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IN VIVO EFFECT OF TUNICAMYCIN ON THE EXPRESSION OF RAT SMALL INTESTINAL BRUSH BORDER MEMBRANE GLYCOPROTEINS AND GLYCOENZYMES

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Abstract—Tunicamycin, a known inhibitor of the lipid-dependent glycosylation of proteins, was used *in vivo* to study the biosynthesis of rat intestinal brush border membrane aminopeptidase N and dipeptidyl aminopeptidase IV. The incorporation of [^{3}H]glucosamine into newly synthesized total protein of mucosal cell homogenates was inhibited by 60%, whereas incorporation of [^{3}H]leucine was decreased only 21% by tunicamycin. This effect was much more pronounced in the brush border membrane fraction isolated from intestinal mucosal cells where incorporation of radiolabled leucine and glucosamine was reduced to 50 and 82% of control values respectively. An examination of the brush border membrane protein profile by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that there was a marked selective decrease in the amount of glycoproteins of molecular weights greater than 130 kD. In addition, there were decreased levels of assayable aminopeptidase N, dipeptidyl aminopeptidase IV and disaccharidase activity in intestinal mucosal cell homogenates and brush border membranes of tunicamycin-treated rats. Though tunicamycin decreased incorporation of newly synthesized aminopeptidase N and dipeptidyl aminopeptidase IV protein into brush border membranes by 70–75%, the newly synthesized enzyme that was incorporated was indistinguishable from that of controls. Further, non-glycoslyated forms of both enzymes were not detected in any other subcellular fractions. These results show that tunicamycin, an inhibitor of glycosylation, significantly affected the expression of brush border membrane glycoproteins, suggesting that both polypeptide synthesis and degradation of these proteins may be altered in the presence of this drug.

The brush border membrane of intestinal mucosal cells contains a wide variety of enzymes that are involved in the digestion of protein, carbohydrate and lipid. Studies have shown that these enzymes are integral membrane proteins and are glycosylated to varying degrees [1–2]. During their biosynthesis, N-linked oligosaccharide moieties are added and processed during translocation of the polypeptide chain through the ER–Golgi complex [4, 5]. The completed glycoenzyme is eventually transported and inserted into the brush border membrane by a still poorly defined process that may involve the oligosaccharide side chains [6, 7].

Tunicamycin is an antibiotic that specifically inhibits the glycosylation of asparagine residues in glycoproteins. It has been reported in a variety of systems to reduce the carbohydrate content of specific glycoproteins [8, 9], to decrease the number of glycoproteins expressed at the cell surface [10, 12], and to alter their subcellular distribution [13]. Thus, studies with tunicamycin have proven to be of value in elucidating the biochemical steps and the importance of N-linked carbohydrate in the biosynthesis and intracellular processing of membrane glycoproteins.

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Previous studies from our laboratory have shown that purified rat intestinal brush border membrane aminopeptidase N [14] and dipeptidyl aminopeptidase IV [15] are glycoproteins containing 20 and 8% carbohydrate by weight, respectively, which is primarily N-linked. Very little is known, however, regarding its specific biologic function. Therefore, the following studies were undertaken in an effort to begin to elucidate the role of carbohydrate and the *in vivo* effect of tunicamycin on the biosynthesis and intracellular processing of these two brush border membrane glycoenzymes.

MATERIALS AND METHODS

Chemicals. Microvillus membrane aminopeptidase N and dipeptidyl aminopeptidase IV were purified from rat intestinal brush border membranes as described previously [14, 15], and rabbit polyclonal antisera to the purified enzymes was prepared [15, 16]. Carrier free Na[^{125}I] (20 Ci/mg), tritiated sodium borohydride (54 Ci/mmol), tritiated D-[6-^{3}H]glucosamine hydrochloride (20.2 Ci/mmol), and tritiated L-[1-^{3}H]leucine (59.2 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Tunicamycin was obtained from the Calbiochem-Behring Co. (La Jolla, CA), dimethyl sulfoxide from the J. T. Baker Chemical Co. (Phillipsburg, NJ), Nonidet P-40 (NP-40) from the Particle Data Laboratory (Elmhurst, IL), and fixed *Staphylococcus aureus*

(Zysorbin) from the Zymed Co. (South San Francisco, CA). Leucyl-β-naphthylamide and Gly-Pro-β-naphthylamide were from the Bachem Co. (Torrance, CA), Concanavalin A (ConA) coupled to Sepharose 4B was from Vector Laboratories (Burlingame, CA), and galactose oxidase, Type V from Sigma (St. Louis, MO). Myosin, β-galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin for molecular weight standards were from Bio-Rad Laboratories (Richmond, CA); sodium dodecyl sulfate was from Matheson, Coleman & Bell (Norwood, OH), NCS tissue solubilizer from Amersham (Arlington Heights, IL), and Autofluor from National Diagnostics (Somerville, NJ). All other chemicals used in this study were of reagent grade quality.

