TUNICAMYCIN INHIBITION OF POLYISOPRENYL N-ACETYLGLUCOSAMINYL PYROPHOSPHATE FORMATION IN CALF-LIVER MICROSOMES[1]

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SUMMARY. Calf-liver rough microsomes incorporated N-acetyl[^{3}H] glucosamine from UDP-[^{3}H]GlcNAc or [^{14}C]mannose from GDP-[^{14}C]Man into products soluble in chloroform-methanol (2:1). The [^{3}H]GlcNAc-labeled material had the hydrolytic and chromatographic properties of a polyisoprenyl N-acetylglucosaminyl pyrophosphate whereas the ^{14}C-mannolipid exhibited the characteristics of a polyisoprenyl mannosyl phosphate. Tunicamycin inhibited the production of the [^{3}H]GlcNAc-lipid but did not affect the synthesis of the ^{14}C-mannolipid. This inhibition accounts for the effect of the antibiotic upon glycoprotein formation in vivo since polyprenyl sugar phosphates are intermediates in the biosynthesis of certain glycoproteins.

Tunicamycin is a glucosamine-containing antibiotic (1) that interferes with glycoprotein biosynthesis in mammalian (2) and microbial cells (3, 4). Treatment of yeast protoplasts with the antibiotic rapidly stops the formation of exported glycoproteins such as invertase, acid phosphatase and mannan peptides but does not inhibit the production of intracellular non-glycosylated proteins (e.g., α-glucosidase) or wall polysaccharides, such as glucan or chitin, which are not covalently associated with protein (4, 5). TM does not exert its effect on protoplasts by blocking the uptake of mannose (4) or glucosamine, the two sugars that

[1]Abbreviations: TM, tunicamycin; UDP, uridine diphosphate; GDP, guanosine diphosphate; GlcNAc, N-acetylglucosamine; Man, mannose; CM, chloroform-methanol (2:1, v/v); tlc, thin-layer chromatography. All sugars mentioned in this report are in the D configuration.

comprise the polysaccharide portions of the exported glycoproteins, or by inhibiting the formation of UDP- GlcNAc (Kuo and Lampen, unpublished results).

This evidence suggests that TM interferes directly with the glycosylation reactions leading to the formation of Man- and GlcNAc-containing glycoproteins. Since polyisoprenyl sugar phosphates function as sugar donors in these reactions (6, 7, 8), we have investigated the influence of TM on the synthesis of the polyisoprenyl derivatives of Man and GlcNAc.

MATERIALS AND METHODS. Tunicamycin (lot T-11-05) was supplied by G. Tamura, Dept. of Agricultural Chemistry, University of Tokyo, Japan. GDP-[^{14}C]Man (160 mCi/mmole) and UDP-[^{3}H]GlcNAc (6.6 Ci/mmole) were purchased from New England Nuclear Corp., Boston, Mass. Whatman microgranular DEAE-cellulose (DE-52, H. Reeve Angel and Co., Clifton, N. J.) was converted to the acetate form as described by Rouser et al. (9). Silica gel (60 - 200 mesh) was obtained from J. T. Baker Co., Phillipsburgh, N. J. Other chemicals and solvents were reagent grade commercial products.

Microsomes were prepared from calf liver by the procedure of Tetas et al. (10) and further fractionated into smooth and rough microsomes as described previously (11). The rough microsomes were washed by gentle homogenization in 10 mM Tris-maleate buffer, pH 7.5, containing 0.1 M KCl, followed by centrifugation at 106,000 x g_{max} for 60 min. Washed pellets were stored at -70 C.

