ENZYMATIC SYNTHESIS AND STRUCTURE OF TWO GLYCOLIPIDS FROM TYPE XIV PNEUMOCOCCUS

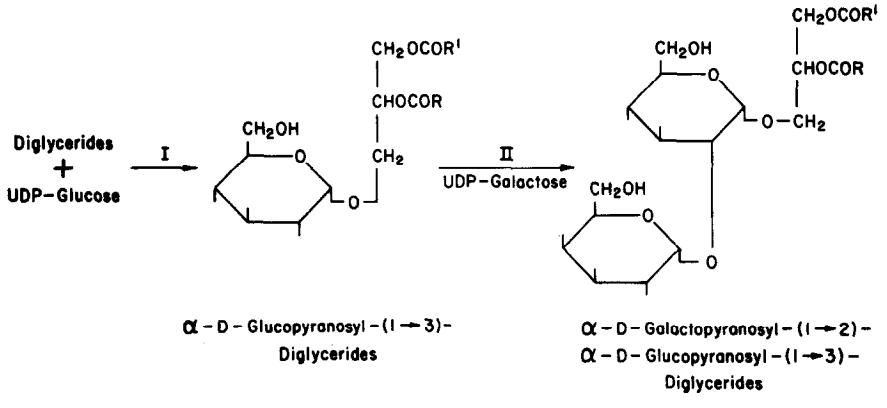
Bernard Kaufman, F. Dodyk Kundig, Jack Distler, and Saul Roseman

Rackham Arthritis Research Unit and the Dept. of Biological Chemistry, The University of Michigan, Ann Arbor, Mich.

Received November 30, 1964

We recently reported (Distler and Roseman, 1964) that a particulate preparation from Diplococcus pneumoniae Type XIV catalyzed the synthesis of antigenic polysaccharides and of two glycolipids.

We have now established the structures of the glycolipids and suggest that they are synthesized as follows:



Similar glycosyl diglycerides, first obtained from wheat flour (Carter et al., 1956), have been isolated from a variety of sources.[1]

[1]Some of the sources include chloroplasts (Benson et al., 1958; Carter et al., 1961; Sastry and Kates, 1963), Streptococcus faecalis (Vorbeck and Marinetti, 1964), and Micrococcus lysodeikticus (Lennarz, 1964). The glycolipids described in the present report are similar or identical to those isolated from Diplococcus pneumoniae Type I by Brundish et al. (1964; adjoining communication). The enzymatic synthesis of galactosyl glycerides in chloroplasts has been reported by Neufeld and Hall (1964) and the synthesis of mannosyl diglycerides has been demonstrated in M. lysodeikticus (Lennarz, 1964).

Materials and Methods.[2] The crude extract, prepared as previously described, was centrifuged at 10^5 x g for 1 hr, giving a particulate and a soluble enzyme system. The particulate fraction was washed and suspended in 0.05 M K PO_4 buffer, pH 7.2, containing 0.15 M KCl and 0.01 M Na thioglycolate; the soluble fraction was adjusted to 80% of saturation with $(NH_4)_2SO_4$, the precipitate dissolved, dialyzed against the same buffer, and centrifuged (10^5 x g, 1 hr). Both the soluble and particulate preparations exhibited the activities described below, but the latter contained high concentrations of endogenous lipid and was therefore unsuitable for substrate specificity studies.

[2]Substrates were prepared as described (Distler and Roseman, 1964). Incubation mixtures were assayed by extracting the lipids with butanol or with chloroform-methanol (C:M) in a ratio of 2:1 (v/v). Non-lipid contaminants were removed by washing the organic phase with water, the samples concentrated to dryness, dissolved in chloroform, and chromatographed on silicic acid columns (5 g). Each column was eluted sequentially with C:M mixtures of the following composition (v/v): 20 ml, 98:2; 25 ml, 95:5 (Lipid A); 20 ml, 93:7; 50 ml, 90:10 (Lipid B). The substrates were UDP-glucose-^{14}C and/or UDP-galactose-^{14}C (UDP-glucose-4-epimerase was present in both enzyme fractions), and product formation was measured by counting the appropriate fractions using liquid scintillation techniques.

The following methods were used: hexose by an anthrone procedure, glucose by a hexokinase-glucose-6-P dehydrogenase assay, galactose by galactose oxidase, glycerol by glycerol dehydrogenase (in the presence of 0.25 M hydrazine at pH 9.5), periodate oxidation by a standard arsenite titrimetric method on a micro scale, and formaldehyde by a chromotropic acid method.

