss ZK 4 Gel Scanner connected to a Zeiss PMQ II spectrophotometer at 565 nm.

*4. Processing of culture media (Table II)*

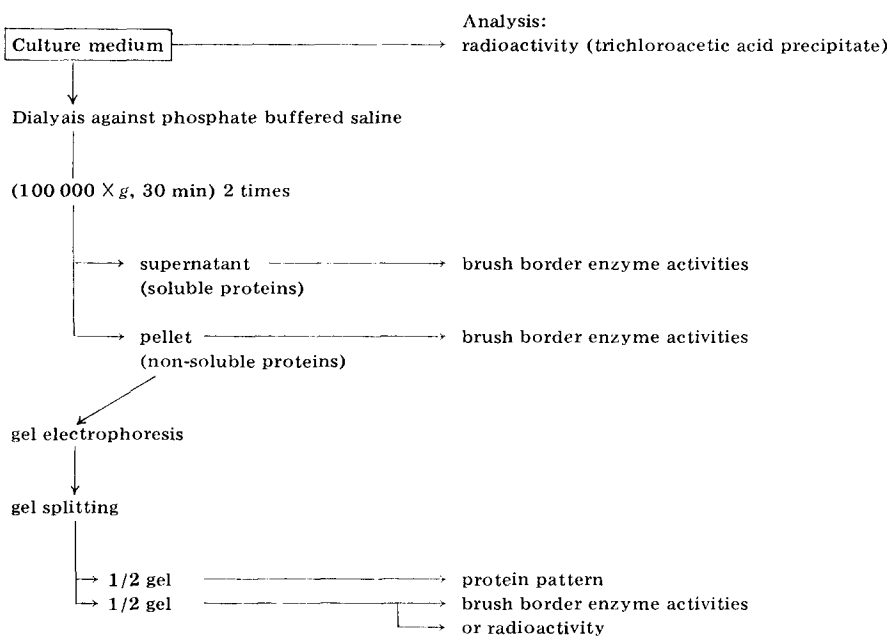
The media were dialysed against 5 washings of phosphate-buffered saline (pH 7.2) in order to remove glucose which would interfere with the assay of glycosidases. In order to separate soluble from non soluble proteins, the media were ultracentrifuged and washed once with distilled water thus removing serum proteins from the non-soluble fraction. The final pellet was resuspended in distilled water and frozen not longer than one week prior to analysis.

*5. Determination of protein and enzyme activities*

Protein was determined according to Lowry et al. [15]. Overall activity of brush border enzymes was determined at different stages during the purification of the brush border membranes and in the culture medium (Tables I and II). Enzyme activities in tissue homogenates and in the medium were assayed after overnight incubation with 0.1% Triton X-100 (w/v final concentration) at 4°C. Enzymes were eluted from gel slices by incubating each slice in 1 ml of distilled water at 0—4°C for at least 4 h and their activities were determined after overnight incubation with the different substrates. Glycosidases were

TABLE II

Flow diagram of the methods for the analysis of the culture media.




assayed according to Messer and Dahlqvist [16], alkaline phosphatase (EC 3.1.3.1.) according to Garen and Levinthal [17] with *p*-nitro-phenyl-phosphate as substrate and enterokinase (enteropeptidase EC 3.4.21.9.) according to Hadorn et al. [18]. Lactase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was always measured in the presence of *p*-chloro-mercuri-benzoate according to Asp and Dahlqvist [19].

*6. Administration of [$^{14}$C]glucosamine and measurement of radioactivity*

Overall incorporation of [1-$^{14}$C]glucosamine (57 mCi/mM, CEA France) into protein was measured in two different ways: either the precursor (5 μCi/ml) was present permanently during the culture period (permanent labelling) or was added for 5 h to the medium after various time intervals (pulse labelling). The washed explants were homogenized and the incorporated radioactivity was measured in the acid precipitable fraction of the specimens and of the corresponding media as described for [$^{3}$H]leucine incorporation [11]. Radioactivity was expressed as dpm per mg of tissue protein.

In order to demonstrate incorporation of [$^{14}$C]glucosamine into brush border glycoproteins this precursor was added to the culture medium to give a final concentration of 10 μCi or 20 μCi per ml. After different time intervals the explants were harvested. Brush border membranes were purified and separated by gel electrophoresis as described above. One half-gel was stained for proteins and the other corresponding half -gel sliced. Each slice was dissolved in 100 μl of 30% $H_2O_2$ at 50°C prior to the addition of scintillation fluid (Aquasol, New

England Nuclear) and the vials were kept in the dark until the counts had stabilized.

