Effect of Ethanol on Mucus Glycoprotein Fatty Acyltransferase from Gastric Mucosa†

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ABSTRACT: The enzyme activity that catalyzes the transfer of palmitic acid from palmitoyl coenzyme A to the deacylated intact or deglycosylated gastric mucus glycoprotein was demonstrated in the detergent extracts of the microsomal fraction of antral and body mucosa of the rat stomach. Both types of mucosa exhibited similar acyltransferase activities and acceptor specificities. A 10–14% decrease in the fatty acyltransferase activity was observed with the reduced and S-carboxymethylated mucus glycoprotein, but the proteolytically degraded glycoprotein showed no acceptor capacity. This indicated that the acylation of mucus glycoprotein with fatty acids occurs at its nonglycosylated polypeptide regions and that some of the fatty acids may be linked via thiol esters. Optimum enzyme activity was obtained at pH 7.4 with the detergent Triton X-100, NaF, and dithiothreitol. The apparent K_m values for the intact and deglycosylated mucus glycoproteins were 0.45 and 0.89 μM, respectively. The acyltransferase activity of the microsomal enzyme was inhibited by ethanol. With both intact and deglycosylated glycoprotein substrates, the rate of inhibition was proportional to the ethanol concentration up to 0.4 M and was of the competitive type. The K_I values were 0.80 μM for the intact mucus glycoprotein and 1.82 μM for the deglycosylated glycoprotein. Preincubation of the microsomal enzyme with low concentrations of ethanol (up to 0.5 M) did not seem to exert any additional deterrent effect on acyltransferase activity. Higher concentrations of ethanol (1.0 M and above), however, caused substantial reduction in the transferase activity due to denaturation of the enzyme.

The viscous and slimy layer of mucus that tenaciously adheres to the epithelial surfaces of the gastrointestinal tract is a heterogeneous mixture of the molecules that find their way into the mucosal surface either by process of active secretion or by passive transudation (Glass & Slomiany, 1977; Allen, 1981). In the stomach, this layer constitutes part of a barrier that protects the underlying epithelium against various noxious agents either introduced into the stomach or formed endogenously. Among the exogenous agents that, when introduced into the stomach, can affect the integrity of gastric mucosa and cause the formation of mucosal lesions is ethanol (Glass & Slomiany, 1977; Pitchumoni & Glass, 1977, Glass et al., 1979; Fromm, 1981). One of the features of ethanol consumption is a marked alteration in the function of gastric mucus that is reflected in its decreased ability to protect the underlying epithelium from the damaging effects of acid and pepsin of luminal contents. The loss of this protective capacity of mucus is thought to be associated with the impairment of its glycoprotein constituent, but the nature of this alteration is not clear.

Studies in this laboratory on gastric mucus glycoprotein in man, dog, and rat established that this important mucus constituent, in addition to protein and carbohydrate, also contains covalently bound fatty acid (Slomiany et al., 1983, 1984a; Sarosiek et al., 1984; Takagi et al., 1984a,b). These ester-bound fatty acids play a significant role in the protection of mucus glycoprotein from peptic digestion and contribute to glycoprotein viscosity and its ability to retard the hydrogen ion diffusion (Murty et al., 1984; Sarosiek et al., 1984; Slomiany et al., 1984a). The interference by ethanol with mucus glycoprotein acylation, therefore, could significantly affect the physiological function of gastric mucus. Hence, a study of the enzymatic processes leading to mucus glycoprotein acylation may aid in our understanding of the mechanism of gastric mucosal damage as a consequence of ethanol ingestion. Recently, we presented evidence for the presence in rat gastric mucosa of an acyltransferase enzyme that catalyzes the transfer of fatty acids from fatty acyl coenzyme A (CoA)[1] to mucus glycoprotein (Slomiany et al., 1984b). Here, we report the effect of ethanol on this transferase activity.

Experimental Procedures

Isolation of Gastric Mucus Glycoprotein. Gastric mucus was obtained by instillation of the freshly dissected rat stomachs with 2 M NaCl–10 mM sodium phosphate buffer, pH 7.0 (Slomiany et al., 1978). The mucus contained in the recovered instillates was dialyzed against distilled water, lyophilized, dissolved in 6 M urea (at 10 mg/mL), and chromatographed on a Bio-Gel A-50 column (2.0 × 150 cm), equilibrated in and eluted with 6 M urea–10 mM sodium phosphate buffer, pH 7.0. The eluted fractions were monitored for protein and carbohydrate (Witas et al., 1983), and the excluded mucus glycoprotein peak was collected. Following rechromatography, the dialyzed and lyophilized glycoprotein was suspended in 0.05 M sodium phosphate buffer–0.15 M NaCl, pH 6.9, containing 38% w/w CsCl and centrifuged for 48 h at 12 °C and 46000 rpm in a Beckman 50Ti rotor. After centrifugation, the tubes were placed in a Beckman fraction recovery system and 1-mL fractions collected. The densities of the individual fractions were determined by refractive index measurement, and each fraction was screened for protein and

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[1] Abbreviations: CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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carbohydrate (Witas et al., 1983). Fractions containing mucus glycoprotein were pooled, dialyzed against distilled water, and lyophilized.

Preparation of Enzyme. Male rats (200–250 g), fasted overnight prior to experiment, were killed by cervical dislocation and their stomachs removed, opened along the lesser curvature, and washed thoroughly with cold 0.15 M NaCl. The mucosa was scraped with a glass slide, suspended in 5 volumes of 3 mM sodium phosphate buffer, pH 7.0, containing 0.25 M sucrose and 1 mM EDTA, and disintegrated with a Potter–Elvehjem glass–Teflon homogenizer (900 rpm, three strokes). The homogenate was centrifuged at 800*g* for 10 min, and the pellet was discarded. The supernatant was filtered through a layer of Miracloth and then centrifuged at 10000*g* for 20 min to sediment the crude mitochondrial fraction. Centrifugation of the resulting supernatant at 100000*g* for 1 h produced the microsomal (pellet) and cytosol (supernatant) fractions. The microsomal pellet was suspended in 0.25 M buffered sucrose, pH 7.0, containing 0.5% Triton X-100, and the content of the tube was stirred at 4 °C for 30 min. The mixture was then centrifuged at 100000*g* for 1 h, and the supernatant was used as an enzyme source.

