Glycoprotein Biosynthesis in Small Intestinal Mucosa

I. A STUDY OF GLYCOSYLTRANSFERASES IN MICROSOMAL SUBFRACTIONS*

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SUMMARY

An active multiglycosyltransferase system involved in the transfer of N-acetylgalactosamine, N-acetylglucosamine, and galactose to various glycoprotein acceptors was found in the rat small intestinal mucosa. The present studies are concerned with the subcellular localization of these glycosyltransferases. Satisfactory isolation of smooth and rough surfaced microsomes from the intestinal mucosa was obtained by modifying the method of Dallner (Acta Pathol. Microbiol. Scand. Suppl., 166, 1 (1963)). Kinetic studies, performed to establish optimum conditions for quantitative assays of the glycosyltransferases, were linear with respect to enzyme concentration and time. Smooth surfaced microsomes were the main submicrosomal loci for the glycosyltransferases; for example 95% of total microsomal polypeptidyl:N-acetylgalactosaminyltransferase activity was found in the smooth surfaced microsomes. The results of alkaline borohydride treatment and acid hydrolysis indicated that N-acetylgalactosamine was incorporated into the heat-treated rough surfaced microsomal acceptors primarily at the protein to carbohydrate linkages while in the case of the heat-treated smooth surfaced microsomal acceptors, this sugar was probably added to positions more distal in the polysaccharide moiety. Immunodiffusion showed that only the smooth surfaced microsomes contained a component immunologically identical with purified rat small intestinal mucin. These results suggest that both stepwise glycosylation and probably the attainment of an immunological identity of rat small intestinal mucin occur in the smooth surfaced microsomes.

Relatively little is known about the structure, biosynthesis, and catabolism of the intestinal mucins. In most mammalian small intestine, histochemical studies show that there are at least two types of cells that secrete mucins; surface epithelial cells and goblet cells. Intestinal mucins are glycoproteins like mucins from other sources, such as the submaxillary gland, ovarian cysts, and urine (1, 2). Recently we isolated and characterized a solubilize glycoprotein from the small intestinal mucosa of rats (3). This glycoprotein is a macromolecule with its carbohydrate content about 80% of the dry weight. It also shares immunological identity with mucin secreted into the intestinal lumen.1

A specific multienzyme system, designated as glycosyltransferases, which adds each carbohydrate constituent to the polypeptide core and to the growing oligosaccharide side chains (4), has been reported to be present in colostrum and in liver, submaxillary gland, thyroid, and gastric mucosa (4–9).

The subcellular localization of these enzymes transferring carbohydrate groups in the biosynthesis of serum glycoproteins, thyroglobulin, and structural glycoproteins has been studied in various tissues (10–15). Studies of the subcellular localization, however, have not been carried out in the intestinal mucosa. In a recent preliminary communication, we reported that these enzymes were localized predominantly in the microsomal fractions of rat small intestinal mucosa (16). The present studies describe partial characterization and further submicrosomal localization of some of the glycosyltransferases in rat small intestinal mucosa. The data indicate that the enzymes are located predominantly in the smooth surfaced microsomes suggesting that glycosylation of secretory glycoproteins may occur in the smooth surfaced microsomes in the rat small intestinal mucosa.

EXPERIMENTAL PROCEDURE

Subcellular Fractionation Male, Sprague-Dawley albino rats weighing 200 to 250 g were allowed only water for 16 hours before use. The animals were killed by decapitation and subsequent steps were performed at 4°C. The small intestine was rapidly removed from the ligament of Treitz to 2 cm proximal to the ileocecal valve, washed with 60.0 ml of 0.15 M NaCl, everted. The mucosa, scraped off with glass slides, was homogenized in 4 volumes (w/v) of 0.25 M sucrose in a Potter-Elvehjem tissue homogenizer by 6 strokes with a Teflon pestle with a 0.004 to 0.006 cm clearance and driven by a Con-Torque stirrer at medium speed. The homogenate was centrifuged at 3,000 × g for 10 min to yield a pellet containing cell debris and nuclei. The supernatant was filtered through four layers of gauze to remove the fat layer and centrifuged at 7,000 × g for 10 min. This heavy mitochondrial pellet was gently resuspended in 0.25 M sucrose, centrifuged at 7,000 × g for 10 min, and the supernatant discarded.

1 Y. S. Kim and J. Perdomy, unpublished results.

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post-heavy mitochondrial supernatant was centrifuged at 20,000 × g for 10 min to yield a pellet containing light mitochondria which was resuspended in 0.25 M sucrose and recentrifuged for 10 min. Two separate fractionation methods were used on the post-light mitochondrial supernatant depending on whether the total microsomal fraction or submicrosomal fractions (rough or smooth surfaced microsomes) were to be obtained. To obtain the total microsomal fractions, the post-light mitochondrial supernatant was centrifuged at 145,000 × g for 5 hours. The total microsomal pellet was rinsed twice with 0.25 M sucrose. The separation of rough and smooth surfaced microsomes from post-light mitochondrial supernatant was achieved by a modification of Dallner's CsCl method (17), since this method yielded markedly cross-contaminated microsomal fractions of rat small intestinal mucosa. The isolation procedure that yielded the best separation of microsomal subfractions from rat small intestine, as outlined in the Fig. 1, is as follows.