Administration of tunicamycin. Male Wistar rats (Simonsen Laboratory, Gilroy, CA), weighing approximately 300 g, were injected intraperitoneally with tunicamycin dissolved in saline containing 2% dimethyl sulfoxide. The pH of this solution was adjusted to 10 with 10 mM NaOH. Rats received a dose of tunicamycin ranging from 0.05 to 0.5 mg/kg body weight for different time periods prior to killing. Control rats were injected with a similar solution not containing tunicamycin. All experiments were performed using animals that had been fasted overnight.

Preparation of intestinal mucosa. Tunicamycin-treated and control rats were killed by decapitation, at the same time of day (10:00 a.m.). A 20-cm jejunal segment beginning 5 cm distal to the ligament of Treitz was removed. Biopsy specimens were fixed in Bouin's solution, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin-eosin for microscopy. The mucosa was scraped from the segment and weighed, and brush border membranes were prepared by the method of Kessler *et al.* [17]. Purity of brush border membrane preparations was judged by the increase in alkaline phosphatase specific activity.

Incorporation of [^{3}H]*leucine and* [^{3}H]*glucosamine into brush border membrane proteins and enzymes.* Rats were injected with either 0.25 mg/kg of tunicamycin or control solution as described above. After 2 hr they were anesthetized with pentabarbital, and the abdominal cavity was opened. [^{3}H]Leucine (1 mCi) or [^{3}H]glucosamine hydrochloride (500 μCi) was instilled intraluminally into ligated 20-cm segments of jejunum by injection with a 25-gauge needle. The abdominal cavity was closed, and the animals were allowed to recover. The rats were then killed 4 hr after injection of the isotope, and jejunal segments were removed and flushed thoroughly with ice-cold saline. Mucosal scrapings were prepared and homogenized in 40 ml of 2 mM Tris–HCl–50 mM mannitol buffer, pH 7.1. Brush border membranes were subsequently isolated and resuspended in 1 ml of distilled water. Aliquots of the homogenate and brush border membrane fractions were precipitated with an equal volume of an ice-cold mixture containing 20% trichloroacetic acid and 2% phosphotungstic acid. The precipitates were obtained by low-speed centrifugation and washed several times in the same manner. The pellet was dissolved in 0.4 N NaOH and neutralized with HCl, and portions of this solution were used to determine the amounts of radioactivity and protein. Quenching was corrected for by use of an external standard. Brush border membranes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [18].

To determine the amount of radioactivity incorporated into aminopeptidase N and dipeptidyl aminopeptidase IV, an immunoprecipitation technique was used. Mucosal homogenates (6 mg protein) and brush border membranes (1 mg protein) were solubilized with 1% NP-40. Solubilized samples were divided into three equal aliquots and incubated with either 20 μl of anti-aminopeptidase antisera, anti-dipeptidyl aminopeptidase IV antisera, or preimmune rabbit serum, respectively, in test tubes containing 20 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.4. The mixture was incubated at 4° overnight, and a 10% suspension of fixed *Staphylococcus aureus* cells was added and incubated at room temperature for 2 hr. Bacterial pellets were harvested by centrifugation, washed two times with 20 mM HEPES buffer, and dissolved in 0.5 ml of 1 N NaOH, and the radioactivity in the pellet was counted. The value obtained with preimmune rabbit serum was subtracted to correct for non-specific precipitation. To test the specificity of the antibody, the enzyme activity remaining in the supernatant fraction following immunoprecipitation was measured. In all cases, greater than 90% of the enzyme activity was precipitated by its specific antisera.

Brush border membranes (500 μg protein) were suspended in 500 μl of phosphate-buffered pH 7.0 saline and incubated with 10 units of galactose oxidase at 37° for 30 min. After washing twice with phosphate-buffered saline, the brush border membrane was dissolved in 500 μl of phosphate buffer, pH 8.0, and incubated with 2 mCi of tritiated sodium borohydride at room temperature for 15 min. Labeled membranes were then washed three times with phosphate-buffered saline and subjected to SDS-PAGE. Gels containing ^{3}H-labeled proteins were immersed in an Autofluor solution for 2 hr, dried, and exposed to X-ray film (Kodak X-omat R film).