The following chromatographic (and electrophoretic) methods were used to establish identity and/or homogeneity: (a) Lipids A and B by thin layer (silica gel G): $CHCl_3$, MeOH, H_2O, 85, 42, 10; PrOH, H_2O, 7, 3; PrOH, NH_3, H_2O, 70, 17, 13. (b) Glycosyl glycerol compounds, SA and SB, by high-voltage paper electrophoresis (borate buffer) and paper chromatography: pyridine, BuOH, H_2O, 25, 45, 40 (R_f: SA, 0.49; SB, 0.37) or 30, 20, 15; iso-PrOH, AcOH, H_2O, 3, 1, 1 (R_f: SA, 0.45; SB, 0.20). (c) Fatty acid methyl esters by thin layer (Silica gel G): hexane, Et_2O, 85, 15. (d) Glucose, galactose, glycerol and erythritol by high-voltage electrophoresis (borate) and paper chromatography: BuOH, AcOH, H_2O, 4, 1, 5; BuOH, pyridine, H_2O, 3, 1, 1.

Standard glucose trimethyl ethers were kindly provided by Drs. Samuel M. Rosen and Nelson K. Richtmyer. Dr. Rosen indicated that the ethers were separable on thin layer (alumina) chromatography: $CHCl_3$, BuOH, 96, 4. In addition, the tri- and tetramethyl ethers were characterized by borate electrophoresis and paper chromatography (BuOH, EtOH, H_2O, 4, 1, 5) followed by treatment with an aniline phosphate spray reagent that gave characteristic colors with some of the standards. The methylated hexoses derived from SB were separated (borate electrophoresis) and treated with BBr_3 to yield the hexoses; only glucose was detected on demethylation of the trimethylhexose fraction.

Characterization of Lipids A and B. The particulate enzyme system, prepared from 20 g of cell paste (20 l culture), was incubated with UDP-glucose, and the crude lipids isolated by butanol extraction. The glycolipids from three such preparations were purified and separated by chromatography on silicic acid (300 g).[2] Phospholipids, contaminating lipids A and B, were removed by chromatography on florisil (Floridin Co., 10 g; C:M, 2:1); the yields of A and B were 86 and 160 μmoles, respectively.

Thin layer chromatography in three solvent systems[2] indicated that A and B were homogeneous; only the compounds shown in Table I were detected after vigorous acid hydrolysis. The analytical data indicate that A is a glucosyl diglyceride, while B is a galactosyl-glucosyl-diglyceride. Partial hydrolysis of B (0.1 M HCl; 100° for 40 min)

TABLE I. Analysis of Lipids A and B

Component*	Molar Ratio		Per Cent of Dry Weight**	
	Lipid A	Lipid B	Lipid A	Lipid B
TOTAL CARBOHYDRATE	1.00	1.00	20.8	34.7
Glucose	1.00	0.97	20.8	16.8
Galactose	<0.04	1.10		19.1
GLYCEROL	1.00	1.00	11.8	9.8
FATTY ACIDS	1.97	1.95	59.6	50.2

*P was not detected in either preparation (< 0.03 mole/mole lipid), and galactose was not detected in Lipid A. Carbohydrate was measured by the anthrone method using glucose and lactose as standards for A and B, respectively. Total fatty acids were measured by conversion to hydroxamates and comparison with methyl palmitate treated in the same way. Methanolysis of A and B gave the fatty acid esters; only traces of hydroxy-esters were detectable on thin-layer chromatography. Gas chromatography of the esters showed the following composition for A: 12:0, 3.1%; 14:0, 6.3%; 16:0, 37.5%; 16:1, 20.0%; 16:? or 18:1, 29.3%; 18:0, 3.8%. The esters from B showed the following ratios: 12:0, 7.3%; 14:0, 12.5%; 16:0, 43.1%; 16:1, 19.3%; 16:? or 18:1, 16.0%; 18:0, 1.8%. We are most grateful to Dr. John Law, who also analyzed the esters and detected no cyclopropane or hydroxy-fatty acids.

**The analyses have been corrected for loss of H_2O by formation of ester and glycosidic bonds.

gave galactose as the only detectable monosaccharide.