The post-light mitochondrial supernatant was adjusted to a final concentration of 15 mM CsCl by adding 0.15 ml of 1 M CsCl to 9.85 ml of the supernatant. After mixing by inversion, 6 ml of the resulting suspension were layered over a discontinuous sucrose density gradient formed by 1 ml of 2.0 M, 3 ml of 1.3 M, and 3 ml of 1.2 M sucrose, all containing CsCl to a final concentration of 15 mM, and were centrifuged at 200,000 × g for 4 hours in a Spinco type SW 41 rotor. At the end of this centrifugation three bands were observed, an upper band (Band I) suspended over 1.2 M sucrose, a middle band (Band II) over 1.3 M sucrose, and a lower band (Band III) over 2.0 M sucrose. Band I was carefully transferred, diluted to a final sucrose concentration of approximately 0.25 M sucrose and 15 mM CsCl, and layered over 5 ml of 1.2 M sucrose (15 mM in CsCl). Band III was pipetted off and diluted in the same manner as for Band I and layered over 5 ml of 1.3 M sucrose (15 mM in CsCl). Band II was pipetted off, diluted with several volumes of 0.25 M sucrose (15 mM in CsCl), and centrifuged at 200,000 × g for 140 min to obtain a ribosomal pellet. The tubes containing either bands I or II were also centrifuged at 200,000 × g for 140 min. From the tube containing the Band I, a fluffy floating fraction above 1.9 M sucrose was pipetted off, diluted with several volumes of 0.25 M sucrose (15 mM in CsCl), and centrifuged at 200,000 × g for 90 min to obtain smooth surfaced microsomes as a pellet. From the tube containing Band III, a dense white rough surfaced microsomal pellet was obtained. Both submicrosomal pellets were rinsed three times with 0.25 M sucrose (15 mM in CsCl).

**Extraction of Submicrosomal and Total Microsomal Fractions**—Submicrosomal pellets were resuspended in 0.25 M sucrose (5 mM in MgCl2, 20 mM in Tris-maleate, pH 7.4, and 25 mM in KCl) adjusted to a final concentration of 0.5% in Triton X-100, homogenized with five strokes in the Teflon Potter-Elvehjem homogenizer, and allowed to stand for 20 min. The resultant submicrosomal suspensions were sonicated (Heat-system-ultrasonic) with a probe terminating in a 3-mm diameter at 80 watts. Two sonic bursts of 15-sec duration were applied and the temperature did not go above 5° at any time. Each extract was extensively dialyzed against distilled water and lyophilized for use as a source of glycosyltransferases. The extract from the total microsomal pellet was also prepared as described above.

**Preparation of Microsomal, Submicrosomal, and Glycoprotein**
Acceptors—When total microsomal or submicrosomal fractions were used as acceptors, each was suspended in a solution containing 0.33% (v/v) of Triton X-100 in water in a concentration of 10 mg of protein per ml. The fractions were immersed in a water bath at 100° for 15 min and were cooled on ice. All acceptors were stored at -20° until used.

Fetuin free of N-acetylneuraminic acid was prepared by treating fetuin (Calbiochem) with neuraminidase (Vibrio cholerae, Calbiochem). Fetuin was dissolved in 0.05 M sodium acetate buffer, pH 5.5, containing 0.05 M CaCl2 at a concentration of 3 mg per ml. Neuraminidase was added to this solution and the mixture was incubated at 37° for 48 hours after addition of several drops of toluene. The reaction mixture was heated at 100° for 15 min to inactivate neuraminidase activity, dialyzed against water, and lyophilized.

Fetuin free of both N-acetylneuraminic acid and galactose was prepared by mild acid hydrolysis followed by Smith degradation, according to a slightly modified method of Spiro (18). Fetuin was heated at 80° for 1 hour in 0.1 N H2SO4 to remove N-acetylneuraminic acid, neutralized with 1 N NaOH, and dialyzed. The nondialyzable material was treated with 0.01 M sodium metaperiodate in 0.05 M sodium acetate, pH 4.5, in the dark for 24 hours at 4°. The reaction was stopped by addition of glycerol and dialyzed against distilled water. The dialyzed material was incubated for 13 hours at 4° in a 0.15 M sodium borohydride and potassium tetraborate buffer, pH 8.0. The reaction was terminated by adjusting the pH to 5.0 with 1 N acetic acid and the sample dialyzed against water. The dialysate was made 0.05 N in H2SO4, heated at 80° for 1 hour, cooled, neutralized with 1 N NaOH, then dialyzed and lyophilized.