Radioimmunoassay of aminopeptidase N and dipeptidyl aminopeptidase IV. Purified rat intestinal brush border aminopeptidase N and dipeptidyl aminopeptidase IV were iodinated with ^{125}I by the chloramine-T method of Klinman and Taylor [19]. Mucosal homogenates and brush border membranes were solubilized with 1% NP-40, bovine serum albumin and purified enzyme standard or unknown. To each tube was added 50 μl of ^{125}I-labeled enzyme (15,000 cpm) and 100 μl of diluted antibody sufficient to bind 50% of the labeled enzyme. Following incubation at room temperature for 1 hr, antibody bound and free enzyme were separated with fixed *S. aureus* cells containing protein A. The bacterial pellet was washed twice with phosphate-buffered saline containing 0.5% BSA, and the radioactivity in the pellet was measured.

Binding of enzymes to Concanavalin A-Sepharose. Mucosal homogenates and brush border membranes were solubilized in 1% NP-40, 20 mM HEPES (pH

7.4). The solubilized samples containing known units of enzyme activity were incubated in test tubes with ConA-Sepharose in 20 mM HEPES buffer, pH 7.4, at room temperature for 1 hr. After incubation, the mixture was centrifuged at low-speed for 5 min, and the supernatant fraction was removed carefully. The amount of enzyme activity remaining in the supernatant fraction was assayed and compared to original samples. The percent decrease in activity in the supernatant with ConA-Sepharose was designated as the amount of enzyme bound.

Membrane solubilization and polyacrylamide gel electrophoresis. SDS-polyacrylamide slab gel electrophoresis in a discontinuous buffer system was carried out using 7% gels according to the method of Laemmli [18]. Membranes were first solubilized by heating in 2% SDS with 5% 2-mercaptoethanol for 5 min at 100° and then applied to the gel. Following electrophoresis at 25 mA/gel, the gel was stained with Coomassie blue. After destaining, the gel was sliced into 3-mm slices and dissolved in NCS solution by incubating at 50° for 2 hr, and the amount of radioactivity was determined. The following proteins were used as molecular weight standards: myosin, β-galactosidase, phosphorylase B, bovine serum albumin and ovalbumin.

Enzyme assays. L-Leucyl-β-naphthylamide and Gly-L-Pro-β-naphthamide were used as substrates for the assay of aminopeptidase N and dipeptidyl aminopeptidase IV respectively. The released β-naphthylamine was assayed as described previously [20]. Activities of intestinal disaccharidases were determined according to Dahlqvist [21]. The amount of total hexose sugars in brush border membrane was assayed by the phenol sulfuric acid method [22]. Protein was determined by the method of Lowry *et al.* [23]. Data were analyzed statistically by use of Student's *t*-test for unpaired observations.

RESULTS

Following a single injection of tunicamycin (0.05 to 0.5 mg/kg), the body weight of the treated rats at the time they were killed was the same as that of the controls regardless of the time of administration of the drug prior to sacrifice. Examination of tissue by light microscopy revealed no histological changes in the intestinal mucosa 12 hr after a single injection of tunicamycin (0.25 and 0.5 mg/kg). However, a second injection of tunicamycin (0.25 and 0.5 mg/kg), 12 hr after the first, caused marked changes in villus height in both the jejunum and ileum and also resulted in a dramatic body weight loss at 48 hr. Therefore, a single injection at doses less than 0.5 mg/kg was used throughout the study. Table 1 shows the effect of various doses of tunicamycin on jejunal brush border membrane enzyme activities examined for a 12-hr period. At doses of more than 0.1 mg/kg, tunicamycin caused a decrease in all enzyme activities examined; however, the extent of decrease was different for each enzyme. For example, aminopeptidase N and sucrase activities (group 1) were reduced by 55 and 47%, respectively, when compared to controls, whereas dipeptidyl aminopeptidase IV and maltase activities (group 2) were reduced by only 23 and 24%, respectively, at a tunicamycin dose of 0.25 mg/kg. These differences were statistically significant between the two groups (1 and 2) of enzymes ($P < 0.05$). Table 2 shows that the effect of tunicamycin was reversible with time. After a single injection of tunicamycin (0.25 mg/kg), a nadir was reached between 6 and 24 hr for each enzyme activity. At 48 hr, all enzyme activities had recovered to control values. As before, aminopeptidase N and sucrase activities were more sensitive to tunicamycin than dipeptidyl aminopeptidase IV and maltase.