Non soluble labelled glycoproteins released into the medium were also analysed by gel electrophoresis. Radioactivity was determined with a Packard Tri Carb Liquid Scinitillation Counter connected to an absolute activity analyzer.

## Results

*1. Protein and enzyme pattern of the brush border membrane*

Representative protein and enzyme scans of the brush border membrane after gel electrophoresis are given in Fig. 1. After the analysis of brush border membranes prepared from more than 30 different biopsies and operation explants the following correspondence of protein and enzyme peaks has been established: Protein peak 2 with maltase (α-glucosidase, EC 3.2.1.20) and the main glucoamylase activity (exo-1,4-α-glucosidase, EC 3.2.1.3), protein peak 3 with lactase activity; protein peak 4 with maltase, sucrase (sucrose α-glucohydrolase, EC 3.2.1.48), palatinase (oligo-1,6-glycosidase, EC 3.2.1.10) and sometimes glucomylase activity and protein peak 5 with enterokinase activity (not

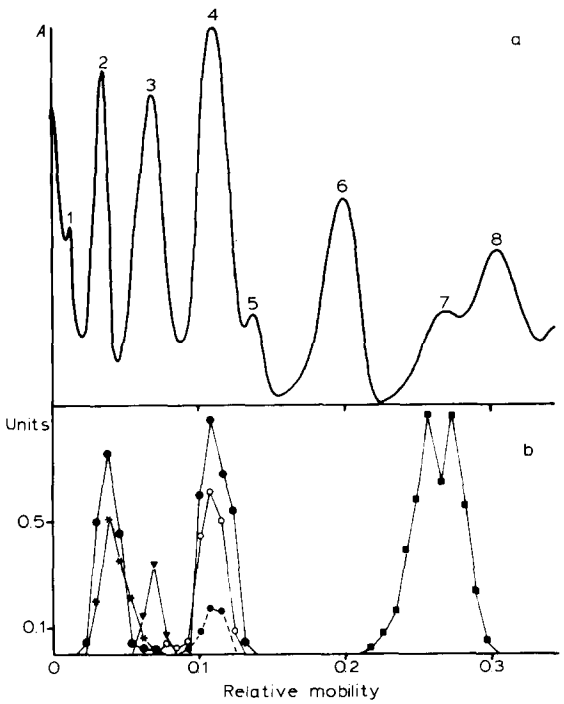



Fig. 1. (a) Representative protein pattern of brush border membrane fragments of a non cultured jejunal explant separated on a 7.5% sodium dodecyl sulfate/polyacrylamide gel (longitudinally split half-gel). The gel was stained with Coomassie Brilliant Blue and scanned at 565 nm with a Zeiss Gel Scanner. 1—8: protein bands. (b) Distribution of brush border enzyme activities on the corresponding half-gel. Activities are indicated as arbitrary units ●———●, maltase; ○———○, sucrase; ●- - - - -●, palatinase; *———*, glucoamylase; ▼———▼, lactase; ■———■, alkaline phosphatase.