Ovine submaxillary mucin was a gift of Dr. W. Pigman. Ovine submaxillary mucin free of N-acetylneuraminic acid and N-acetylgalactosamine was prepared by treatment with neuraminidase and N-acetylgalactosaminidase prepared from jack bean meal. The sample was kindly supplied by Dr. E. H. Eylar.

Enzyme Assays—Unless otherwise stated, the standard assay for glycosyltransferase activities was carried out as described below, with either rough or smooth surfaced microsomal fraction as source of enzyme. Heat-treated microsomal fractions were used as acceptors for the assay of glycosyltransferases except for polypeptidyl-N-acetylgalactosaminyltransferase. All enzyme assays were performed under conditions in which the reaction was linear with respect to enzyme concentration and time.

N-Acetylgalactosaminyltransferase—The incubation mixture for the assay of the N-acetylgalactosaminyltransferase contained the following: 2.63 nmol (250,000 dpm) of UDP-[3H]galactose (92 Ci/mmol, New England Nuclear), 1 mg of microsomal acceptor protein, 3.25 µmol of MnCl2, 0.1 ml of 1% Triton X-100, 2 µmol of β-mercaptoethanol, 17.5 µmol of Tris-maleate, pH 6.9, and 0.50 mg of enzyme protein in a final volume of 0.33 ml. After incubation at 37° for 30 min, the reaction was terminated and the protein-bound radioactivity was determined as described above.

UDP-N-Acetylgalactosamine—The incubation mixture contained: 2.63 nmol (250,000 dpm) of UDP-N-[3H]galactosamine (43 Ci/mmol, New England Nuclear), 1 mg of microsomal acceptor protein, 3.25 µmol of MnCl2, 0.1 ml of 1% Triton X-100, 2 µmol of β-mercaptoethanol, 17.5 µmol of Tris-maleate, pH 6.9, and 0.1 mg of enzyme protein in a final volume of 0.33 ml. The conditions for incubation and the determination of the protein-bound radioactivity were as described above.

Polypeptidyl-N-acetylgalactosaminyltransferase—This enzyme transfers N-acetylgalactosamine to the hydroxy amino acids of protein acceptor (19) and is probably responsible for the attachment of the first sugar to the protein core in the biosynthesis of small intestinal mucin.2 The incubation mixtures and conditions for the assay of this enzyme were similar to those used for N-acetylgalactosaminyltransferase except that ovine submaxillary mucin (0.1 mg of protein) free of N-acetylneuraminic acid and N-acetylgalactosamine was used as an acceptor.

Paper Chromatography—Labeled monosaccharides were identified by descending paper chromatography on Whatman No. 3MM paper in ethyl acetate-pyridine-water (12:5:4, v/v) for 19 hours at 25°. Radioactive areas on the paper chromatograms were cut and counted in a liquid scintillation spectrometer as described above. Reducing sugars on paper chromatograms were detected by the silver nitrate reagent (20).

Alkaline Borohydride Treatment of Reaction Products of Submicrosomal Acceptors and UDP-N-[3H]Acetylgalactosamine—Heat-treated smooth or rough surfaced microsomes were incubated with UDP-N-[3H]acetylgalactosamine and an enzyme (smooth surfaced microsomes) under the standard assay condition as described above. To determine the radioactivity incorporated into endogenous acceptors, smooth surfaced microsomes were incubated in the absence of an exogenous acceptor. Following incubation as described previously, the phosphotungstic acid precipitate was suspended in water, neutralized with 0.2 N NaOH, adjusted to a final concentration of 0.2 N NaOH and 1.0 M NaBH4. The mixtures were kept for 15 hours at 45° (21, 22) and then were acidified with 0.3 N acetic acid. The excess boric acid was removed with methanol in vacuo as methylborate. Following the addition of unlabeled galactosamine and galactosaminol (0.14 µmol each) as carriers, the samples were hydrolyzed in 2 N HCl for 6 hours at 100°. The hydrolysate was dried in vacuo at 45°, dissolved in distilled water, and applied to a Dowex 50-X8 column as described by Boas (23). Both radioactive galactosamine and galactosaminol were eluted

2 A. M. Bella, Jr., and Y. S. Kim, unpublished results.
with 10 ml of 2.0 N HCl and then taken to dryness in vacuo at 45°. The dried eluate was dissolved in a pH 5.05 citrate-borate buffer and fractionated on a column, 0.9 x 56 cm, of Beckman UR-30 resin on a Beckman 120C amino acid analyzer according to the method of Bella and Kim (24). The column effluent was diverted to test tubes in a fraction collector and 5-ml fractions collected. After drying in a vacuum at 45°, the radioactivity of each fraction was determined in Insta-gel emulsifier (Packard Instrument) with a liquid scintillation spectrometer. A standard mixture containing galactosaminol, glucosaminol, galactosamine, and glucosamine (1.5 μmoles each) was treated and fractionated as above.