The profiles of brush border membrane proteins and glycoproteins following tunicamycin treatment were analyzed by SDS-PAGE. As shown in Fig. 1 (short daggers), there was a significant decrease in the amount of protein of molecular weights greater than 130,000 following tunicamycin administration. No major shifts in the electrophoretic mobilities of

Table 1. Effect of different doses of tunicamycin on the levels of assayable brush border membrane enzyme activity in rat small intestine

	Enzyme activity (mol/min/mg brush border protein)				
		Tunicamycin treated* (mg/kg of body weight)			
	Control [13]†	0.05 [4]	0.1 [5]	0.25 [7]	0.5 [5]
Aminopeptidase N	1.08 ± 0.09	0.92 ± 0.15¶ (15)‡	0.71 ± 0.29§ (34)	0.49 ± 0.09§ (55)	0.52 ± 0.26§ (52)
Dipeptidyl aminopeptidase IV	0.80 ± 0.07	0.74 ± 0.05 (7)	0.64 ± 0.10‖ (20)	0.62 ± 0.10§ (23)	0.55 ± 0.11§ (32)
Sucrase	1.46 ± 0.16	1.33 ± 0.19 (8)	0.93 ± 0.29§ (36)	0.78 ± 0.15§ (47)	0.70 ± 0.30§ (52)
Maltase	11.8 ± 1.0	10.9 ± 2.2 (8)	9.5 ± 1.6‖ (19)	9.0 ± 1.1§ (24)	7.4 ± 1.7§ (37)

Data are expressed as mean ± SD.
* Tunicamycin was injected intraperitoneally in different doses, as described, 12 hr before killing the rats.
† Numbers of animals are given in brackets.
‡ Numbers in parentheses are the percent reduction of enzyme activity when compared to controls.
§–¶ Significantly different from control value: § $P < 0.001$, ‖ $P < 0.01$ and ¶ $P < 0.05$.

Table 2. Effect of different time periods of tunicamycin treatment on the activity of rat intestinal brush border membrane enzymes

	Enzyme activity (nmol/min/mg brush border protein)					
		Tunicamycin treated*				
	Control [13]†	3 hr [4]	6 hr [6]	12 hr [7]	24 hr [5]	48 hr [4]
Aminopeptidase N	1.08 ± 0.09	1.10 ± 0.17 (0)‡	0.68 ± 0.25§ (37)	0.49 ± 0.09§ (55)	0.81 ± 0.13§ (25)	1.02 ± 0.11 (8)
Dipeptidyl aminopeptidase IV	0.80 ± 0.07	0.79 ± 0.09 (1)	0.63 ± 0.08§ (21)	0.62 ± 0.10§ (23)	0.68 ± 0.05‖ (15)	0.80 ± 0.08 (0)
Sucrase	1.46 ± 0.16	1.46 ± 0.12 (0)	1.03 ± 0.24§ (29)	0.78 ± 0.15§ (47)	1.10 ± 0.28‖ (25)	1.34 ± 0.19 (8)
Maltase	11.8 ± 1.0	11.9 ± 7 (0)	8.9 ± 2.0 (25)	9.0 ± 1.1§ (24)	10.6 ± 0.6¶ (11)	11.7 ± 0.6 (1)

Data are expressed as mean ± SD.

* Tunicamycin (0.25 mg/kg body weight) was injected intraperitoneally into rats, and animals were killed at different time intervals as described.

† Numbers of animals are given in brackets.

‡ Numbers in parentheses are the percent reduction of enzyme activity when compared to controls.

§–‖ Significantly different from control value: § $P < 0.001$, ‖ $P < 0.01$ and ¶ $P < 0.05$.