Preparation of Acceptor. Mucus glycopeptide was obtained from the purified mucus glycoprotein by digestion with pepsin. For this, 20 mg of mucus glycoprotein was dissolved in 40 mL of 0.1 M citrate buffer, pH 2.2, and incubated at 37 °C for 72 h under a layer of thymol crystals with pepsin, in an enzyme-to-protein ratio of 1:30 (w/w) (Slomiany et al., 1984a). The soluble digest was lyophilized, dissolved in 6 M urea–10 mM sodium phosphate buffer, pH 7.0, and chromatographed on a Bio-Gel A-50 column under the conditions described above. The included peak that contained the mucus glycopeptide was dialyzed, lyophilized, and subjected to equilibrium density gradient centrifugation in CsCl (Witas et al., 1983). Following centrifugation, fractions containing glycopeptide were pooled, dialyzed against distilled water, and lyophilized. The deglycosylated glycoprotein was obtained from the purified mucus glycoprotein by the treatment with trifluoromethanesulfonic acid (Edge et al., 1983). The deglycosylated glycoprotein was freed of reagents and the released sugars by extraction with diethyl ether followed by dialysis against distilled water. Reduction of disulfide bonds in the mucus glycoprotein was performed with β-mercaptoethanol. For this, mucus glycoprotein was dissolved in 2.5% β-mercaptoethanol–1% SDS, pH 8.7, incubated at 37 °C for 2 h, and alkylated with 0.4 M iodoacetamide at room temperature for 16 h in the dark. Following dialysis and lyophilization, the glycoprotein was dissolved in 6 M urea–10 mM sodium phosphate buffer, pH 7.0, and separated from the undegraded mucus glycoprotein by chromatography on a Bio-Gel A-50 column under the conditions described above. Fractions containing the included reduced glycoprotein peak were pooled, dialyzed against distilled water, and lyophilized. Removal of the covalently bound fatty acids from the isolated mucus glycoprotein in its intact and modified forms was accomplished with hydroxylamine. The glycoprotein samples were incubated at room temperature for 5 h with 1.0 M hydroxylamine, pH 7.0 (Slomiany et al., 1984a). The released fatty acids were removed by three consecutive extractions with hexane, and the deacylated glycoprotein samples were dialyzed against distilled water and lyophilized.

Incubation System. The fatty acyltransferase assay mixtures contained the following components: acceptor glycoprotein, deacylated mucus glycoprotein, 100 μg, or deglycosylated and deacylated glycoprotein, 16 μg; [1-^{14}C]palmitoyl-CoA, 100 μM (300000 cpm); Triton X-100, 0.5%; NaF, 25 mM; dithiothreitol, 2 mM; ethanol, 0–2.0 M; imidazole hydrochloride buffer at pH 7.4, 100 mM; enzyme protein, 50–100 μg, in a final volume of 0.10 mL. The glycoprotein substrates and palmitoyl-CoA were prepared as concentrated stock solutions in 0.1 M imidazole hydrochloride buffer, pH 7.4. The tubes containing the complete incubation mixtures were briefly sonicated, and the reaction was initiated by the addition of enzyme preparation. Assays were run for 30 min at 37 °C, and the reactions were stopped by immersing the tubes for 5 min in a boiling water bath. Following sonication, the tubes were centrifuged, sediments were washed twice with 0.1 mL of incubation buffer, and the combined supernatant and wash from each assay were spotted on Whatman 3MM paper strips (2.5 × 20 cm). The ^{14}C-labeled glycoprotein products were separated from the components of the reaction mixtures by ascending chromatography in 1-butanol/acetic acid/water (5:2:3 v/v/v). After the paper strips were dried, the application zone (1.5 cm) was cut and washed twice, each time for 30 min, with 20% trichloroacetic acid, 5% trichloroacetic acid, ethanol, ethanol/diethyl ether (2:1 v/v), and diethyl ether (Baxter & Durham, 1979). The dried paper strips were than placed in vials containing liquid scintillation solution and counted in a scintillation counter. The enzyme activity was expressed as nanomoles of ^{14}C-labeled fatty acid transferred to glycoprotein per milligram of enzyme protein every 30 min. All experiments were performed in the presence of controls that contained the incubation mixture minus the glycoprotein acceptor or boiled enzyme in order to determine endogenous activity and any nonspecific adsorption. Incubations were performed in triplicate.

Product Identification. The incubates from 28 assay tubes containing the deacylated mucus glycoprotein and 31 tubes containing the deglycosylated and deacylated glycoprotein were combined separately and treated with 10 volumes of 20% trichloroacetic acid in 5% phosphotungstic acid. After 4 h at 4 °C, the precipitates were collected by centrifugation, washed with 5% trichloroacetic acid, and thoroughly delipidated by extraction with organic solvents (Slomiany et al., 1983, 1984a). The delipidated materials were then subjected to SDS–polyacrylamide gel electrophoresis and column chromatography. The ^{14}C-labeled glycoprotein formed in the presence of the deacylated mucus glycoprotein was combined with the cold gastric mucus glycoprotein and subjected to gel filtration on Bio-Gel A-50 column followed by CsCl density gradient centrifugation (Witas et al., 1983). Fractions containing ^{14}C-labeled mucus glycoprotein were pooled, dialyzed, lyophilized, and treated with 0.3 M ethanolic KOH at 37 °C for 30 min (Slomiany et al., 1983). Following acidification, the mixture was extracted with hexane, and the recovered ^{14}C-labeled fatty acid methyl esters were chromatographed on thin-layer plates. After chromatography, the zones corresponding to the position of authentic methyl palmitate were scraped into scintillation vials, suspended in liquid scintillation solution, and counted. The ^{14}C-labeled product formed in the presence of the deglycosylated and deacylated glycoprotein was electrophoresed on SDS–polyacrylamide gels. The gels were scanned for [^{14}C]palmitic acid, and the material contained in radioactive slices was recovered. Following dialysis and lyophilization such obtained ^{14}C-labeled protein was treated with methanolic KOH and analyzed for the liberated ^{14}C-labeled fatty acid methyl esters as described above.