**Chemical Determination**—Protein was determined by the method of Lowry et al. (25). The method of Scott, Fracastoro, and Taft (26) was used for the analysis of RNA and phospholipid as described by Chen, Taribara, and Warner (27).

**Electron Microscopy**—Microsomal pellets were fixed 18 hours in cold 0.05 M phosphate-buffered 1% OsO₄ at 4°, dehydrated in increasing concentration of ethanol, embedded, and left at 0° for 16 hours in Epon 812 according to Luft (28). Thin sections were examined with a Phillips EM 300 electron microscope.

**Immunological Methods**—Purified rat small intestinal mucin was prepared as previously described (3). Rabbit antisera were obtained from three random bred New Zealand rabbits (6 kg, male) which were immunized by weekly subcutaneous injections for 4 weeks of 0.5 ml of the purified mucin (2 mg per ml) emulsified in an equal volume of complete Freund's adjuvant. One week after the last injection, the rabbits were injected intravenously with 1 mg of mucin in 1 ml of Tris buffer, pH 7.4, and 5 days later blood was obtained by cardiac puncture. Merthiolate was added to the antisera to a final concentration of 1:10,000 and kept at -20° in small aliquots until used.

**Immunodiffusion** was performed by the technique of Ouchterlony (29) on immuno plates (Hyland Company, California). Extracts of smooth and rough surfaced microsomes were prepared by solubilizing each submicrosomal fraction with Triton X-100 with constant stirring at 4° for 16 hours. Insoluble material was removed by centrifugation for 90 min at 40,000 x g and the soluble extract dialyzed and lyophilized.

**RESULTS**

**Subcellular Fractionation**—Dallner's method (17) of subcellular fractionation with CsCl yielded unsatisfactory separation of rough and smooth surfaced microsomes, when applied to rat small intestinal homogenates. However, with some modifications...
of the method, effective separation of microsomal fraction of rat small intestinal mucosa into smooth and rough surfaced microsomal subfractions could be achieved as shown by electron micrographs (Fig. 2, A and B) and chemical composition (Table I). Fig. 2, A and B illustrates the degree of homogeneity of rough and smooth surfaced microsomal subfractions. Rough

**Table II**

**Requirements for glycosyltransferases of rat small intestinal mucosa**

Complete incubation mixtures contained the following in final volumes of 0.33 ml: heat-treated microsomal acceptor, 1 mg; 1% Triton X-100, 0.1 ml; MnCl₂, 3.25 μmol; and β-mercaptoethanol, 2 μmol. The reaction mixture also contained the following: UDP-N-acetylglucosamine (2.63 × 10⁻⁴ μmol), 17.5 μmol of Tris-maleate buffer, pH 6.9, and the smooth surfaced microsomes (0.5 mg of protein) for N-acetylglucosaminyltransferase assay. The incubation mixture also contained the following: UDP-[¹⁴C]galactose (4.5 × 10⁻⁴ μmol), 17.5 μmol of Tris-maleate buffer, pH 6.9, and the smooth surfaced microsomes (0.5 mg of protein) for galactosyltransferase assay; UDP-[¹⁴C]N-acetylgalactosamine (2.63 × 10⁻⁴ μmol), 17.5 μmol of Tris-maleate buffer, pH 6.9, and the smooth surfaced microsomes (0.5 mg of protein) for N-acetylgalactosaminyltransferase assay. The incubation was for 30 min at 37°C after which the radioactivity was determined as described in the text.

<table>
<thead>
<tr>
<th>Components omitted from complete incubation mixture</th>
<th>Enzyme activity with nucleotide-[¹⁴C] sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UDP-GlcNAc⁻</td>
</tr>
<tr>
<td></td>
<td>Microsomal acceptor</td>
</tr>
<tr>
<td>None</td>
<td>%</td>
</tr>
<tr>
<td>Enzyme</td>
<td>100</td>
</tr>
<tr>
<td>Acceptor</td>
<td>18.7</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>105.0</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>100.5</td>
</tr>
<tr>
<td>Mn⁺⁺ + EDTA (3.25 μmol)</td>
<td>68.4</td>
</tr>
<tr>
<td></td>
<td>65.7</td>
</tr>
</tbody>
</table>

a The abbreviations used are: UDP-GlcNAc, UDP-N-acetylglucosamine; UDP-Gal, UDP-galactose; UDP-GalNAc, UDP-N-acetylgalactosamine.