Incubation mixtures containing 200 µl rough microsomes (3.35 mg protein) suspended in 50 mM Tris-maleate buffer, pH 7.5, 5 µl 0.5 M $MnCl_2$, 10 µl GDP-[^{14}C]Man (40,000 cpm) or UDP-[^{3}H]GlcNAc (70,000 cpm), 30 µl H_2O, and 5 µl 50 mM NaOH with or without 1.25 µg TM were incubated at 30 C for various intervals. Labeled lipids were extracted directly by the addition of 1.0 ml-quantities of chloroform-methanol (2:1, v/v) followed by thorough mixing. After phase separation, the aqueous phases were discarded, and the organic phases were washed three times with 0.5 ml portions of methanol-water (1:1, v/v). To obtain CM-extracts containing sufficient radioactivity to permit further characterization of the labeled components, larger reaction mixtures (with H_2O replacing the NaOH and TM) were prepared and processed maintaining the volume ratios given.

Protein was estimated by the Hartree modification of the Lowry technique (12) with bovine serum albumin as standard. For radioactivity measurements, liquid samples were quantitatively transferred to counting vials, and the solvent was removed by evaporation. The residues were suspended in 10-ml portions of Aquasol (New England Nuclear Corp.) and the ^{3}H or ^{14}C determined with a Packard liquid scintillation spectrometer (Model 3320, Packard Instrument Co., Inc., Downers Grove, Ill.).

Thin-layer chromatography was performed on Merck precoated plates of Silica Gel G (0.25 mm thick, without fluorescence indicator, Brinkmann Instruments, Inc., Westbury, N.Y.) in chloroform-methanol-water (60:25:4, solvent A or 60:35:6, solvent B). ^{14}C-Labeled components were located by radioautography with Kodak Blue-Sensitive medical X-ray film (SB-54); ^{3}H-products were detected either by solid scintillation fluorography (13) using the same x-ray film or by scraping 0.5 cm bands of silica gel from the tlc plate into counting vials and measuring the radioactivity by liquid scintillation in Aquasol. Whatman no. 1 paper was employed for descending chromatography with ethyl acetate-pyridine-water (8:2:1, solvent C) or n-butanol-pyridine-water (6:4:3, solvent D). Authentic Man and GlcNAc were detected with alkaline silver nitrate (14), and the ^{14}C-labeled material was located with a Packard Radiochromatogram Scanner, Model 7201. Chromatograms bearing ^{3}H-labelled material were cut into 1 cm strips, and the radioactivity was measured by liquid scintillation after conversion of the labeled product to $^{3}H_2O$ in a Packard Sample Oxidizer, Model 306.

RESULTS. Incubation of calf-liver rough microsomes with either UDP-[^{3}H]GlcNAc or GDP-[^{14}C]Man at pH 7.5 in the presence of 10 mM Mn^{2+} led to the rapid incorporation of radioactivity into CM-soluble products (Fig. 1A and 1B). CM-extracts from 15-minute incubations were used for characterization of the labeled materials. Examination of the ^{14}C-labeled extract by tlc in solvent A revealed the presence of a single radioactive compound that had an R_f of 0.25 and that migrated with calf-pancreas dolichyl[^{14}C]mannosyl phosphate prepared as described previously (11). One radioactive component with an R_f of 0.15 was found when the ^{3}H-labeled extract was subjected to tlc in solvent B.

As shown in Table 1, treatment of the CM-extracts under mild alkaline conditions which bring about the deacylation of glycosyl diglycerides did not convert the labeled lipids to radioactive water-soluble products. In contrast, both radioactive compounds were rapidly degraded by mild acid hydrolysis (Fig. 2A and 2B); incubation at 80°C in 50% n-propanol containing 0.01 N HCl converted 90 to 95% of the radioactivity to water-soluble material within 20 min. Upon paper chromatography (solvent D) of the ^{3}H-labeled material released in that interval, a single radioactive component that