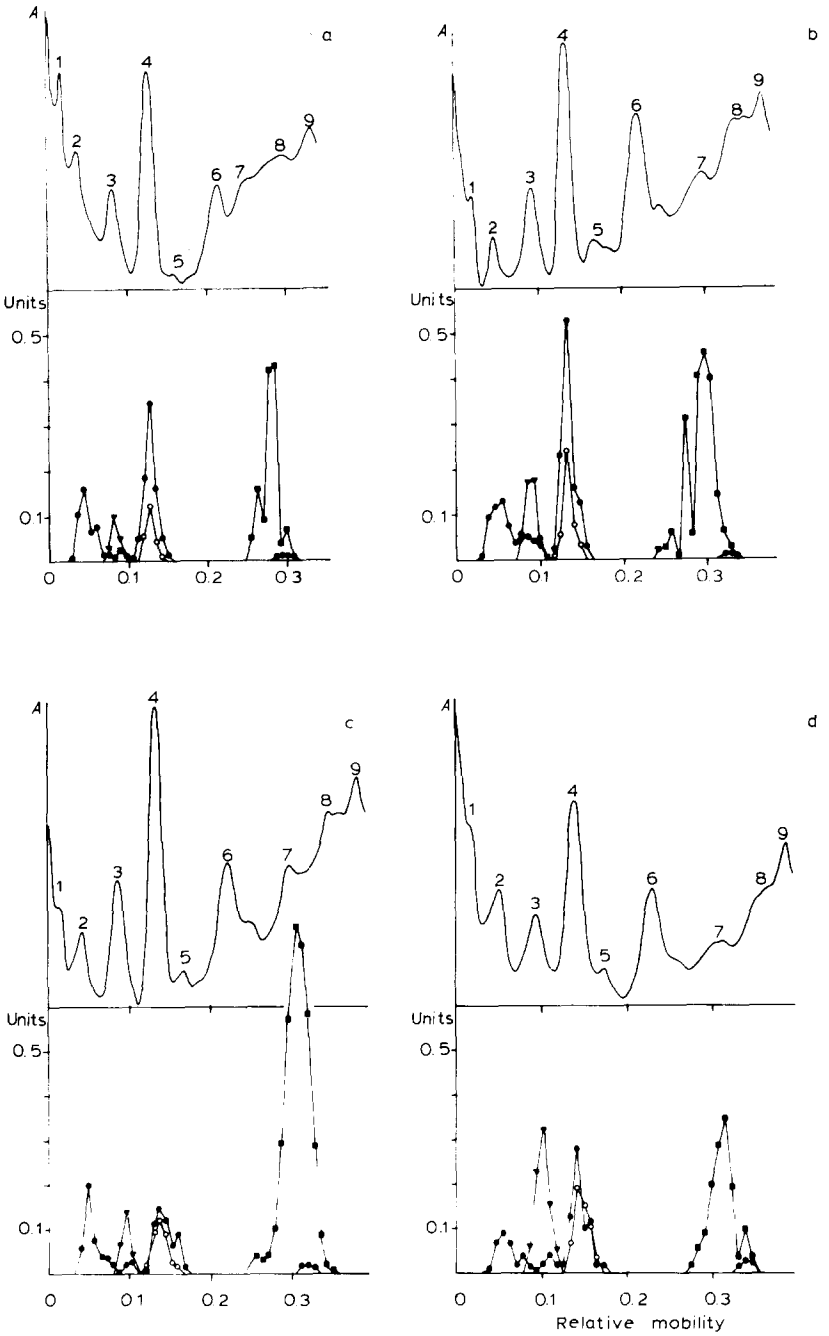



Fig. 2. Protein and enzyme pattern of brush border membrane fragments of jejunal explants separated on sodium dodecyl sulfate/polyacrylamide gels after 0 h, (a); 5 h, (b); 12 h, (c); and 24 h (d) in organ culture. One half of the gel was stained for protein and the other half was sliced and assayed for enzymatic activity. ●——●, maltase; ○—— ——○, sucrase; ▼——▼, lactase; ■——■, alkaline phosphatase.

shown). Between the first and the second maltase, low maltase activity very often appeared together with a little sucrase and glucoamylase activity. Alkaline phosphatase activity appeared as a broad main peak which migrated approximately to the position of protein band 7 but its correspondence was less exact than that obtained for the disaccharidases. 1 to 3 minor peaks of alkaline phosphatase activity with variable mobilities were sometimes found between the origin and the main alkaline phosphatase activity. In some experiments an additional maltase activity was found in the distal shoulder of the main alkaline phosphatase activity (Fig. 2).

In order to investigate the functional preservation of the brush border in vitro we cultured mucosal explants and analyzed the protein and enzyme pattern of their brush border membranes by gel electrophoresis after various time intervals. As shown in Fig. 2 there was no obvious change in the protein pattern (only high molecular weight region shown) either qualitatively or quantitatively when protein peaks were compared relative to each other. The pattern of brush border enzymes did not change during culture and the correlation with protein bands was maintained. Brush border enzymes were also determined in the homogenates prior to membrane purification to obtain a quantitative estimation of total enzyme activity during culture (Table III). Disaccharidases of the explants remained essentially constant up to 12 h while alkaline phosphatase activity increased up to 24 h.