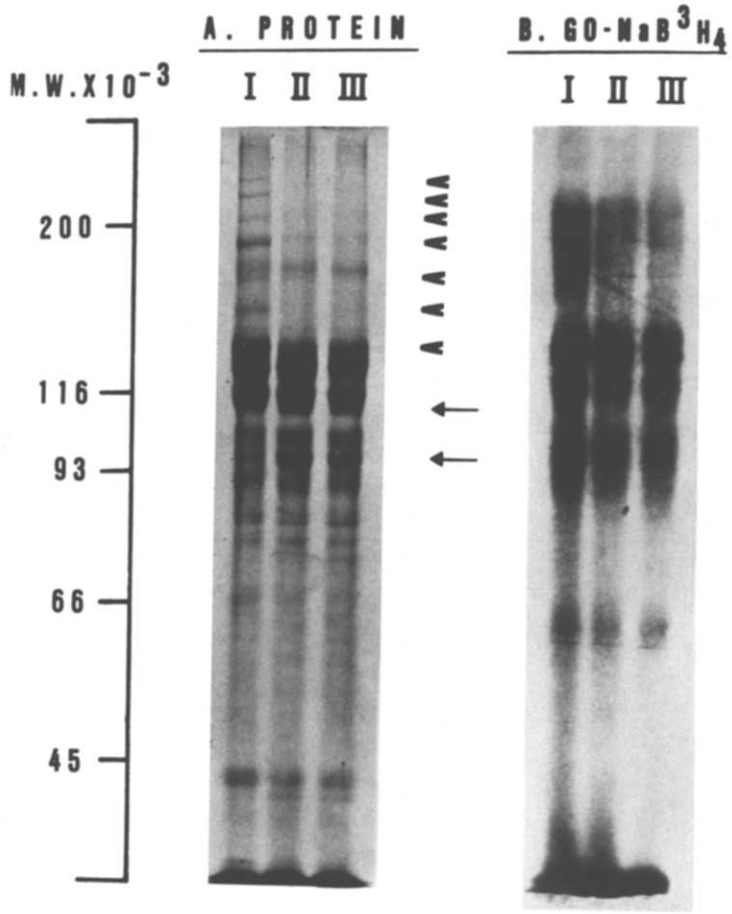

Fig. 1. Effect of tunicamycin on the SDS-PAGE profiles of brush border membrane proteins and glycoproteins. A. Protein stained with Coomassie blue. B. Radioautography of brush border membrane glycoproteins labeled with ^{3}H (galactose oxidase). Key: (I) controls, (II) 6 hr (tunicamycin), and (III) 12 hr (tunicamycin). In all cases 50 μg of protein was applied to each lane. Arrows and short daggers indicate increased or decreased staining of protein bands respectively. Experimental details are described in the text.

Table 3. Effect of tunicamycin on the incorporation of [^{3}H]leucine and [^{3}H]glucosamine into the trichloroacetic acid insoluble fraction of mucosal cell homogenates and purified brush border membranes

	[^{3}H]Leucine (dpm × 10^5/mg protein)			[^{3}H]Glucosamine (dpm × 10^5/mg protein)		
	Control	Tunicamycin	% Reduction	Control	Tunicamycin	% Reduction
Homogenate	1.27 ± 0.12*	1.01 ± 0.15	20.5	5.08 ± 0.52	2.05 ± 0.26	59.6
Brush border membrane	6.73 ± 0.75	3.37 ± 0.57	49.9	41.69 ±3.14	7.57 ± 0.40	81.8

[^{3}H]Leucine (1 mCi) or [^{3}H]glucosamine (500 μCi) was injected into a 20-cm loop of rat jejunum 2 hr after intraperitoneal injection of tunicamycin (0.25 mg/kg) or control solution. After 4 hr of *in vivo* labeling, rats were killed, and incorporation of label into different fractions was determined.

* Data are expressed as the mean ± SD of five experiments.

protein bands were observed with the possible exception of two bands of molecular weight 116,000 and 100,000, that were somewhat enhanced after tunicamycin treatment as denoted by the two arrows in Fig. 1. When membrane glycoproteins were labeled by the galactose oxidase–sodium borotritide procedure, essentially the same pattern was observed. It was also found that tunicamycin treatment (0.25 mg/kg) caused a decrease in the content of brush border membrane total neutral sugars to 63–69% of control values. To examine the cause of the decreased amount of glycoproteins after tunicamycin treatment, newly synthesized proteins and glycoproteins were examined *in vivo* by use of labeled precursor amino acids and sugars. As shown in Table 3, following a 6-hr treatment with tunicamycin (0.25 mg/kg) there was 60% decrease of [^{3}H]glucosamine incorporation into the total acid-precipitable protein of mucosal homogenates, and total cellular protein synthesis was inhibited by 21%. Thus, while there was some inhibition of *de novo* protein synthesis, tunicamycin inhibited the incorporation of sugar into total protein to a much greater extent. In the brush border membrane fraction, however, the reduced incorporation of labeled leucine and glucosamine into total protein was much more pronounced with 50 and 82% reduction respectively.