The α anomeric configuration was assigned to the glycose units on the following basis: After saponification (1 N KOH, 37°, 16 hr), the fatty acids were removed, and the products obtained from A and B (SA and SB, respectively), were shown to be homogeneous and separable.[2] Hydrolysis of SA and SB with 1 N HCl (90 min, 100°) gave equimolar proportions of the expected products, glycerol, galactose, and/or glucose. Glucosyl glycerol (SA) was hydrolyzed by yeast α-glucosidase but not by emulsin β-glucosidase. It showed a molar rotation of +34,600°. The reported values for isomers of glucosyl-glycerol are: α-glucosyl-1-glycerol, +32,500° (Sawai and Hehre, 1962); α-glucosyl-2-glycerol, +30,700° and β-glucosyl-2-glycerol, -7,620° (Charlson, et al., 1962). The glycosidic bonds in SB were resistant to α- and β-galactosidases and glucosidases; however, the molar rotation, +67,200°, strongly suggests that both the galactose and glucose linkages are α (cf., α-melibiose, +61,300°; α-lactose, +30,600°).

Periodate oxidation studies, under a variety of conditions, showed that SA and SB were both rapidly cleaved, each yielding 1.0 mole of formaldehyde. By contrast, periodate consumption continuously increased, suggesting over-oxidation. These results are explicable by postulating the formation of active methylene groups; e.g., SA → glucosyl-glycolaldehyde → hemi-acetal (C-2 hydroxyl of glucose and the CHO group), followed by cleavage between C-3 and C-4. For this reason, periodate studies were repeated with intact lipids A and B (in 70% EtOH); no formaldehyde was liberated and the reaction was complete in 24 hr at 0°. Lipid A consumed 2.18, and B, 3.00 moles of periodate; the hexoses were completely destroyed. Finally, the periodate oxidized products were reduced with $NaBH_4$, and hydrolyzed with HCl; glycerol, but no erythritol, was detected after borate electrophoresis.

To conclusively establish the linkage of galactose to glucose, the

glycerol glycosides were methylated (Kuhn and Trischmann, 1963), and acid hydrolyzed. SA gave tetramethyl-glucose while SB gave tetramethyl-galactose and 3,4,6-trimethylglucose as the only detectable hexoses (yields = 60 to 90%).[2]

The endogenous lipids, A and B, in the particulate preparations were present at 5 to 20-fold the concentration of enzymatically-synthesized materials. However, the ^{14}C-products were characterized by the methods outlined above, and gave the same results as those obtained with the endogenous compounds; e.g., $NaIO_4$ oxidation of ^{14}C-SB followed by $NaBH_4$ reduction and acid hydrolysis gave ^{14}C-glycerol, but no ^{14}C-erythritol.

The structures proposed for lipids A and B are based on these data.

Enzymatic Synthesis of A and B. As shown in Fig. 1, the soluble enzyme system catalyzed a rapid transfer of hexoses from the UDP-hexoses-^{14}C

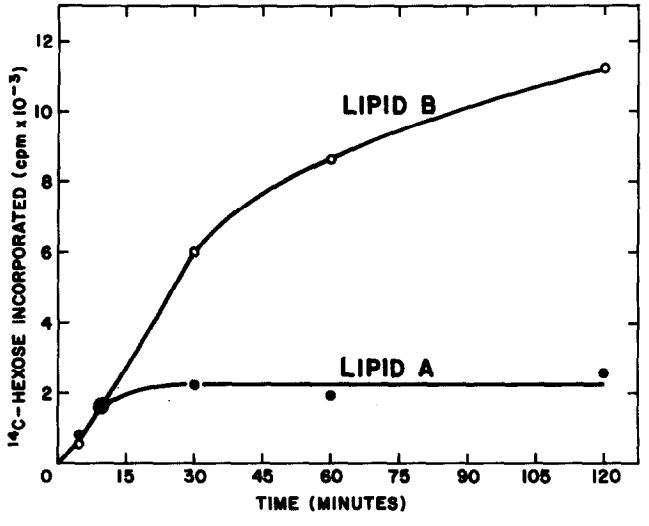


Fig. 1. Effect of time on the synthesis of Lipids A and B. Each sample contained the following components in 0.50 ml: 0.25 ml buffer containing the soluble enzyme system (4.2 mg protein); 0.125 ml boiled particulate preparation (1.6 mg protein); 0.40 µmole each of UDP-glucose and UDP-galactose (spec. act. of each sugar, 1.6 x 10^6 cpm/µmole); and $MgCl_2$, 5 µmoles. Samples were incubated at 37° for the indicated times and the reactions stopped by adding C:M (2:1). Controls contained heat-inactivated enzyme; < 200 cpm were detected in the lipid fractions. When the intact particulate preparation was substituted for the soluble system and for the lipid acceptor, the relative rates were comparable to those shown, except that the incorporation of ^{14}C-hexose was about 15-fold greater.