*Fig. 3. The influence of pH on the incorporation of N-acetylgalactosamine (A), galactose (B), and N-acetylgalactosamine (C) into heat-treated microsomal acceptors (1 mg of protein) (●—●) and into endogenous acceptor (○—○) prepared from rat small intestinal mucosa. Smooth surfaced microsomes were used as the source of glycosyltransferases and the assay conditions are described fully in the text.*
**Acceptor Specificity**—The heat-treated total microsomal fraction was active as acceptor for both N-acetylgalactosamine and galactose but was relatively inactive for N-acetylglucosamine. Both heat-treated rough and smooth surfaced microsomes accepted N-acetylgalactosamine, although the latter incorporated about 2 times as much radioactivity as the former (Table III). This would suggest that N-acetylgalactosamine incorporated into the rough surfaced microsomal acceptor may represent mainly the polypeptidyl:N-acetylgalactosaminyltransferase activity and the radioactivity incorporated into the smooth surfaced microsomal acceptor may represent both this enzyme and the N-acetylgalactosaminyltransferase activity. Fetalin free of N-acetylenuraminic acid and galactose having N-acetylglucosamine exposed at nonreducing positions of the carbohydrate chains, served as an effective acceptor for the galactosyltransferase. Ovine submaxillary mucin free of N-acetylenuraminic acid with N-acetylgalactosamine exposed, also accepted galactose. Ovine submaxillary mucin from which both N-acetylenuraminic acid and N-acetylgalactosamine had been removed was found to be a suitable acceptor for the polypeptidyl:N-acetylgalactosaminyltransferase. This acceptor also incorporated galactose. These results are shown in Table III.

**Distribution of Glycosyltransferase in Submicrosomal Fractions**—As shown in Table IV, all glycosyltransferase activities studied were significantly higher in the smooth surfaced than in the rough surfaced microsomal fraction. The ratios of enzyme activities between these fractions varied from 8 to 54. These were similar for both endogenous enzyme activity and the enzyme activity with exogenous acceptors. It is of particular interest that the enzyme involved in the transfer of the first sugar, an N-acetylgalactosamine residue, to a protein acceptor in the biosynthesis of rat small intestinal mucin was localized almost entirely in the smooth surfaced microsomes.

The specificity of the N-acetylgalactosaminyltransferase in submicrosomal fractions for heat-treated submicrosomal acceptors was investigated (Table V). Whether the acceptor was prepared from heat-treated smooth or rough surfaced microsomes, N-acetylgalactosaminyltransferase activity was located mainly in the smooth surfaced microsomes. A possible presence of inhibitors for glycosyltransferases in the rough surfaced microsomes was studied by determining the effect of mixing both smooth and rough surfaced microsomes on the N-acetylgalactosaminyltransferase activity. A typical result of this study, as shown in Table VI, did not indicate the presence of inhibitors in rough surfaced microsomes, since the enzyme activity determined from the mixture of both smooth and rough surfaced microsomes was the same as the additive value of each submicrosomal fraction assayed separately.

**Identification of Labeled Sugars Incorporated into Protein**—The respective reaction products of N-acetylgalactosaminyl, galactosyl, and N-acetylgalactosaminyltransferases were precipitated and washed with phosphotungstic acid and then were hydrolyzed in

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**Fig. 4.** Effect of enzyme concentration on the incorporation of N-acetylglucosamine (A), galactose (B), and N-acetylgalactosamine (C) into heat-treated microsomal acceptors (1 mg of protein) (O-O) and into endogenous acceptors (O----O) prepared from rat small intestinal mucosa as described in the text. The total counts and the specific activity of each nucleotide sugar used and the incubation conditions are as given in the text. Smooth surfaced microsomes were used as the source of the glycosyltransferases.

**Fig. 5.** Effect of length of incubation on the incorporation of N-acetylglucosamine (A), galactose (B), and N-acetylgalactosamine (C) into heat-treated microsomal acceptors (1 mg of protein) (O-O) and into endogenous acceptors (O----O) prepared from rat small intestinal mucosa as described in the text. Smooth surfaced microsomes were used as the source of the glycosyltransferases and the incubation mixtures and conditions are given in the text.
$3 \times 10^{-1}$ under nitrogen at $100^\circ$ for 4 hours. The hydrolysates were filtered through glass wool, dried at $50^\circ$, dissolved in water, and chromatographed. In all cases, the only radioactive peak corresponded to the respective labeled sugar precursor as shown in Fig. 6, indicating that conversion to other nucleotide sugars probably did not occur during the reaction.

**TABLE III**

Acceptor specificities of rat small intestinal glycosyltransferases

The complete assay systems and conditions are given in Table II. All of the values shown represent total radioactivity incorporated and have not been corrected for endogenous activity.