Analytical Methods. The protein content of samples was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard. The phenol/H_2SO_4 method was

Table I: Amino Acid Composition of the Intact and Deglycosylated Rat Gastric Mucus Glycoprotein[a]

amino acid	mol/100 mol	
	intact	deglycosylated
Lys	4.7	4.2
His	2.9	2.6
Arg	3.1	3.5
Asp	6.8	7.3
Thr	18.2	17.5
Ser	12.6	13.2
Glu	9.8	9.0
Pro	10.1	11.6
Gly	7.3	6.5
Ala	6.2	6.2
Cys	1.8	1.5
Val	4.6	4.8
Met	0.9	0.6
Ile	3.1	3.4
Leu	4.5	4.7
Tyr	1.8	1.5
Phe	1.6	1.9

[a] Values represent the means of duplicate analyses performed on each sample.

Table II: Chemical Composition of the Deacylated Intact and Degraded Rat Gastric Mucus Glycoprotein[a]

component	relative weight (%)			
	glycoprotein	reduced and alkylated glycoprotein	glycopeptide	deglycosylated glycoprotein
fucose	9.8	9.6	10.5	
galactose	28.7	29.0	31.2	0.5
N-acetylglucosamine	17.2	17.1	18.6	1.2
N-acetylgalactosamine	14.5	14.7	15.0	3.4
sialic acid (as NeuAc)	6.3	6.5	7.1	
sulfate	3.4	3.2	3.5	
protein	14.6	14.3	11.2	87.5

[a] Values represent the means of triplicate analyses performed on each sample.

Table III: Mucus Glycoprotein Fatty Acyltransferase Activity in Subcellular Fractions of Rat Gastric Mucosa[a]

fraction	specific activity [nmol (mg of protein)$^{-1}$ (30 min)$^{-1}$]	
	mucus glycoprotein	deglycosylated glycoprotein
total homogenate	0.08 ± 0.02	0.07 ± 0.01
mitochondrial	0.20 ± 0.03	0.18 ± 0.02
microsomal	0.84 ± 0.07	0.81 ± 0.06
cytosol	0.02 ± 0.00	0.03 ± 0.00

[a] Incubation mixtures contained the following components in a final volume of 0.10 mL: 100 μg of deacylated mucus glycoprotein or 16 μg of deglycosylated and deacylated glycoprotein, 80 μM [1-^{14}C]palmitoyl-CoA, 100 μg of enzyme protein, 0.5% Triton X-100, 2 mM dithiothreitol, 25 mM NaF, and 100 mM imidazole hydrochloride buffer, pH 7.4. Enzyme activities were measured as described in the text. Each value represents the means ± SD of four separate experiments performed in triplicate.

used for monitoring the carbohydrate in column and density gradient fractions (Dubois et al., 1956). The content and composition of carbohydrates in various mucus glycoprotein preparations were determined by gas–liquid chromatography following methanolysis, re-N-acetylation, and derivatization with silylating reagent (Hughes & Clamp, 1972). A Beckman Model 120B analyzer was used for the analysis of amino acids. Samples were hydrolyzed for 22 h at 110 °C in 6 M HCl under a nitrogen atmosphere (Wu & Pigman, 1977). The ^{14}C-labeled fatty acid methyl esters were chromatographed on thin-layer plates developed in *n*-hexane/diethyl ether/acetic acid (90:10:1 v/v/v). Gel electrophoresis in 1% SDS was performed with 7.5% polyacrylamide gels (Laemmli, 1970). Samples of glycoprotein (150–200 μg) were incubated for 3 min at 100 °C in a sample buffer, pH 6.8, devoid of β-mercaptoethanol and then applied to the gels. After electrophoresis, gels were stained for protein with Coomassie brilliant blue and for carbohydrate by the periodate–Schiff method (Fairbanks et al., 1971). In some instances the Eastman Kodak's "Kodavue" electrophoresis visualization kit for protein was also used. Electroimmunoassays for albumin were performed according to the method of Laurell (1972). Various preparations of mucus glycoprotein and rat albumin standards were placed in the wells of agarose gel containing rabbit anti-rat albumin antiserum. Following electrophoresis, the agarose plates were dried and stained with Thiazine red reagent. Calibration curve was constructed from the heights of the albumin standard peaks.

Results

Application of the solubilized rat gastric mucus to a Bio-Gel A-50 column led to separation of mucus glycoprotein from the bulk of protein material. Following rechromatography, the glycoprotein was freed to the residue protein contaminants by equilibrium CsCl density gradient centrifugation. The purified mucus glycoprotein gave on a Bio-Gel A-50 column a well-defined peak that emerged just after the void volume (Figure 1A). SDS–polyacrylamide gel electrophoresis of this glycoprotein revealed a strong carbohydrate staining band at the origin, and no extract protein staining bands were detected. The results of electroimmunoassays, while showing the presence of albumin in the whole gastric mucus, did not reveal this serum protein in the purified glycoprotein (Figure 2). Such obtained undergraded mucus glycoprotein was used for preparation of various substrates for the assays of gastric mucosal fatty acyltransferase activity. Treatment of the purified mucus glycoprotein with β-mercaptoethanol followed by alkylation resulted in a reduced glycyprotein which on Bio-Gel A-50 column emerged in the included volume (Figure 1B). Deglycosylation of the undegraded mucus glycoprotein produced a protein which on SDS–polyacrylamide gel electrophoresis gave a band with an apparent molecular weight of 66 000 (Figure 3). Results of analysis revealed that the amino acid composition of this protein was similar to that of the intact glycoprotein (Table I). Both preparations contained amino acids characteristic to mucin-type glycoproteins. They were rich in threonine, serine, proline, glutamate, aspartate, and glycine and low in aromatic and sulfur-containing amino acids. Neither the reduced and alkylated glycoprotein nor the deglycosylated glycoprotein reacted with anti-rat albumin antiserum (Figure 2). The chemical compositions of the various substrate preparations are given in Table II.