*2. Overall incorporation of [$^{14}$C]glucosamine*

The incorporation of [$^{14}$C]glucosamine into jejunal tissue glycoproteins as

TABLE III

Brush border enzyme activities of cultured jejunal explants and corresponding media (same cultures as in Fig. 2). Enzyme determinations were done in the homogenate of 18 pooled explants out of 3 dishes cultured in parallel and in the pooled media.

| Enzyme | Hours in culture | Activity (I.U./g wet mucosa) | | | |
|---|---|---|---|---|---|
| | | Tissue | Medium pellet | Medium supernatant | Total |
| Maltase | 0 | 15.7 | | | 15.7 |
| | 5 | 14.1 | 4.3 | 0.7 | 19.1 |
| | 12 | 16.4 | 7.5 | 1.6 | 25.5 |
| | 24 | 10.8 | 11.0 | 3.9 | 25.7 |
| Sucrase | 0 | 6.9 | | | 6.9 |
| | 5 | 6.2 | 1.8 | 0.8 | 8.8 |
| | 12 | 6.1 | 3.1 | 1.0 | 10.2 |
| | 24 | 4.4 | 4.3 | 1.2 | 9.9 |
| Lactase | 0 | 3.0 | | | 3.0 |
| | 5 | 2.5 | 0.9 | 0.9 | 4.3 |
| | 12 | 2.7 | 1.9 | 1.2 | 5.8 |
| | 24 | 2.1 | 1.8 | 1.2 | 5.1 |
| Alkaline phosphatase | 0 | 92.1 | | | 92.1 |
| | 5 | 86.2 | 23.6 | 6.0 | 115.7 |
| | 12 | 113.4 | 36.5 | 11.3 | 161.2 |
| | 24 | 131.6 | 57.4 | 15.9 | 204.9 |

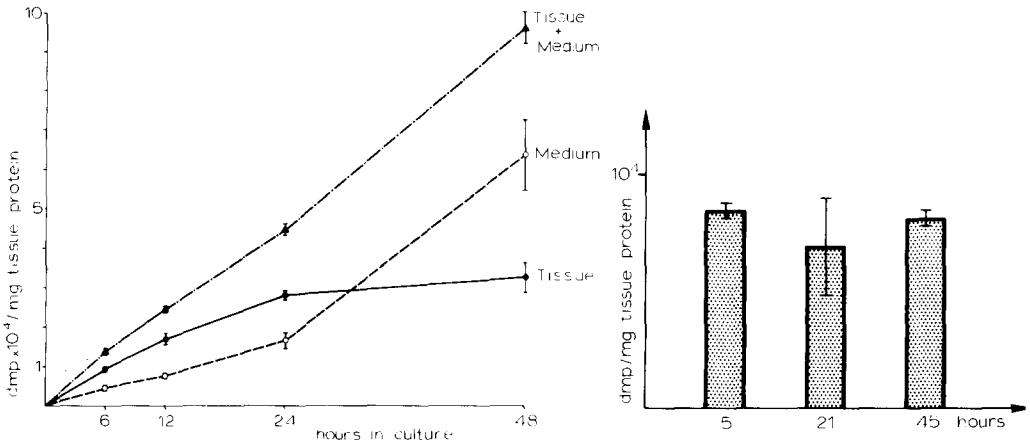



Fig. 3. Permanent labelling experiment. Incorporation of radioactivity by jejunal explants exposed permanently to 5 μCi/ml of [$^{14}$C]glucosamine. Radioactivity was determined in trichloroacetic acid precipitates of tissue homogenates and of the corresponding media. The brackets represent ±1 standard error of the mean.

Fig. 4. Pulse labelling experiment. Incorporation of [$^{14}$C]glucosamine into acid precipitable tissue protein by jejunal explants in culture. The specimens were cultured in isotope-free medium for 0, 21 or 45 h and then transferred to medium containing [$^{14}$C]glucosamine (5 μCi/ml) for 5 h. The brackets represent ±1 standard error of the mean.

well as the release of labelled glycoproteins increased essentially linearly with time up to 24 h (permanent labelling) as indicated in Fig. 3. Thereafter, the radioactivity remained constant in the tissue, whereas it increased more rapidly in the medium. Total radioactivity (explants plus medium) showed a linear increase up to 48 h indicating that glycoprotein synthesis remained constant up to this time. This was confirmed by the pulse experiments which demonstrated an unchanged incorporation rate of radioactive glucosamine into tissue protein (Fig. 4).