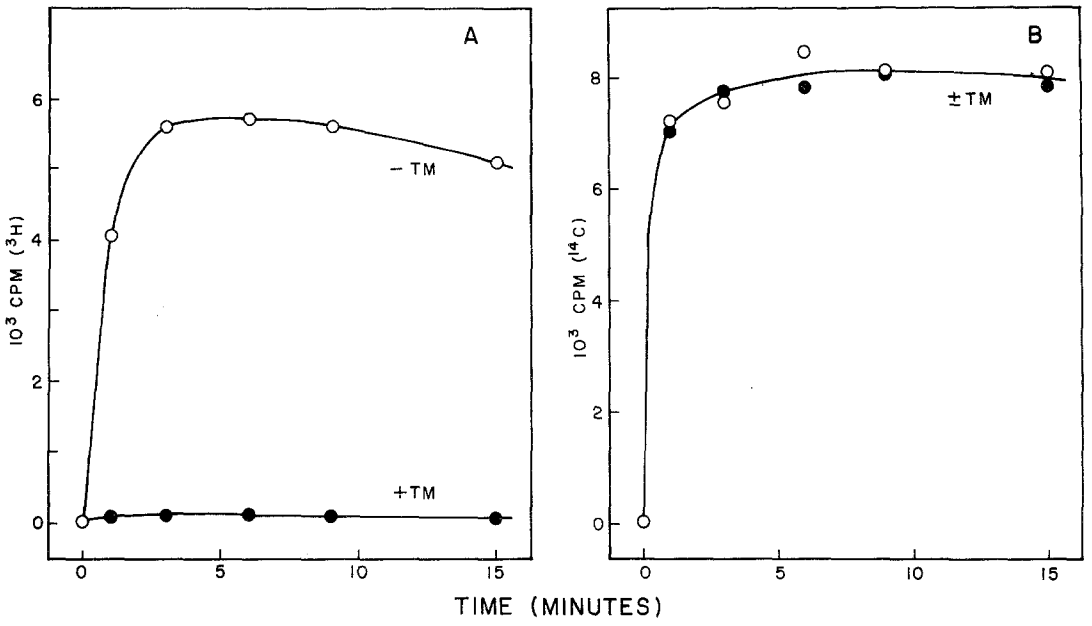

FIG. 1. Incorporation of radioactivity from UDP-[^{3}H]GlcNAc (A) and GDP-[^{14}C]Man (B) into CM-soluble products in the presence (---●---●---) or absence (---O---O---) of tunicamycin (5 μg/ml). Each point represents the average of duplicate determinations. Reaction mixtures containing boiled microsomes were processed to obtain the zero-time values.

migrated with authentic GlcNAc was found. Similarly, 20-minute mild acid hydrolysis of the ^{14}C-labeled lipid yielded one radioactive water-soluble compound that behaved as mannose during chromatography in solvent C.

When the CM-extracts were subjected to silicic acid column chromatography, the labeled lipids were not eluted by acetone which removes neutral glycolipids (9), but were recovered in the chloroform-methanol eluate along with phosphatides (Table 2). The acidic character of the radioactive lipids was also evident by their behavior during DEAE-cellulose chromatography (Table 2); both lipids remained bound to the column packing in chloroform-methanol-water (10:10:3) but were eluted when ammonium

TABLE 1. Stability of the radioactive lipids to mild alkaline alcoholysis [a]

Sample	Percent of Recovered Radioactivity	
	In the Aqueous Phase	In the Organic Phase
[^{3}H]GlcNAc-labeled CM extract		
before alcoholysis	5.2	94.8
after alcoholysis	3.8	96.2
[^{14}C]Man-labeled CM extract		
before alcoholysis	1.4	98.6
after alcoholysis	3.4	96.6

[a] The procedure of Lahav et al. (15) was employed with the exception that the incubation of lipids in alkali was extended to 30 min. Controls not exposed to alkali were included for comparison.

formate was added to the solvent. The [^{14}C]-mannolipid was released at a salt concentration of 1 mM whereas the [^{3}H]GlcNAc-compound was obtained with 20 mM ammonium formate.