The profiles of newly synthesized proteins and glycoproteins which were inserted into the brush border membrane in the presence of tunicamycin were examined by SDS-PAGE (Figs. 2 and 3). The incorporation patterns of radiolabeled precursor sugar and amino acids in control rats suggest that, in general, proteins of molecular weights greater than 85,000 were glycosylated, especially those in the 125–200 kD range. Alternatively, proteins with molecular weights less than 85,000 contained less carbohydrate and those of low molecular weight (43,000) had little or no associated carbohydrates. While tunicamycin inhibited the incorporation of precursor

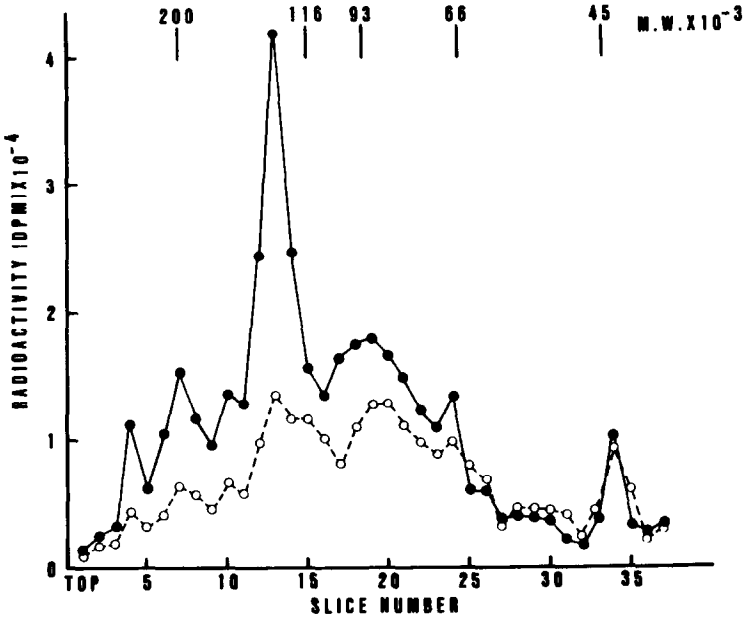

Fig. 2. Effect of tunicamycin on the SDS-PAGE profile of newly synthesized brush border membrane glycoproteins labeled *in vivo* with [^{3}H]leucine. Gels were sliced into pieces (3 mm), and the radioactivity was counted in each slice. Protein (200 μg) was applied to the gel. Key: (●—●) controls; and (○---○) tunicamycin.

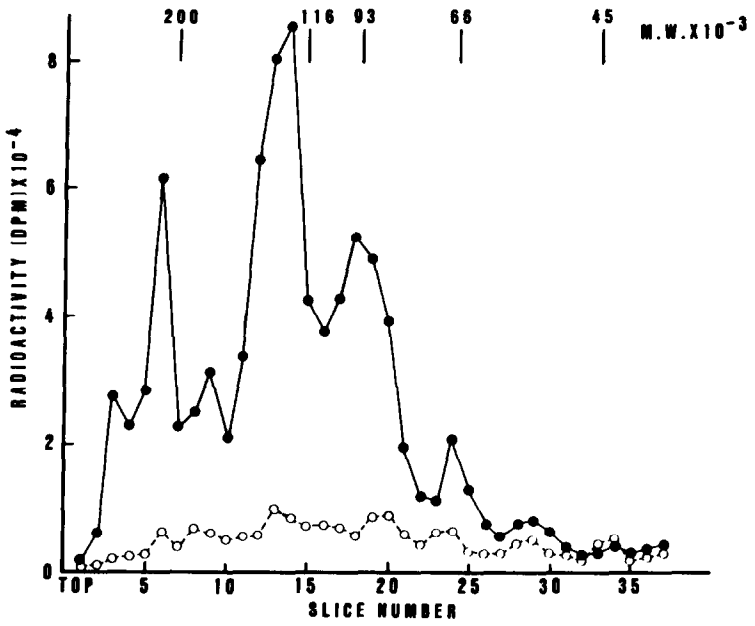

Fig. 3. Effect of tunicamycin on the SDS-PAGE profile of newly synthesized brush border membrane glycoproteins labeled *in vivo* with [^{3}H]glucosamine. Experimental details are in the legend of Fig. 2. Key: (●—●) controls; and (○---○) tunicamycin.

sugar into newly synthesized brush border membrane protein as shown in Fig. 3 and described above, it also inibited the incorporation of [^{3}H]leucine into brush border membrane proteins with molecular weights greater than 100,000. This effect was much less noticeable with lower molecular weight proteins (less than 90,000), and those with molecular weights of less than 43 kD apparently were not affected.