to lipid in the boiled particles. The synthesis of A apparently ceased after 10 min, while that of B continued for at least 2 hr. Preliminary results suggested that the lipid fraction (C:M extracts) of the particles were efficient glycose acceptors, although dipalmitin, for example, was not. The lipid acceptor in Reaction I must therefore be characterized. Here, as in the lipopolysaccharides (Rothfield and Horecker, 1964), physical state may determine whether lipids can serve as acceptors, this state being a function of many factors such as fatty acid composition and the presence of other "inert" lipids (e.g., phospholipids). Reaction II, on the other hand, was demonstrated by the enzymatic conversion of lipid A to B (Table II).

Current studies are directed toward securing unequivocal evidence for the existence of two specific transferases, I and II, and determining the function of the glycolipids and their possible role in polysaccharide biosynthesis.

TABLE II. Enzymatic conversion of Lipid A to Lipid B

Tube	Substrates	^{14}C- Lipid B Synthesized cpm	μmoles
1	^{14}C-Lipid A + UDP-gal	41,700	8.5
2	^{14}C-Lipid A	< 50	-
3	UDP-gal-^{14}C + Lipid A	39,800	14.7
4	UDP-gal-^{14}C	5,000	1.9

*Tubes 1 and 2 contained the following components in 0.11 ml: 0.16 μmole ^{14}C-glucose labeled Lipid A (4.9×10^6 cpm/μmole); 1.2 μmoles $MgCl_2$; 0.25 mg Tween-20; 1.7 mg protein (soluble enzyme preparation). Tube 1 also contained 0.10 μmole UDP-galactose. Tubes 3 and 4 contained the same components except that 0.19 μmole unlabeled Lipid A and 0.30 μmole of UDP-galactose-^{14}C (2.7×10^6 cpm/μmole) were used as indicated. After 2 hr at 37°, the lipids were separated and purified[2] and further characterized by: (a) saponification followed by paper chromatography; and (b) acid hydrolysis and identification of the ^{14}C-sugars. The soluble enzyme preparations contained endogenous lipid acceptors for galactose (Tube 4) and glucose (i.e., Lipid A fractions from Tubes 3 and 4 exhibited 1100 and 1800 cpm respectively). ^{14}C was not detected in the sugar-nucleotides isolated from the incubation mixtures in Tubes 1 and 2; therefore, the ^{14}C-glucose in ^{14}C-Lipid A did not exchange with the UDP-hexoses.

REFERENCES

Benson, A. A , Wiser, R., Ferrari, R. A., and Miller, J. A. J. Am. Chem. Soc., 80, 4740 (1958).
Brundish, D. E., Shaw, N., and Baddiley, J., Biochem. Biophys. Res. Comm., 18, 308 (1965).
Carter, H. E., McCluer, R. H., and Slifer, E. D. J. Am. Chem. Soc., 78, 3735 (1956).
Carter, H. E., Hendry, R. A., and Stanacev, N Z. J. Lipid Res., 2, 223 (1961).
Charlson, P. A., et al. in Methods in Carbohydrate Chemistry, Whistler, R. L., and Wolfrom, M. L. (ed.), Academic Press, New York, Vol. I, p. 422 (1962).
Distler, J., and Roseman, S. Proc. Natl. Acad. Sci., 5, 897 (1964).
Kuhn, R., and Trischmann, H. Chem. Ber., 96, 284 (1963).
Lennarz, W. J. J. Biol. Chem., 239, PC 3110 (1964).
Neufeld, E. F., and Hall, C. W. Biochem. Biophys. Res. Comm., 14, 503 (1964).
Rothfield, L., and Horecker, B. L. Proc. Natl. Acad. Sci., 52, 939 (1964).
Sastry, P. S., and Kates, M. Biochem. Biophys. Acta, 70, 214 (1963).
Sawai, T., and Hehre, E. J. J. Biol. Chem., 237, 2047 (1962).
Vorbeck, M. L., and Marinetti, G. V. Federation Proc., 23, 375 (1964).