Table III shows the distribution of fatty acyltransferase activity for the synthesis of acylated mucus glycoprotein in the various subcellular fractions of rat gastric mucosa. The enzyme activity for fatty acylation of the deacylated intact or deglycosylated mucus glycoprotein was located mainly in the microsomal fraction. This fraction contained from 45 to 50% of the total fatty acyltransferase activity of gastric mucosal homogenate and also had the highest specific activity. The fatty acyltransferase activity of the microsomal fraction was readily extractable with Triton X-100, and after centrifugation the solubilized enzyme contained 89–92% of the total acyltransferase activity. The specific activity of the solubilized enzyme for the acylation of the intact mucus glycoprotein was

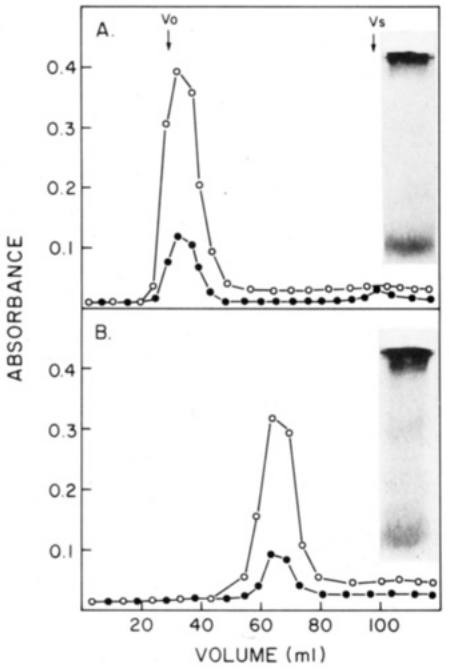

FIGURE 1: Bio-Gel A-50 column chromatography in 6 M urea–10 mM sodium phosphate buffer, pH 7.0, of the purified undegraded (A) and reduced (B) rat gastric mucus glycoprotein. The glycoprotein samples (10 mg each) were separately dissolved in 2 mL of the buffered 6 M urea and applied individually to a column (0.9 × 105 cm). Fractions of 2 mL were collected and monitored for neutral sugar (phenol/H_2SO_4 method) (○) and protein (absorbance at 280nm) (●). The insert in (A) shows the SDS–polyacrylamide gel electrophoresis of the undegraded mucus glycoprotein following deacylation with hydroxylamine. The insert in (B) shows the SDS–polyacrylamide gel electrophoresis of the mucus glycoprotein following reduction with β-mercaptoethanol. The glycoprotein samples were incubated at 100 °C for 3 min in a sample buffer, pH 6.8, devoid of β-mercaptoethanol and then applied (150–200 μg) to the gels. Following electrophoresis the gels were visualized with KODAVUE stain (minimum sensitivity of this stain for protein is 1–2 ng). In both cases (A and B), the glycoprotein gave a strong protein band at the top of the gels. The bands moving with the front of the gels represent the dissociated lipids.

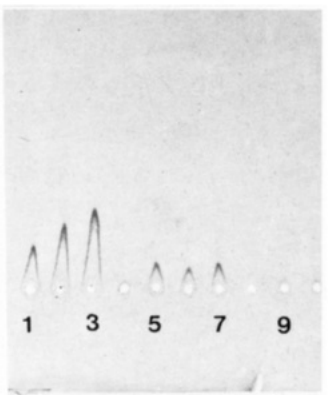

FIGURE 2: Electroimmunoassay of albumin in various preparations of rat gastric mucus glycoprotein using diluted rat serum albumin as standard (0.1, 0.2, and 0.4 μg/mL). Each well contained 1.5 μL of standard or sample solution. Wells 1–3, standard dilutions of albumin; well 4, purified undegraded mucus glycoprotein (20μg); wells 5–7, solubilized mucus used for glycoprotein preparation (20 μg); well 8, deacylated mucus glycoprotein (20 μg); well 9, deacylated reduced mucus glycoprotein (20 μg); well 10, deacylated deglycosylated mucus glycoprotein (12 μg).

only slightly (≃4%) higher than that for the acylation of the deglycosylated glycoprotein.

Figure 4 shows the effects of pH on the activity of mucus glycoprotein fatty acyltransferase. The pH optimum for acylation of mucus glycoprotein, in its deacylated or de-

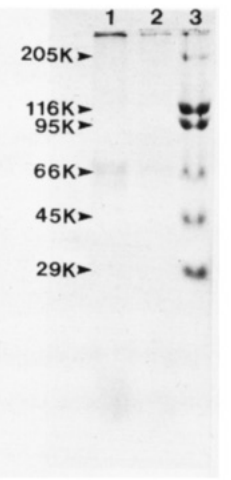

FIGURE 3: SDS–polyacrylamide gel electrophoresis of the gastric mucus glycoprotein following the deglycosylation with trifluoromethanesulfonic acid (1), and ^{14}C-labeled protein (2) purified from the fatty acyltransferase assay mixtures run in the presence of deglycosylated and deacylated glycoprotein. (3) Molecular weight standards. A 25–30-μg sample of the deglycosylated glycoprotein or ^{14}C-labeled protein, dissolved in the sample buffer devoid of β-mercaptoethanol, was applied to 7.5% SDS–polyacrylamide gels for electrophoresis. The gels were stained for protein with Coomassie blue. The bands on the top of all three gels including the marker gel (3) represent the material trapped in the sample gel.

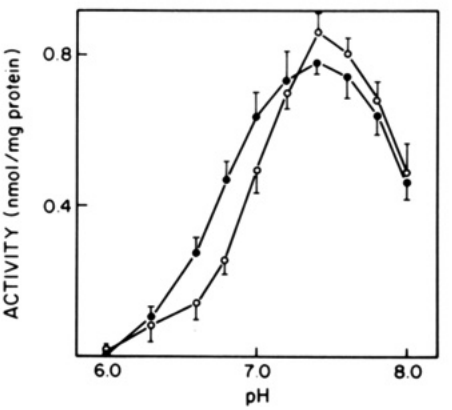

FIGURE 4: Effect of pH on gastric mucosa mucus glycoprotein fatty acyltransferase activity. Acceptor: deacylated mucus glycoprotein (○); deglycosylated and deacylated glycoprotein (●). The composition of the incubation mixtures was the same as in Table IV, except that a different pH buffer was used. The data show the mean ± SD of three experiments performed in triplicate.