The *in vivo* incorporation of [^{3}H]glucosamine and [^{3}H]leucine into newly synthesized aminopeptidase N and dipeptidyl aminopeptidase IV is shown in Table 4. Although incorporation of [^{3}H]glucosamine into both enzymes was reduced by tunicamycin, [^{3}H]leucine incorporation into both enzymes was also reduced in brush border membranes and mucosal cell homogenates. The difference between the percent reduction of leucine versus glucosamine incorporation was statistically significant for aminopeptidase ($P < 0.01$) but not for dipeptidyl aminopeptidase IV in both cell preparations. As can be seen, the percent reduction of incorporation of both radiolabeled precursors was much higher for each enzyme than for total protein in both cell preparations. The fact that percent reduction values for both enzymes in homogenates and brush border membranes were very similar suggested that there was no accumulation of newly synthesized non-glycosylated enzymes in other subcellular compartments. To examine this possiblity further, the amount of enzyme protein was determined by a

Table 4. Effect of tunicamycin on the incorporation of [^{3}H]leucine and [^{3}H]glucosamine into newly synthesized immunoprecipitable aminopeptidase N and dipeptidyl aminopeptidase IV from mucosal cell homogenates and purified brush border membranes

	[^{3}H]Leucine*			[^{3}H]Glucosamine*		
	Control	Tunicamycin	% Reduction	Control	Tunicamycin	% Reduction
Cell homogenate						
Aminopeptidase N	8.27 ± 0.53†	2.50 ± 0.54	69.8	13.43 ± 1.09	1.57 ± 0.69	88.3
DAP IV‡	1.66 ± 0.19	0.49 ± 0.11	70.5	3.21 ± 0.78	0.52 ± 0.24	83.8
Brush border membrane						
Aminopeptidase N	4.64 ± 1.19	1.19 ± 0.28	74.4	10.07 ± 1.92	0.95 ± 0.32	90.6
DAP IV‡	0.92 ± 0.08	0.27 ± 0.08	70.7	2.26 ± 0.73	0.39 ± 0.15	82.7

See Table 3 for legend and description.

* In the cell homogenate, units for control and tunicamycin values are dpm × 10^3/mg protein; in the brush border membrane, units are dpm × 10^4/mg protein.

† Data are expressed as the mean ± SD of five experiments.

‡ DAP IV, dipeptidyl aminopeptidase IV.

competitive radioimmunoassay in homogenates and brush border membranes and expressed as the ratio of enzymatic activity per milligram of enzyme protein (specific enzyme activity, SEA). The results indicated that there was no change in the SEA value for both enzymes in control and tunicamycin-treated rats.

In both brush border membranes and cell homogenates, the binding of enzyme activities to ConA-Sepharose was not affected by treatment with tunicamycin, indicating that there were no significant changes in the carbohydrate moieties of newly synthesized enzymes. With all four enzymes, about 40–60% of the enzyme activity bound to ConA-Sepharose, and there was no difference between enzymes from brush border membranes and mucosal cell homogenates.

DISCUSSION

Although glycoproteins are present in abundance in eukaryotic cells and are implicated in a number of diverse cellular functions, the precise biological significance of the constituent oligosaccharide is not fully understood. The carbohydrate moieties of glycoproteins are thought to: (a) serve as a chemical "tag" for intracellular recognition during their biosynthesis or as passports for export from the cell, (b) participate in the mediation of specific biological activity of the protein, or (c) stabilize the conformation of the glycoprotein and protect it from proteolytic degradation. The brush border membrane of rat intestinal epithelial cells contains a number of integral membrane hydrolytic enzymes that are glycoproteins. Aminopeptidase N and dipeptidyl aminopeptidase IV are peptidases that have been purified recently from the brush border membrane of rat small intestine by our laboratory, each containing 20 and 8% carbohydrate by weight respectively [14, 15]. A preliminary carbohydrate analysis has shown that the oligosaccharides are primarily N-linked and can be cleaved from the peptide backbone by hydrazinolysis [15]. Beyond this, however, very little is known regarding the possible role that these carbohydrate units play in the biosynthesis, localization and activity of these brush border membrane enzymes.

Tunicamycin was used in this investigation to study its effect on the biosynthesis and expression of these two brush border membrane peptidases *in vivo*. This antibiotic has been shown to inhibit the formation of *N*-acetylglucosamine–lipid intermediates which serve as the initial donors for synthesis of glycoprotein carbohydrate units [24–26]. Numerous studies in cultured cell systems have shown that it is generally effective in inhibiting glycosylation without significantly inhibiting protein synthesis [6, 8, 27]. In contrast, however, there have been relatively few reports in the literature when tunicamycin has been administered to experimental animals [9, 13]. Our initial experiments were designed, therefore, to determine an optimum dose and time regimen for conducting this study. Under the conditions chosen, there were significantly decreased levels of several hydrolases associated with the intestinal brush border membrane. Interestingly, sucrase and aminopeptidase N activities were decreased to 50% of control values after 12 hr (Table 2) whereas dipeptidyl aminopeptidase IV and maltase were affected to a lesser extent (75% of control). While the explanation for these apparent differences in susceptibility to tunicamycin is not clear at the present time and must await further investigation, it may be related to differences in the synthetic and turnover rates of individual proteins.