*3. Incorporation of [$^{14}$C]glucosamine into brush border proteins*

Based on the stability of the brush border membrane and the steady state of total glycoprotein synthesis during culture, we investigated the biosynthesis of brush border membrane glycoproteins. Jejunal specimens were cultured in the presence of 10 μCi/ml or 20 μCi/ml of [$^{14}$C]glucosamine, harvested after different time intervals and their brush border membrane was separated and analysed for radiocativity. After 5 h of culture (Fig. 5a) hexosamine incorporation could be found in the position of protein peak 2 (maltase-glucoamylase), peak 3 (lactase), peak 4 (sucrase-maltase) peak 5 (enterokinase) and peak 6 (no known enzyme activities). Highest radioactivity corresponded to the lactase region. After 24 h (Fig. 5b) the radioactivity profile was more pronounced showing also radioactivity corresponding to peak 1 (unidentified), peak 7 (region of alkaline phosphatase) and peak 9 (unidentified). It was surprising to find highest radioactivity in the lactase position after short incubation, since lactase activity measured in the homogenate of the explants did not behave differently from other disaccharidases during culture (Table III). It is impor-

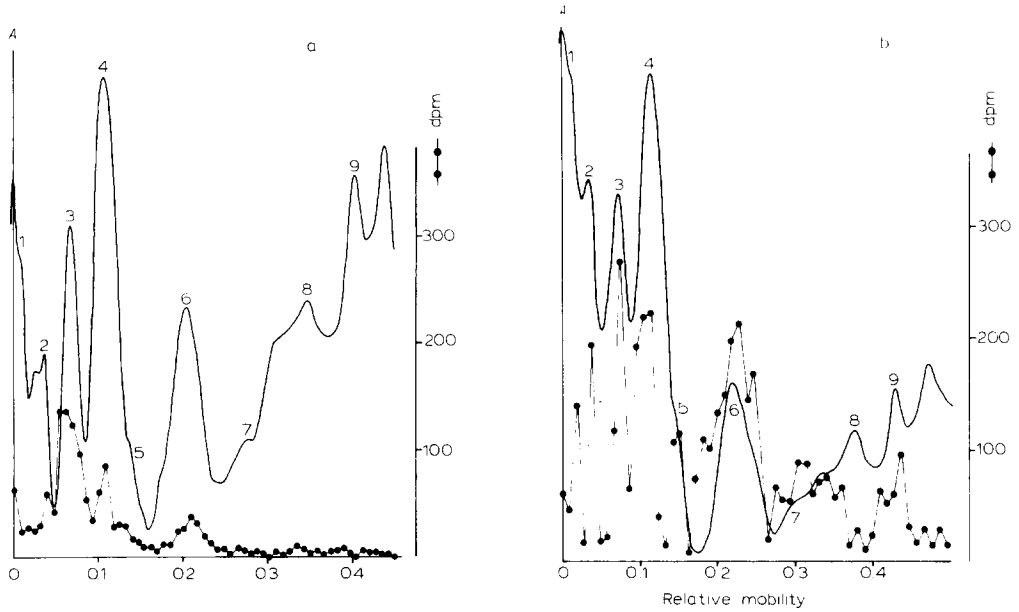



Fig. 5. Sodium dodecyl sulfate/polyacrylamide gels of brush border membrane proteins (50 μg per gel) labelled with [$^{14}$C]glucosamine (10 μCi/ml) for 5 h, (a); 24 h (b) in organ culture. Protein scans and determinations of radioactivity were performed with corresponding half-gels.

tant to note that no incorporation occurred in the region of the smaller molecular weight proteins.

*4. Analysis of [$^{14}$C]glucosamine labelled proteins released into the culture medium (non soluble fraction)*

As is evident from Table III, an increasing amount of disaccharidase activities was released into the medium with time. After 24 h these activities reached similar levels to those found in the corresponding explants, and they were mainly particulate bound suggesting a continuous desquamation of enterocytes. We analysed this non-soluble fraction by sodium dodecyl sulfate polyacrylamide gel electrophoresis after various time intervals. Its protein pattern (Fig. 6) was essentially the same after 5 or 24 h. After 5 h, four distinct peaks of radioactivity appeared: peak I remaining practically at the origin of the gel, peak II corresponding to protein band A, peak III corresponding roughly to protein band C and peak IV corresponding to protein band F. The rest of the gel did not show any radioactivity peaks. After 24 h the radioactivity peaks were more pronounced. Peak I was relatively increased and peak III exhibited a tail covering the region of protein band D. No new peaks appeared. In order to characterize the protein bands of the high molecular weight region we determined brush border enzyme activities on gels run in parallel (Fig. 6). After 5 h we found lactase, sucrase and the main alkaline phosphatase activities in the same positions as was the case for the purified brush border membranes. The first maltase was not measurable, the second was present. There was a good correspondence of sucrase with protein band D and of lactase with protein band C. After 24 h lactase was no longer measurable, sucrase was very weak and