The effect of TM on the formation of the radioactive lipids is shown in Fig. 1. At a level of 5 μg/ml (ca. 6 μM), the antibiotic completely inhibited the formation of the [^{3}H]GlcNAc-labeled compound but did not affect the synthesis of the ^{14}C-mannolipid.

DISCUSSION. In addition to sphingo- and glycero-glycolipids, mammalian tissues produce glycosylated derivatives of dolichol, a linear polyprenyl

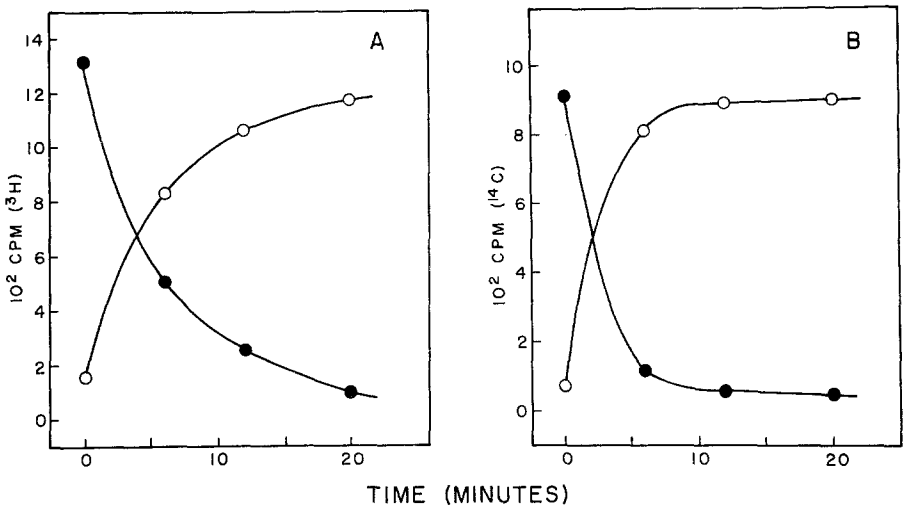

FIG. 2. Mild acid hydrolysis of the [^{3}H]GlcNAc-labeled lipids (A) and the [^{14}C]Man-labeled lipid (B). Lipid residues obtained by evaporation under N_2 were dissolved in 100 µl portions of 50% n-propanol containing 0.01 N HCl. After heating the mixtures to 80° C for the intervals indicated, the samples were neutralized with 100 µl quantities of 50% n-propanol containing 0.01 N NaOH and thoroughly mixed with 200 µl H_2O, 300 µl methanol and 800 µl chloroform. Upon phase separation, the aqueous phases were transferred to counting vials, and the organic phases were washed with 200 µl portions of methanol-water (1:1, v/v). The washes were combined with the appropriate aqueous extracts, and the organic phases were placed in counting vials. The radioactivity in the aqueous (---O---O---) and organic (---●---●---) phases was measured as described in Materials and Methods.

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alcohol that has a saturated isoprenyl residue at the hydroxy terminus. These derivatives are stable to mild alkaline conditions under which the esterified fatty acids in glycosyl diglycerides are released and, unlike sphingo-glycolipids, are rapidly degraded in dilute acid (6). Instability in acid exists because the saccharide moieties are bound to the polyprenyl chains via phosphodiester or pyrophosphate linkages.

Beyond the hydrolytic differences, the chromatographic properties of the dolichyl derivatives distinguish them from neutral

TABLE 2. Column chromatography of the radioactive lipids [a]

Chromatographic Medium and Procedure	[^{3}H]GlcNAc-Labeled CM Extract		[^{14}C]Man-Labeled CM Extract	
	CPM Eluted	Percent Recovery	CPM Eluted	Percent Recovery
Silicic acid eluted with:				
chloroform	14	0.4	7	0.3
acetone	34	1.0	22	1.0
chloroform-methanol	2800	86.5	1900	89.4
Total		87.9		90.7
DEAE-cellulose eluted with chloroform-methanol-water (10:10:3) plus				
no ammonium formate	126	3.9	21	0.8
1 mM ammonium formate	43	1.3	2390	90.6
20 mM ammonium formate	2510	77.4	140	5.3
Total		82.6		96.7