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Enzyme activity with nucleotide-$^{14}$C sugar</th>
<th>dpm/mg protein/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UDP-GalNAc</td>
<td>UDP-Gal</td>
</tr>
<tr>
<td>None (endogenous accep-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tor)</td>
<td>1,250</td>
<td>2,750</td>
</tr>
<tr>
<td>Total microsomes (heat-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>treated)</td>
<td>2,190</td>
<td>9,790</td>
</tr>
<tr>
<td>Rough surfaced micro-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>somes (heat-treated) ...</td>
<td>1,426</td>
<td>4,410</td>
</tr>
<tr>
<td>Smooth surfaced micro-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>somes (heat-treated) ...</td>
<td>3,970</td>
<td>12,390</td>
</tr>
<tr>
<td>NAN and galactose-free</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fetuin ...</td>
<td>1,820</td>
<td>36,390</td>
</tr>
<tr>
<td>NAN-free fetuin ...</td>
<td>1,930</td>
<td>12,070</td>
</tr>
<tr>
<td>NAN and GalNAc-free</td>
<td>1,470</td>
<td>17,330</td>
</tr>
<tr>
<td>OSM ...</td>
<td>1,390</td>
<td>72,210</td>
</tr>
</tbody>
</table>

* One milligram of protein of each acceptor prepared as described in the text was used except for NAN and galactose-free ovine submaxillary mucin, 0.1 mg of protein of which was used.

**TABLE IV**

Distribution of glycosyltransferases in smooth and rough surfaced microsomes

Microsomes were subfractionated into rough and smooth surfaced microsomes according to the modification of the method of Dallner (17), as described in the text. Heat-treated total microsomes prepared from homogenates of rat small intestinal mucosa were used as exogenous acceptors for all glycosyltransferases except polypeptidyl:$N$-acetylgalactosaminyltransferase, for which ovine submaxillary mucin free of $N$-acetylgalactosaminic acid and $N$-acytetylgalactosamine was used. The assay mixtures and conditions are as described in Tables I and II and in the text. Endogenous enzyme activity represents the amount of radioactivity incorporated in the absence of added acceptor. Exogenous values have been corrected for the presence of endogenous activity. The numbers represent the mean values with the standard error of the mean obtained from six experiments (18 animals).

<table>
<thead>
<tr>
<th>Glycosyltransferase</th>
<th>RSM$^a$ enzyme activity</th>
<th>SSM enzyme activity</th>
<th>Ratio of enzyme activity (SSM/RSM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endogenous</td>
<td>Exogenous</td>
<td>Endogenous</td>
</tr>
<tr>
<td>$N$-Acetylgalactosaminyltransferase</td>
<td>70 ± 19</td>
<td>102 ± 62</td>
<td>1,992 ± 306</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>124 ± 13</td>
<td>332 ± 54</td>
<td>2,852 ± 330</td>
</tr>
<tr>
<td>$N$-Acetylgalactosaminyltransferase</td>
<td>1,670 ± 510</td>
<td>17,260 ± 3,260</td>
<td>35,980 ± 5,810</td>
</tr>
<tr>
<td>Polypeptidyl:$N$-acetylgalactosaminyltransferase</td>
<td>750 ± 140</td>
<td>40,500 ± 4,780</td>
<td>54</td>
</tr>
</tbody>
</table>

* The abbreviations used are: RSM, rough surfaced microsomes; SSM, smooth surfaced microsomes.
TABLE VI
Effect of mixing rough and smooth surfaced microsomes on incorporation of UDP-N-[14C]acetylgalactosamine

One milligram of heat-treated microsomal protein prepared as described in the text was used as exogenous acceptor except when the mixture of rough and smooth microsomes were used as a source of the enzyme. In the latter study, 2 mg of heat-treated microsomal protein were used. Incubations were carried out as described in the Tables I, II, and III, and in the text.

<table>
<thead>
<tr>
<th>Source of N-acetylgalactosaminyltransferase</th>
<th>Endogenous activity&lt;sup&gt;a&lt;/sup&gt; (dpm/hr)</th>
<th>Exogenous activity&lt;sup&gt;a&lt;/sup&gt; (dpm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rough surfaced microsomes (0.1mg of protein)</td>
<td>320</td>
<td>1,810</td>
</tr>
<tr>
<td>Smooth surfaced microsomes (0.1mg of protein)</td>
<td>4,775</td>
<td>19,840</td>
</tr>
<tr>
<td>Mixture of rough and smooth surfaced microsomes (0.1 mg of protein each)</td>
<td>5,190</td>
<td>22,160</td>
</tr>
</tbody>
</table>

<sup>a</sup> Both endogenous and exogenous activity were calculated as described in Table IV and in the text.

ably represent the enzyme linking N-acetylgalactosamine to sugar acceptors at more distal sites from the linkage region.<sup>b</sup>

Fig. 7. Chromatographic separation of labeled galactosamine and galactosaminitol from rough (A) and smooth (B) surfaced microsomal acceptors after incubation with UDP-N-[14C]acetylgalactosamine and alkaline borohydride treatment. See text for conditions. Radioactivity in each eluent fraction represents the total radioactivity in each fraction minus the endogenous radioactivity. The standards shown on the chromatogram are: GalN-tol (galactosaminitol), GlcN-tol (glucosaminitol), GlcN (glucosamine), and GalN (galactosamine).