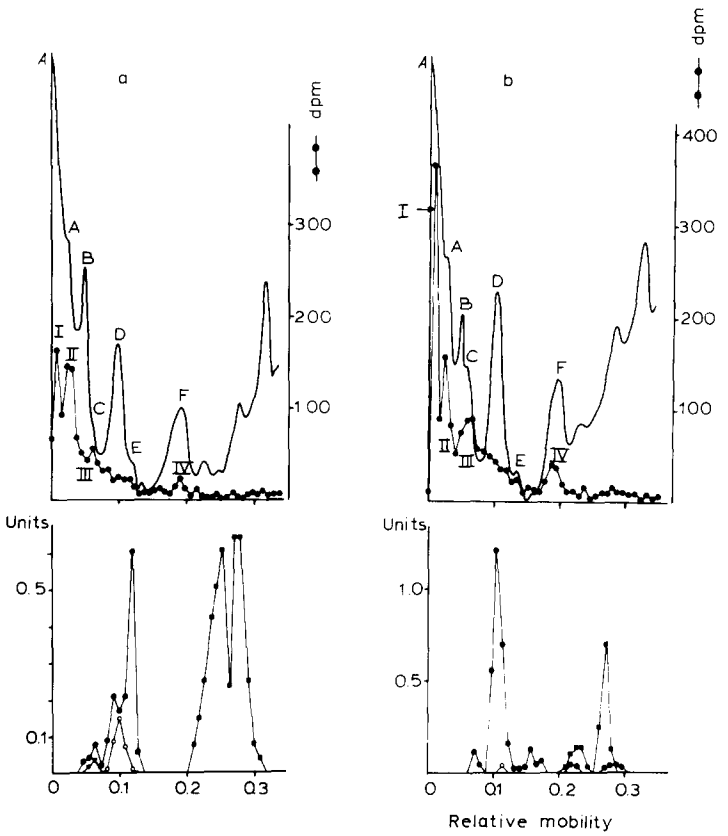



Fig. 6. Sodium dodecyl sulfate/polyacrylamide gels of non-soluble proteins (100 μg per gel) released into the medium for 5 h (a); and 24 h (b) by jejunal explants cultured in the presence of 10 μCi/ml of [$^{14}$C]-glucosamine. The radioactivity and protein patterns (two corresponding half-gels) are related to enzyme activities determined in gels run in parallel. Symbols for enzyme activities as in Fig. 2. A, B, C, D, E, F: protein bands. I, II, III, IV: radioactivity peaks.

between the second maltase and the main alkaline phosphatase activity new maltase peaks appeared, probably degradation products.

The third maltase was present. This analysis demonstrated that radioactivity peaks I and II were not due to membrane glycoproteins which had been identified as brush border enzymes.

## Discussion

The gel electrophoresis system for the separation of human brush border membranes originally described [2] was based on the techniques established by Neville [20]. In our hands this method gave a good separation of the high molecular weight region [21], but often an unsatisfactory resolution of protein bands migrating to the centre of the gel. This was mainly due to the absence of sodium dodecyl sulfate in the running gel, since its addition to the gel improved separation (unpublished observations). Even sharper bands were obtained by using the Laemmli system [14] instead of the Neville system. The protein pattern obtained was comparable to that described previously [2]. The pattern of

brush border enzyme activities was essentially the same as established by Maestracci [12]. A new maltase activity not described so far, very often appeared as a low peak in the distal shoulder of the main alkaline phosphatase activity peak. Its nature is as yet unclear. No known enzyme activities could be attributed to protein bands 1 and 6.

The application of these techniques to cultured mucosa demonstrated the functional stability of the brush border membrane, agreeing well with its structural integrity observed in the electron microscope [11].