glycosylated forms, with palmitoyl-CoA was 7.4. The fatty acyltransferase activity toward both glycoprotein substrates was stimulated by Triton X-100, NaF, and dithiothreitol, while $MgCl_2$ and $MnCl_2$ have inhibitory effects at the concentrations examined. The transferase activity was only slightly affected by ATP, NaCl, and EDTA (Table IV). Under optimal conditions, the rate of [^{14}C]palmitic acid transfer to both types of glycoprotein acceptors was proportional to microsomal enzyme protein concentration up to 200 μg and remained constant with time of incubation for at least 1 h. Addition of the deacylated mucus glycoprotein or the deglycosylated and deacylated glycoprotein to the incubation mixture produced 3–4-fold stimulation of the synthesis of ^{14}C-labeled glycoprotein (Table IV). No incorporation of palmitic acid to mucus glycoprotein acceptors was observed in the absence of enzyme or with the boiled enzyme. The transfer of palmitic acid into both glycoproteins was proportionally enhanced, over a given range with increase concentrations of the substrates, intact or deglycosylated mucus glycoprotein, and palmitoyl-

Table IV: Requirements for Gastric Mucosa Mucus Glycoprotein Fatty Acyltransferase[a]

incubation mixture	specific activity [nmol (mg of protein)$^{-1}$ (30 min)$^{-1}$]	
	mucus glycoprotein	deglycosylated glycoprotein
complete	0.83 ± 0.09	0.80 ± 0.07
minus acceptor glycoprotein	0.24 ± 0.03	0.22 ± 0.03
minus Triton X-100	0.36 ± 0.04	0.35 ± 0.04
minus NaF	0.32 ± 0.04	0.34 ± 0.03
minus dithiothreitol	0.27 ± 0.03	0.30 ± 0.04
minus Triton X-100 plus ethanol (0.1 M)	0.25 ± 0.04	0.27 ± 0.03
minus Triton X-100 plus ethanol (0.5 M)	0.18 ± 0.02	0.16 ± 0.02
minus Triton X-100 plus ethanol (1.5 M)	0.08 ± 0.01	0.04 ± 0.01
plus NaCl (20 mM)	0.80 ± 0.07	0.79 ± 0.09
plus $MgCl_2$ (10 mM)	0.58 ± 0.06	0.58 ± 0.07
plus $MnCl_2$ (10 mM)	0.55 ± 0.04	0.51 ± 0.05
plus ATP (10 mM)	0.82 ± 0.07	0.79 ± 0.08
plus EDTA (10 mM)	0.66 ± 0.07	0.60 ± 0.05

[a] The complete incubation mixture contained, in 0.10 mL, 100 μg of deacylated mucus glycoprotein or 16 μg of deglycosylated and deacylated glycoprotein, 100 μM [1-^{14}C]palmitoyl-CoA, 50 μg of enzyme protein, 0.5% Triton X-100, 2 mM dithiothreitol, 25 mM NaF, and 100 mM imidazole hydrochloride buffer, pH 7.4. Following incubation at 37 °C for 30 min, the activity of mucus glycoprotein fatty acyltransferase was assayed. Values represent the means ± SD of two to five separate experiments performed in triplicate.

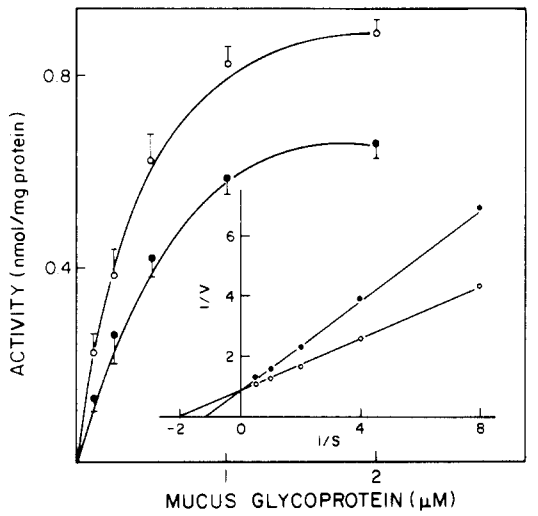

FIGURE 5: Effect of glycoprotein concentration on mucus glycoprotein fatty acyltransferase activity in the absence (○) and in the presence (●) of 0.1 M ethanol. The composition of the incubation mixtures was the same as in Table IV, except that varied concentrations (10–200 μg) of deacylated glycoprotein substrate were used. The data show the mean ± SD of four experiments performed in triplicate. $1/S$ = (μM)$^{-1}$ and $1/V$ = (nmol of palmitic acid transferred) (mg of protein)$^{-1}$ (30 min)$^{-1}$.

CoA. The apparent K_m value for the deacylated mucus glycoprotein, based on the molecular weight of 1.0×10^6 (Decaens et al., 1981), calculated from Lineweaver–Burk plots was 0.45 μM (Figure 5). When the acyltransferase assays were performed in the presence of the deglycosylated and deacylated glycoprotein, molecular weight of which was estimated to be 66 000 (Figure 3), the apparent K_m for this glycoprotein was 0.89 μM (Figure 6).

Figure 7 shows the effect of ethanol concentration on the activity of gastric mucosa fatty acyltransferase. The results with both glycoprotein acceptors indicated that the introduction of ethanol to the incubation mixtures resulted in the reduction of the rate of glycoprotein acylation. The rate of inhibition

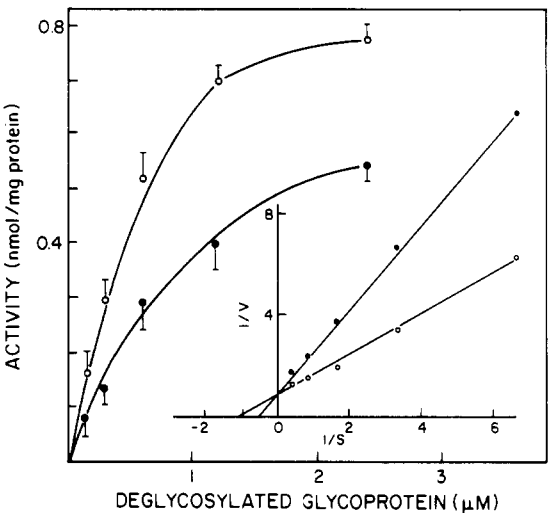

FIGURE 6: Effect of deglycosylated glycoprotein concentration on the fatty acyltransferase activity in the absence (○) and in the presence (●) of 0.2 M ethanol. The composition of the incubation mixtures was the same as in Table IV, except that varied concentrations (1–32 μg) of deglycosylated and deacylated mucus glycoprotein substrate were used. The data show the mean ± SD of three separate experiments performed in triplicate. $1/S$ = (μM)$^{-1}$ and $1/V$ = (nmol of palmitic acid transferred) (mg of protein)$^{-1}$ (30 min)$^{-1}$.