[a] The CM-extracts were dried under N_2, and the residues were dissolved in chloroform for silicic acid chromatography or chloroform-methanol-water (10:10:3) for DEAE-cellulose column chromatography. Four-ml columns of silicic acid were prepared in chloroform, charged with lipids, and eluted successively with 5 bed volumes of chloroform, 5 bed volumes of acetone, and 10 bed volumes of chloroform-methanol (1:1, v/v). A slurry of DEAE-cellulose (acetate form) in chloroform-methanol-water (10:10:3) was used to prepare 4-ml columns. After the application of the lipids, the columns were eluted with 2.5 bed volumes of chloroform-methanol-water (10:10:3) followed by 2.5 bed volumes each of 1 mM and 20 mM ammonium formate in the same solvent.

glycolipids. Thus, on silicic acid column chromatography, neutral glycolipids are eluted with acetone (9), whereas the dolichyl sugar phosphates elute with chloroform-methanol (1:1) along with other phosphatides. Behrens et al. (16) have shown that dolichyl phosphodiester compounds may be differentiated from the corresponding pyrophosphates by chromatography on DEAE-cellulose in chloroform-methanol-water (10:10:3) containing ammonium formate; elution of derivatives having single phosphate residues occurs at salt concentrations of 1 - 3 mM, but those with two phosphate residues remain bound until the salt concentration is raised to 8 - 10 mM.

The [^{14}C]Man- and [^{3}H]GlcNAc-labeled lipids formed in calf-liver rough microsomes were identified as polyisoprenyl derivatives on the basis of their resistance to mild alkaline treatment (Table 1), susceptibility to mild acid hydrolysis (Fig. 2), and behavior on silicic acid column chromatography (Table 2). Paper chromatography of the radioactive products of mild acid hydrolysis indicated that the saccharide moiety of each glycolipid consisted of a single sugar residue, and the results of column chromatography on DEAE-cellulose (Table 2) suggest that the sugar was attached through a phosphodiester linkage in the [^{14}C]Man-lipid^{2} and a pyrophosphate linkage in the [^{3}H]GlcNAc-lipid. Further support for the conclusion that the ^{14}C-mannolipid from calf liver is a polyisoprenyl mannosyl phosphate was provided by the finding that it had a mobility on tlc identical to that of calf pancreas dolichyl [^{14}C] mannosyl phosphate. The detailed characterization of a polyisoprenyl mannosyl phosphate from bovine liver (18) and the formation of polyisoprenyl N-acetylglucosaminyl pyrophosphate in particulate preparations from rabbit and rat liver (10, 19, 20, 21) have been reported previously.

Evidence from several laboratories suggests that polyisoprenyl sugar phosphates participate in the biosynthesis of Man- and GlcNAc-containing glycoproteins (6, 7, 8), and the experiments reported here have shown that tunicamycin prevents the formation of polyisoprenyl N-acetyl-glucosaminyl pyrophosphate. Although this reaction may not be the only GlcNAc transfer reaction affected by the antibiotic, its inhibition accounts for the action of TM on glycoprotein synthesis in vivo.

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[2]Hsu et al. (8) and Ghalambor et al. (17) have reported that dolichyl mannosyl phosphate is eluted from DEAE-cellulose by chloroform-methanol-water (10:10:3); however, under the experimental conditions employed here, both the calf-liver ^{14}C-mannolipid and authentic dolichyl [^{14}C]mannosyl phosphate (provided by C.D. Warren, Mass. General Hospital, Boston, Mass.) remained bound until 1 mM ammonium formate was added to the elution solvent.

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