The experiments dealing with the measurement of total glycoprotein synthesis resulted in an unchanged incorporation rate of [$^{14}$C]glucosamine into acid precipitable proteins up to 48 h (pulse labelling). When this precursor was present permanently in the medium overall radioactivity, tissue plus medium, increased linearly with time up to 48 h. While a plateau of incorporation was reached in the tissue between 24 and 48 h, release of labelled proteins was more pronounced during this time. These findings corroborate our previous results obtained with incorporation of [$^{3}$H] leucine into protein, confirming that small intestinal mucosa in organ culture represents a steady-state system of (glyco-) protein synthesis and release. Similar results were obtained by McDermott et al. [22] for mucosal biopsies of rabbit colon and human rectum up to 24 h. In contrast to our results, the plateau of hexosamine incorporation was observed already after 12 h of culture. Moreover, with large intestine, glucosamine was predominantly incorporated into macromolecules ultimately secreted, whereas leucine was mainly incorporated into tissue macromolecules. This may be due to the relative abundance of goblet cells in large intestinal as compared to small intestinal mucosa. It must however be noted that mucus is also regularly produced by jejunal explants maintained in vitro.

The stability of brush border membrane and the constant rate of overall glycoprotein synthesis represented a firm base for studying the incorporation of [$^{14}$C]glucosamine into single brush border glycoproteins in vitro. It was found that all protein bands detectable in the high molecular weight region could be associated with radioactivity peaks due to hexosamine incorporation, indicating that at least the last step of their synthesis had occurred in vitro. Some of these bands having incorporated [$^{14}$C]glucosamine could be identified as brush border enzymes. However, the question arises as to whether these bands represent single proteins. This can only be answered for some of the bands by the indirect method of comparing patterns obtained from normal biopsies with those of enzyme deficient cases. The protein band associated with maltase-isomaltase-sucrase was found to be absent in sucrase-isomaltase deficiency [23,24], whereas in congenital or adult type lactase deficiency the protein band corresponding to lactase was strongly reduced, but still visible [21]. In cases with low or no detectable maltase-glucoamylase activity on the gel, the corresponding protein band was strongly reduced (unpublished). Less clear is the situation for alkaline phosphatase, showing no significant correspondance to a defined protein band. We conclude therefore that at least the radioactivity peak corresponding to sucrase-isomaltase activity was totally due to this enzyme and that sucrase is synthęsized and incorporated into the brush border membrane within 5 h in vitro. The possibility exists however that all other radioactivity peaks could be contaminated by minor protein bands migrating

with the same speed as the identified brush border enzyme. This could be the case especially for the most prominent peak seen after a 5 h culture corresponding to lactase activity which was not always separated completely from the more proximal one in the position of maltase-glucoamylase. Nevertheless, we cannot rule out the possibility that lactase contains more hexosamine sugars and/or its rate of synthesis is much higher in vitro than that of other brush border membrane glycoproteins.

The analysis of the non-soluble medium fraction by gel electrophoresis showed no distinct radioactivity peak in the position of sucrase even after 24 h, although sucrase was present and its specific activity reached similar levels as found in the explants. Evidently enterocytes extruded from the tip of villi had incorporated only little [$^{14}$C]glucosamine into brush border proteins during culture, whereas newly synthesized brush border enzymes remained predominantly associated with the brush border of non-released cells. The main radioactivity due to [$^{14}$C]glucosamine incorporation was observed between the origin of the gel and the sucrase band as three distinct peaks. Peak I and peak II could not be attributed to any known brush border enzyme activity. Their corresponding proteins are likely to have a molecular weight higher than 440 000 since they migrated more slowly than maltase-glucoamylase [13]. They could be due to mucus as well as to intracellular proteins. Radioactivity peak III appeared in the position of lactase activity and could therefore correspond to the main hexosamine peak of the brush border membrane.

The present study represents a first attempt to investigate the brush border glycoprotein synthesis of human small intestinal mucosa in organ culture. Our results showing continuous incorporation of glucosamine into brush border glycoproteins up to 24 h may prove to be useful in two ways. First, it will be possible to investigate the mode of action of hormones involved in the regulation of normal brush border glycoprotein synthesis in man. Second, glycoprotein synthesis in culture will help to further characterize the molecular nature of altered glycoprotein bands found on gels from patients with congenital brush border enzyme deficiencies [21,25].

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