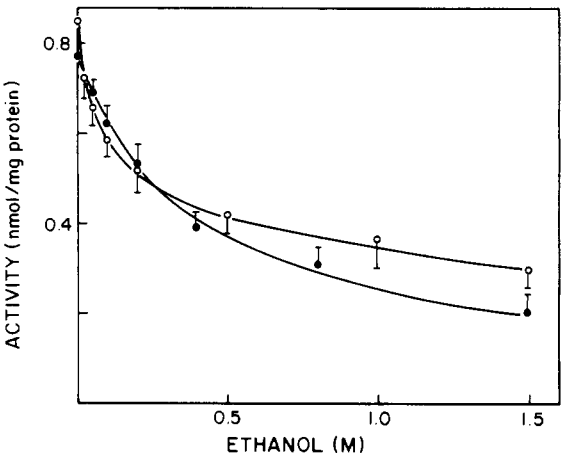

FIGURE 7: Effect of ethanol on the activity of rat gastric mucosa mucus glycoprotein fatty acyltransferase. Acceptor: deacylated mucus glycoprotein (○); deglycosylated and deacylated glycoprotein (●). The composition of the incubation mixtures was the same as in Table IV, except that varied concentrations (0–1.5 M) of ethanol were included. The data show the mean ± SD of four experiments performed in triplicate.

was proportional to the ethanol concentratioin up to 0.4 M but deviated from linearity at higher concentrations. From the results of parallel experiments in which the microsomal enzyme was preincubated (1 h at 37 °C) with ethanol prior to the addition of acceptor glycoprotein, it would appear that ethanol at low concentrations (up to 0.5 M) does not exert additional deterrent effect on the fatty acyltransferase activity (Table V). Higher concentrations of ethanol (1.0 M and above), however, caused a substantial reduction in the transferase activity. This indicated that ethanol at higher concentrations caused denaturation of the enzyme protein. The detrimental effect of high ethanol concentrations on fatty acyltransferase activity appeared somewhat more severe in the absence of Triton X-100 (Table IV). Under optimal conditions, the inhibition of glycoprotein acylation caused by ethanol at concentrations up to 0.4 M was of the competitive type. The K_I values calculated from the double-reciprocal plots were 0.80 μM for the deacylated mucus glycoprotein (Figure 5) and 1.82

Table V: Effect of Preincubation with Ethanol on the Activity of Gastric Mucosa Mucus Glycoprotein Fatty Acyltransferase[a]

ethanol concn (M)	specific activity: control [nmol (mg of protein)$^{-1}$ (30 min)$^{-1}$]	specific activity: following preincubation [nmol (mg of protein)$^{-1}$ (30 min)$^{-1}$]	% of control
0	0.83 ± 0.10	0.82 ± 0.08	98.8
0.1	0.58 ± 0.07	0.59 ± 0.05	101.7
0.5	0.42 ± 0.5	0.42 ± 0.04	100.0
1.0	0.37 ± 0.4	0.34 ± 0.04	91.9
1.5	0.30 ± 0.3	0.25 ± 0.3	83.3
2.0	0.24 ± 0.3	0.16 ± 0.2	67.5

[a] The assay mixtures contained, in 0.10 mL, 100 μg of deacylated mucus glycoprotein, 100 μM [1-^{14}C]palmitoyl-CoA, 50 μg of enzyme protein, 0–2.0 M ethanol, 0.5% Triton X-100, 2 mM dithiothreitol, 25 mM NaF, and 100 mM imidazole hydrochloride buffer, pH 7.4. Preincubation of the enzyme with ethanol was carried out at 37 °C for 1 h at pH 7.4. Values represent the means ± SD of three separate experiments performed in triplicate.

Table VI: Distribution and Substrate Specificity of Gastric Mucosa Mucus Glycoprotein Fatty Acyltransferase[a]

acceptor	concn (μg)	specific activity [nmol (mg of protein)$^{-1}$ (30 min)$^{-1}$]: antrum	specific activity [nmol (mg of protein)$^{-1}$ (30 min)$^{-1}$]: body
deacylated mucus glycoprotein	100	0.80 ± 0.07	0.85 ± 0.08
deacylated mucus glycoprotein following reduction and alkylation	100	0.72 ± 0.08	0.73 ± 0.10
deglycosylated and deacylated glycoprotein	16	0.77 ± 0.06	0.83 ± 0.09
deacylated glycopeptide	90	0.09 ± 0.01	0.07 ± 0.01

[a] The conditions of the assay were the same as described in Table II, except that microsomal enzyme was prepared from gastric mucosa of antrum and body. Each value represents the means ± SD of three separate experiments performed in triplicate.

μM for the deglycosylated and deacylated glycoprotein (Figure 6).

The distribution of fatty acyltransferase activity for the acylation of mucus glycoprotein in antral and body mucose of rat stomach is given in Table VI. The data indicated that both areas of the stomach exhibit essentially similar enzyme activity. The antrum and body enzyme preparations also exhibited similar substrate specificity. The microsomal fatty acyltransferase exhibited high specificity for the deacylated intact or deglycosylated mucus glycoprotein, but the deacylated glycopeptide failed to serve as acceptor of the palmitic acid from palmitoyl-CoA (Table VI). Treatment of the deacylated mucus glycoprotein with β-mercaptoethanol followed by alkylation with iodoacetamide caused only a 10–14% decrease in the ability of S-carboxylmethylated glycoprotein to act as acceptor for the palmitate in the transferase reaction.