The decrease in brush border membrane hydrolase activity was paralleled by a corresponding change in the SDS-PAGE profile of protein from tunicamycin-treated animals. As judged by Coomassie blue staining and galactose oxidase–sodium borotritide labeling, there was a marked decrease in the amount of glycoproteins with molecular weights greater than 130 kD (Fig. 1). Relatively minor changes were observed, however, for glycoproteins of lower molecular weight. This decrease in high molecular weight glycoproteins is consistent with the fact that many of the brush border membrane hydrolases have subunit molecular weights of 130–160 kD [1, 28]. Except for possibly two new protein bands in tunicamycin-treated animals (Fig. 1), there were no major changes in the relative position of individual protein bands in tunicamycin versus control brush border membranes. This suggests that the molecular weight of the individual glycoproteins was not altered significantly by tunicamycin treatment as might be expected if attachment and processing of the oligosaccharide side chains were affected significantly. Further evidence for a normal oligosaccharide content is suggested by the similarity in ConA binding patterns for the individual hydrolases from tunicamycin and control animals. Taken together, these results indicate that *in vivo* administration of tunicamycin caused a selective decrease in the content of high molecular weight glycoproteins associated with the intestinal brush border membrane. However, the individual glycoproteins which are synthesized in the presence of tunicamycin and inserted into the brush border membrane apparently have normal oligosaccharide units.

Tunicamycin had a mild inhibitory (20%) effect on total cellular protein synthesis as judged by the incorporation of [^{3}H]leucine into mucosal cell protein following 4 hr of continuous labeling. Under these conditions, [^{3}H]glucosamine incorporation was inhibited by 60%. These results are in agreement with those of others showing that the major effect of tunicamycin is on glycosylation [24–27]. In contrast, however, for the newly synthesized glycoproteins specifically destined for the brush border membrane tunicamycin had a more profound effect, inhibiting [^{3}H]leucine incorporation by 50%. Incorporation of amino acid precursor into immunoprecipitable brush border membrane aminopeptidase N and dipeptidyl aminopeptidase IV was inhibited 70% by tunicamycin, and identical inhibition values (70%) were observed for these two enzymes immunoprecipitated from total cell homogenates.

One explanation for these results is that, in addition to its known effect on glycosylation, tunicamycin may strongly suppress the synthesis of the polypeptide moiety of these two enzymes. Though many studies have shown that it is possible to

uncouple glycosylation and protein synthesis with tunicamycin, others have indicated that the two processes are interdependent and highly regulated. It has been found in canine kidney cells, for example, that inhibition of protein synthesis also inhibits synthesis of lipid-linked oligosaccharide presumably by end product inhibition [29]. Work by Hasilik and Tanner [30] and Seagar *et al.* [31] has strongly suggested that regulation can also operate in the opposite direction with inhibition of the formation of lipid-linked oligosaccharides leading to inhibition of peptide synthesis. Other workers have shown that tunicamycin suppresses the synthesis of placental alkaline phosphatase by causing a decrease in the amount of translatable phosphatase mRNA [32]. Similarly, results by Trugnan *et al.* [33] have shown that tunicamycin has a strong inhibitory effect on the biosynthesis of sucrase-isomaltase in differentiated Caco-2 cells. These studies show that very little immunoprecipitable sucrase-isomaltase could be detected in pulse-labeled cells, suggesting that polypeptide synthesis may be affected.

Alternatively it is also possible that the newly synthesized, malprocessed brush border membrane enzymes are recognized, rapidly degraded [6], and fail to be incorporated into the brush border membrane. In this regard, Danielsen and coworkers [34, 35], using intestinal explants, have shown that expression of microvillar enzymes is greatly reduced by tunicamycin but could be partially restored by the protease inhibitor leupeptin. However, it has not been reported what effect tunicamycin had on total cellular or brush border membrane protein synthesis and whether increased degradation alone could account for the observed reduction in biosynthesis. Thus, as this study and others suggest, it is important to consider that the effects of tunicamycin on intestinal brush border membrane protein biosynthesis may be 2-fold and lead to changes in both the rate of polypeptide synthesis and degradation.

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