Fig. 8. Double diffusion in agar gel of soluble purified rat small intestinal mucin and smooth and rough surfaced microsomes. Rabbit anti-rat small intestinal mucin was placed in the center well and the peripheral wells contained the following: 1, soluble purified rat small intestinal mucin, 5 mg per ml; 2 and 4, Triton X-100 solubilized rough surfaced microsomes, 20 mg of protein per ml; 3 and 5, Triton X-100 solubilized smooth surfaced microsomes, 10 mg of protein per ml. Similar results were also obtained with different concentrations of Triton X-100 solubilized sub-microsomal fractions (5, 10, and 20 mg of protein per ml).

and this sugar located in distal positions in the carbohydrate chain, although this sugar linked to the aspartamido group of asparagine has not been found in glycoprotein.
As shown in Fig. 7, with the heat-treated rough surfaced microsomal acceptor, N-acetylgalactosamine was primarily incorporated directly to the protein while with the heat-treated smooth surfaced microsomal acceptor, this sugar was attached both directly to the protein and at distal sites on the carbohydrate side chains. The ratio of radioactivity corresponding to galactosamine and galactosaminol was 0.17 with rough surfaced microsomal acceptors and 2.8 with smooth surfaced microsomal acceptors.

**Immunochromatography of Rat Small Intestinal Mucin in Submicromolecular Fractions**—Immunodiffusion of rough and smooth surfaced microsomes and purified rat small intestinal mucin (3) were carried out against rabbit antisera to this mucin (Fig. 8). Both purified rat small intestinal mucin and the smooth surfaced microsomes solubilized in Triton X-100 gave a single immunoprecipitin in the identity against rabbit antisera to purified rat small intestinal mucin, while the similarly solubilized rough surfaced microsomes even at high concentrations failed to give an immunoprecipitin reaction. These findings suggest that a protein of immunodiffusion identity to the rat small intestinal mucin may be formed in the smooth surfaced but not in the rough surfaced microsomes.

**DISCUSSION**

It is generally accepted that the protein backbones of glycoproteins are synthesized according to the established pathway of protein synthesis, i.e. on the polyosomes associated with rough endoplasmic reticulum. Opinions vary among investigators, however, regarding the initial site of the glycosylation of the protein and subsequent elongation of oligosaccharide side chains. Some believe that glycosylation takes place in the Golgi apparatus, smooth surfaced microsomes, or both (10, 15, 22, 32, 33), others present evidence that both rough and smooth surfaced microsomes are sites of carbohydrate addition (13, 34, 35), while some suggest that sugars are added to nascent polypeptides on polyosomes (35-37). These studies were performed in various mammalian tissues involved in the biosynthesis of glycoproteins such as liver and thyroid gland. We have recently isolated and characterized a soluble mucin (3) from rat small intestinal mucin as an immunoprecipitin reaction. These findings suggest that a protein of immunodiffusion identity to the rat small intestinal mucin may be formed in the smooth surfaced but not in the rough surfaced microsomes.

The pH optimum for both galactosyl and N-acetylgalactosaminyltransferases was similar to those obtained in goat colostrum (5) and in thyroid gland (14) for presumably the same enzymes. Absolute cation requirement for these enzymes, clearly indicated in this study, is also seen with the data of others (5, 6, 7, 14). Triton X-100 markedly stimulated the activities of galactosyl and N-acetylgalactosaminyltransferase in this study, a result similar to that reported by others for other tissues (9, 15). In contrast, Triton X-100 failed to stimulate N-acetylgalactosaminyltransferase activity. It is possible that this enzyme may be more tightly bound to the membranes in the intestinal mucosal cells than the other transferases examined and may not be readily solubilized with the concentration of Triton X-100 used. This may account in part for the relatively low activity of this enzyme that we obtained with various acceptors (Table III).

Galactosyltransferase of rat small intestinal mucosa transferred galactose to a glycoprotein acceptor with terminal N-acetylgalactosamine residues (fetuin treated with neuraminidase and Smith degradation procedure). This is consistent with the data obtained with galactosyltransferase in thyroid tissues (7) and in HeLa cells (41). Our results (Table III) suggest in addition that glycoprotein acceptors with other terminal sugars such as N-acetylgalactosamine (ovine submaxillary mucin treated with neuraminidase) and possibly galactose (fetuin treated with neuraminidase) may also be suitable acceptors for the galactosyltransferase from rat small intestinal mucosa. Ovine submaxillary mucin, treated both with neuraminidase and α-N-acetylgalactosaminidase would have most of the carbohydrate groups removed from the protein backbone and therefore should be an acceptor for polypeptide N-acetylgalactosaminyltransferase. Indeed our results (Tables III and IV) indicated that ovine submaxillary mucin treated in this manner was an excellent acceptor for the measurement of this enzyme.