When the deacylated mucus glycoprotein was used as a substrate, the synthesized ^{14}C-labeled glycoprotein cochromatographed on Bio-Gel A-50 with the intact rat gastric mucus glycoprotein and on CsCl density gradient centrifugation gave a single band at the density of 1.53 in which carbohydrate peak coincided with that of the ^{14}C label (Figure 8). SDS–polyacrylamide gel electrophoresis of the delipidated product formed in the presence of the deglycosylated and deacylated glycoprotein revealed one major [^{14}C]palmitic acid labeled band which corresponded in its migration to that of the deglycosylated mucus glycoprotein (Figure 9). This ^{14}C-labeled protein material was found to have an apparent molecular weight of 66000 (Figure 3). Treatment of the purified ^{14}C-labeled products with methanolic KOH followed

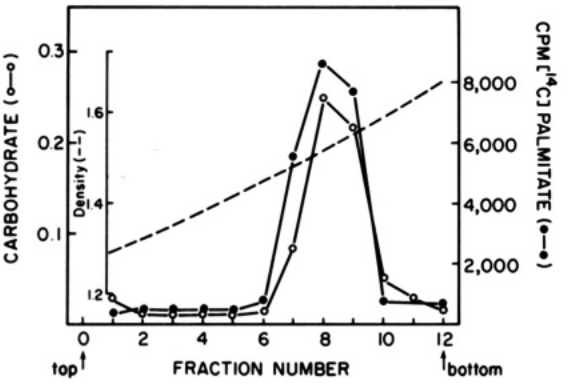

FIGURE 8: Cesium chloride density gradient centrifugation of ^{14}C-labeled glycoprotein synthesized in vitro in the presence of deacylated mucus glycoprotein substrate. The ^{14}C-labeled glycoprotein combined with 5 mg of cold rat gastric mucus glycoprotein was dissolved (2 mg/mL) in the phosphate buffer, pH 7.0, containing CsCl at a loading density of 1.42 g/mL and centrifuged for 48 h at 46000 rpm. Fractions of 1 mL were collected and monitored for carbohydrate (O) and [^{14}C]palmitate (●).

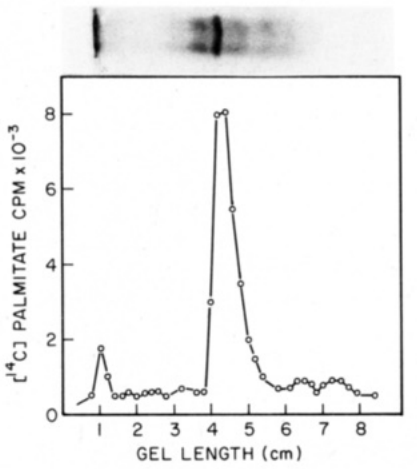

FIGURE 9: SDS–polyacrylamide gel electrophoresis of ^{14}C-labeled product formed in vitro in the presence of deglycosylated and deacylated glycoprotein. The ^{14}C-labeled protein combined with 50 μg of cold deglycosylated rat gastric mucus glycoprotein was incubated at 100 °C for 3 min in a sample buffer, pH 6.8, devoid of β-mercaptoethanol and then applied (20–40 μg) to the gels for electrophoresis. The top panel shows the gels stained for protein with Coomassie blue, and the bottom shows the scan of the ^{14}C label.

by hexane extraction resulted in both cases in the liberation of the label to the hexane phase. This hexane extractable ^{14}C-labeled material cochromatographed on thin-layer plates with the methyl palmitate standard and accounted for 92–97% of the [^{14}C]palmitate incorporated to mucus glycoprotein acceptors in the in vitro system.

Discussion

Gastric complications following ingestion of ethanol are well recognized. This commonly abused intoxicant increases the permeability of gastric mucosa to back-diffusion by H^+, affects the process of active ion transport and secretion, decreases the thickness of the mucus layer, depletes the mucus content of the lining epithelial cells, and causes damage to the mucosa (Pitchumoni & Glass, 1977; Glass et al., 1979; Slomiany et al., 1979; Puurunen, 1982). The constituent that plays a primary role in the maintenance of the gastric mucosal integrity is a mucus glycoprotein. The protective qualities of this densely glycosylated, high molecular weight glycoprotein depend on its polymeric structure and the interaction with other constituents of the mucus gel (Allen, 1981; Witas et al., 1983). The ability of mucus glycoprotein for this interaction

is directly related to the features acquired by the glycoprotein during its posttranslational modifications such as glycosylation, sulfation, and the recently recognized process of acylation with fatty acids (Schachter & Williams, 1982; Slomiany et al., 1983, 1984a). In the light of our findings that the covalently bound fatty acids play an important role in the preservation of the polymeric structure of the mucus glycoprotein which is crucial for the protection of gastric epithelium, we have investigated the effect of ethanol on the acylation of this glycoprotein with fatty acids.

In the previous study, it was shown that ethanol has a detrimental effect on the fatty acid acylation of mucus glycoprotein by gastric mucosal cells in culture (Takagi et al., 1984b). The results of this work show that ethanol affects adversely the in vitro acylation of gastric mucus glycoprotein with fatty acids by inhibiting the enzyme activity which catalyzes the transfer of palmitic acid from palmitoyl-CoA to the glycoprotein. The data indicate that this detrimental effect of ethanol on the fatty acyltransferase activity is dose dependent and occurs below and above its isosmotic concentration to plasma. At the isosmotic concentration (1.7%) ethanol caused about a 47% decrease in transferase activity, while its maximal effect occurred at concentrations of 6.9–9.2% where up to 75% activity was lost. From the preincubation experiments it was ascertained that the loss of fatty acyltransferase activity at low concentrations of ethanol (up to 0.5 M) was due to its inhibitory effect, whereas the high concentrations of ethanol (1.0 M and above) caused denaturation of the enzyme protein. As ethanol concentration in the stomachs of moderate drinkers often reaches values of 5% or more (Glass et al., 1979), the inhibitory effect on the fatty acyltransferase activity noted here in vitro even with low doses of ethanol could also be detrimental to gastric mucosa in vivo. It should also be borne in mind that, in addition to this inhibitory effect, ethanol is known to diffuse rapidly across the gastric epithelium causing damage to the morphology of the mucosa, and to affect the secretion of acid and pepsin (Glass et al., 1979; Fromm, 1981; Puurunen, 1982).