Heat-treated microsomes accepted N-acetylgalactosamine, although it was not determined whether this sugar was being added directly to the protein or to partially synthesized carbohydrate chains of the intestinal mucin. A more specific acceptor
...for this latter enzyme is fetuin treated with neuraminidase, as shown in Table III.

Our data on the distribution of the glycosyltransferase in smooth and rough surfaced microsomes (Table IV) indicate that all four glycosyltransferases were localized primarily in the smooth surfaced microsomes. Although one should be cautious in reaching a conclusion based on subcellular fractionation data, a clear-cut localization of the enzyme responsible for the addition of the first sugar to the protein backbone of mucin in smooth surfaced microsomes strongly suggests that in the small intestinal mucosa of rats the glycosylation of secretory glycoprotein may be initiated in the smooth membranes.

Recently several reports appeared in which some glycosyltransferase were localized in the Golgi apparatus isolated from liver (10, 15, 32), confirming the previous radioautographic studies of Neutra and Leblond (33). The localization of these enzymes in smooth surfaced microsomes in the present study correlates well with the previous studies of these enzymes in other tissues (11, 14, 41). However, it is possible that the isolated smooth surfaced microsomal fraction also contained Golgi membranes. It is of interest that in the study of Schachter et al. (15), the percentage of total activity of glycosyltransferases in microsomal fraction was greater than that of the Golgi fraction.

The low protein content of the Golgi apparatus may be partly responsible for the high specific activity of these enzymes in this organelle. The more precise localization and quantitation of glycosyltransferases in either smooth surfaced microsomes or Golgi apparatus of small intestinal mucosa would help elucidate whether carbohydrate transfer occurs in stepwise manner at a single or multiple subcellular sites in the biosynthesis of the mucin of the small intestine. A definite conclusion in this regard may not be reached until a method to obtain pure fractions of smooth microsomes and Golgi membranes is developed. The methods currently available for separating these fractions are successful for liver and for plants, but not for mucin-secreting tissues such as gastrointestinal mucosa and salivary glands.

It appears that the subcellular step of carbohydrate transfer may be different for various glycoproteins. For instance, Bouchilloux et al. (34) reported that glycosyltransferases were present both rough and smooth surfaced microsomes of thyroid gland. Glycosyltransferases responsible for transfer of the internal sugars to a precursor of thyroglobulin were present in rough surfaced microsomes, while smooth surfaced microsomes rich in Golgi membranes had enzymes catalyzing the transfer of more external sugars, suggesting a multistep addition of carbohydrates in the completion of oligosaccharide side chains of thyroglobulin.

In the present study, the mechanism of carbohydrate addition to protein in rat small intestine was investigated by isolation of submicrosomal fractions from mucosal homogenates and examination of the enzymes and receptor substances involved in this process. Immunodiffusion studies indicated that only the smooth microsomes contained a component of immunological identity to that of the purified mucin of rat small intestinal mucosa. Even at high concentrations of microsomes, no cross-reactive material was observed in the rough membrane, suggesting that the polypeptide backbone of the mucin, which should be present in this fraction, does not have immunological identity with the completed mucin. The localization in the smooth membrane of the glycosyltransferases required for the completion of the mucin structure indicates that the addition of carbohydrate may be essential in attaining this identity. Although an acceptor for the enzyme responsible for the initial sugar attachment was, in fact, found in the rough membrane (Table III and Fig. 7), the polypeptidyl-N-acetylglactosaminyltransferase was localized in the smooth membrane. In addition, the other glycosyltransferases involved in the joining of subsequent sugars to the growing oligosaccharide chain, as well as their acceptors (Table III), are also found in the smooth membranes.

From the data presented in this and other papers on the subject, the following steps may be involved in the biosynthesis of mucin in the rat small intestinal mucosa. The completed protein core of the secretory glycoprotein is released into vesicular channels of the rough surfaced microsomes and is transported through the channels to smooth surfaced microsomes and to the Golgi apparatus. Carbohydrates are added to the protein core sequentially and immunological competence of this glycoprotein is attained at smooth surfaced microsomes, or the Golgi apparatus, or both. The completed mucin then accumulates in Golgi apparatus and finally secreted into the intestinal lumen. The precise role of glycosyltransferases in terminations of the oligosaccharide side chain elongation and the regulation of secretion of glycoproteins in the intestine remains to be elucidated.

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REFERENCES

* D. J. Morré, personal communication.