This study, besides demonstrating the inhibitory effect of ethanol on the mucus glycoprotein fatty acyltransferase, also provides information on the substrate specificity of the enzyme. Since both the intact and deglycosylated preparations of the deacylated mucus glycoprotein had similar acceptor capacities, it appears that enzymatic acylation of mucus glycoprotein with fatty acids precedes or occurs simultaneously with glycosylation. The fact that the proteolytically degraded and deacylated mucus glycoprotein failed to serve as acceptor of the palmitic acid from palmitoyl-CoA suggests that the fatty acyltransferase requires for its activity the nonglycosylated protease-susceptible regions of the glycoprotein. That fatty acids in gastric mucus glycoprotein are ester linked to these nonglycosylated polypeptide regions has been recently demonstrated (Slomiany et al., 1984a); however, the nature of the group to which they are attached is not known. Studies with other proteins containing covalently bound fatty acids indicate the possibilities of the involvement of hydroxyl groups of serine and threonine, or thiol group of cysteine (Cockle et al., 1980; Schmidt, 1982). In an attempt to provide evidence for the type of ester bond in gastric mucus glycoprotein, we have performed the assays of fatty acyltransferase activity using the deacylated β-mercaptoethanol-reduced and S-carboxymethylated mucus glycoprotein as acceptor of palmitic acid from palmitoyl-CoA. The results indicated that the acceptor capacity of such modified glycoprotein was only 10–14% lower than that of the deacylated undegraded mucus glycoprotein. This would suggest that in gastric mucus glycoprotein the fatty acids are linked through hydroxyl esters, but the possibility that some fatty acids may be linked through thiol esters cannot be ruled out.

Another feature furnished by this investigation is the data on the distribution of mucus glycoprotein fatty acyltransferase activity in anatomically distinct and functionally different areas of the stomach. Both antral and body mucosa exhibited similar enzyme activities and substrate specificities. Studies now in progress in our laboratory indicate that fatty acyltransferase activity is also displayed by human gastric mucosa. Measurements of this enzyme activity may provide some insight into the changes taking place in the mucus gel in gastropathy.

Registry No. Acyltransferase, 9054-54-0; ethanol, 64-17-5.

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Fatty Acids Bound to Unilamellar Lipid Vesicles as Substrates for Microsomal Acyl-CoA Ligase†

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ABSTRACT: Palmitate incorporated into single-layered vesicles of phosphatidylcholine was used as a substrate for palmitoyl coenzyme A ligase (palmitoyl-CoA ligase) in microsomes from rat liver. This was done in order to avoid the use of detergents for dispersal of the water-insoluble palmitate and the possibility of precipitating palmitate added to the aqueous assay as a salt suspension. The activity of the ligase measured when palmitate was added to assays as a component of phospholipid vesicles was 10–40-fold greater vs. activities reported in the literature using other methods for adding fatty acids to the assay system. Phospholipids, however, had no direct effect on the activity of palmitoyl-CoA ligase. The data indicate, therefore, that the activity of this enzyme has been underestimated because of the manner in which fatty acid was added to the assay, which has a significant effect on the activity of the ligase. It is shown too that the rate of spontaneous transfer of palmitate from unilamellar vesicles of phosphatidylcholine to microsomes via a hydrated intermediate is far more rapid than the inherent catalytic activity of the fatty acyl-CoA ligase. The data also suggest that the membrane-associated pool of fatty acid and not fatty acid in the aqueous phase of the assay is the pool of substrate interacting with the ligase.

The substrates of many enzymes that are integral components of biological membranes have exceedingly limited solubility in water. These substrates will partition selectively into the lipid phases of membranes, and concentrations in membranes will be high whereas concentrations in water will be low. The implications of the insolubility in water of substrates for the function of membrane-bound enzymes have not been examined in a systematic manner. It is unknown, therefore, whether the selective partitioning of substrates into membranes is important for function of membrane-bound enzymes generally or in certain specific cases. Lack of knowledge on this point seems related in part to the manner in which assays for the activity of membrane-bound enzymes are carried out when the substrates are insoluble in water. These substrates usually are added to assays as complexes with proteins containing nonspecific sites for binding of hydrophobic compounds, or they are added as salt suspensions [e.g., see Aas (1971), Farstad et al. (1967), Pande & Mead (1968), and Pande (1972)]. Detergents are added to assays in some instances for the purpose of dispersing the water-insoluble substrates (Bar-Tana et al., 1971; Tanaka et al., 1979; Suzue & Marcel, 1972). The data are treated as if substrates added by these methods distribute homogeneously in the assay system.

The first reaction in the utilization of fatty acids in cells is synthesis of coenzyme A (CoA) derivatives, which is catalyzed by a long-chain acyl-CoA ligase (EC 6.2.1.3). This enzyme is an integral component of the endoplasmic reticulum of cells. The natural substrates for this enzyme have exceedingly low solubilities in water. The fatty acyl-CoA ligase is assayed routinely by adding fatty acid substrates as salt suspensions to a suspension of microsomes in aqueous buffers (Bar-Tana et al., 1971). Assay systems usually contain detergents, presumably to aid in dispersing fatty acids, even though it is reported that detergents inhibit the activity of the fatty acid acyl-CoA ligase (Pande & Meade, 1968). In the present experiments, we describe an assay for this enzyme in which fatty acids are added to the assay as integral components of unilamellar vesicles of phosphatidylcholine. The data presented show that there are several advantages to an assay system in which fatty acids are introduced in this manner as compared with previous systems. The data appear also to have important implications for thinking about mechanisms for the intracellular movement of water-insoluble substrates of membrane-bound enzymes.

Materials and Methods

Scintillation liquid (Liquescint) was purchased from National Diagnostics. All other chemicals were obtained from Sigma Chemical Co. [^{14}C]Palmitate and [^{14}C]stearate were obtained from New England Nuclear. Microsomes were prepared from male Wistar rat livers (3–4 months old) (Pande & Meade, 1968). Microsomes were suspended in 0.25 M sucrose and stored at −80 °C until used. Fatty acid binding protein was partially purified from male Sprague-Dawley rats weighing 300–400 g (Ockner & Manning, 1982). Microsomal protein was determined by the Lowry method (Lowry & Lopez, 1946). Liposomes were made from egg phosphatidylcholine (Sigma, type III in hexane). After removal of

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