CHEMICAL CONSTITUTION OF A GLYCOLIPID FROM *C.diphtheriae* P.W.8

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Previous analyses of the lipid material from *C.diphtheriae* have revealed the presence of free and esterified forms of corynomycolic acid (2-tetradecyl 3-hydroxystearic acid) (Lederer and Pudles, 1951 and Lederer *et al.* 1952) and corynomycolenic acid (2-tetradecyl 3-hydroxy 9-10-octadecenoic acid) (Pudles and Lederer, 1953, 1953a and 1954). An additional lipid material has also been reported which contained trehalose esterified with unidentified fatty acids (Alimova, 1954 and 1959); the latter was toxic by subcutaneous injection into guinea pigs.

The present communication describes the isolation and characterization of a single glycolipid which contains trehalose, corynomycolic acid and corynomycolenic acid in equal molar amounts.

Isolation of the glycolipid - The fraction of lipids from *C.diphtheriae* which was insoluble in boiling acetone (Pudles 1954) was used as the starting material in this work. This fraction was dissolved in chloroform and passed through a

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column of cellulose powder Whatman n^{o} 1 (Lea and Rhodes, 1953). After evaporation of the eluate to 1/10 of its volume an equal volume of methanol was added and the insoluble fraction removed. Evaporation of the soluble phase left a residue which was dissolved in chloroform and chromatographed on a column of silicic acid (Mallinckrodt) employing increasing concentrations of methanol in chloroform (Hanahan et al. 1957).

The glycolipid, contaminated with some high molecular weight fatty acids, was eluted with chloroform-methanol 19/1. After removal of the solvent this fraction was rechromatographed on a column of silica gel (B.D.H.) with petroleum ether as the developing solvent. The glycolipid emerged as a homogeneous peak after approximately 3 column volume of eluate. Reprecipitation from a ethyl ether solution by the addition of 4 volumes of acetone gave a neutral colorless wax m.p. 110-115^{o}C; α_D =+64^{o} (c=0.605 in chloroform). Determination of the hexose content by a modified anthrone method* (Johanson, 1954), revealed 28 per cent carbohydrate measured as glucose. The infrared spectrum** in chloroform exhibited a strong hydroxyl group band at 3500cm^{-1} and a strong ester carbonyl group at 1720cm^{-1}.

Characterization of the carbohydrate - The glycolipid was saponified by refluxing for 8 hours in 5 percent methanolic potassium hydroxide solution. After acidification of the mixture with acetic acid, 73 percent of the starting material was recovered as ether soluble high molecular weight fatty acids. An additional 24 percent of the mass was recovered as a neutral fraction in the aqueous phase after desalting by passing through

*Prior to the addition of the anthrone reagent we refluxed the sample with 5 percent methanolic potassium hydroxide in a test tube for 30 minutes. After evaporation of the solvent the anthrone procedure was carried out on the residue.
** All infrared spectra were performed in a Infracord model 137B

Dowex AG 50 WX8 and Amberlite IR4B. On evaporation the residue had a glassy appearence. α_D =+156º (c=0,432 in water). No reducing power was found with alkaline silver nitrate or aniline acid phthalate. Hexose content, estimated with anthrone, was 97,3 percent measured as glucose. Chromatography on Whatman paper nº 1 (ascending) with isopropanol-pyridine-acetic acid-water (8:8:1:4) gave one spot as detected with periodic acid-benzidine (Gordon et al., 1956) with an RF 0,52. Its migration was identical to authentic trehalose.

Acetylation with acetic anhydride in pyridine yielded a crystalline compound which did not depress the melting point of authentic trehalose octaacetate (Anderson and Newman, 1933). Their infrared spectra in chloroform were identical.

After acid hydrolysis and deionization with Amberlite IR4B, ascending paper chromatography yielded one spot which corresponded to D-glucose in the following solvent systems (Smith, 1958): ethyl acetate-pyridine-water (8:2:1), n-propanol-ethyl acetate-water (7:1:2) and n-butanol-acetic acid-water (12:3:5). The osazone did not depress the m.p. of authentic D-glucosazone.

Characterization of the fatty acids - The fatty acid fraction from the saponification was methylated with diazomethane. Crystallization from acetone-methanol yielded a solid ester and evaporation of the mother liquor yielded a liquid ester in nearly equal proportions.

Chromatography of the solid ester on aluminium oxide (Merck) with petroleum ether-benzene mixtures resulted in the elution of the methyl ester as a single peak with benzene. Recrystallization from methanol presented a colorless compound m.p. 58ºC α_D = +10º (c=0,47 in chloroform) infrared spectra in chloroform showed a hydroxyl group band at 3500cm^{-1} and a ester carbonyl at

1720cm^{-1}. Alkaline hydrolysis gave a microcrystalline substance, m.p. 68°C, α_D = + 8° (c=1,34 in chloroform), neutralization equivalent, 480; calcd., 496. Infrared spectra in chloroform showed the hydroxyl group band at 3500cm^{-1} and the carboxyl carbonyl group band at 1720cm^{-1}.

Oxidation of the solid hydroxy ester with chromic anhydride in acetic acid at 50°C over 18 hours gave a neutral compound m.p. 50°C without rotatory power but with a weak absorption band at 260mu. The infrared spectra showed two peaks at the carbonyl region at 1700cm^{-1} and at 1720cm^{-1}. With alkaline hydrolysis of the ketoester, a neutral crystalline ketone, m.p. 80°C was obtained which had no rotatory power but showed a keto carbonyl group band at 1700cm^{-1}. This ketone gave a crystalline oxime m.p. 55°C. Since the alkaline hydrolysis of the ketoester yielded a neutral ketone, the position of the original hydroxyl group (prior to chromic anhydride oxidation) must have been beta to the carboxyl group. Comparing the product with palmitone obtained from chromic anhydride oxidation of corynomycolic acid (Lederer and Pudles, 1951) revealed the two materials to be identical. Similarly the hydroxy acid, the methyl ester and the methyl ester of the ketoacid isolated from the glycolipid were identical with the corresponding derivatives from authentic corynomycolic acid.

The liquid ester was distilled under 0,1mm Hg at 220°C as a liquid yellow oil, $n_D^{20°}$=1,4657, α_D=+9,6° (c=0,7 in chloroform). The infrared spectra in chloroform showed the hydroxyl group band at 3500cm^{-1} and ester carbonyl group band at 1720cm^{-1}. Alkaline hydrolysis yielded a liquid acid $n_D^{20°}$= 1,4696 α_D=+8,5° (c=0,845 in chloroform), neutralization equivalent 510; calcd., 494.

One mole of hydrogen was absorpted on catalytic hydrogenation of the liquid methyl ester with PtO_2 (Adams catalyst) in ethyl acetate. A solid methyl ester was recovered which was identical with methyl ester of corynomycolic acid by melting point, infrared spectra and the chromic anhydride oxidation products. Accordingly the liquid ester has the carbon skeleton of corynomycolenic acid or one of its positional isomers.

We wish to conclude from these data that the glycolipid from C.diphtheriae is a ester of trehalose having at least one hydroxyl group esterified with either corynomycolic acid or with corynomycolenic acid or one of its positional isomers. The additional fatty acid group in the glycolipid may esterified a second hydroxyl in the trehalose molecule or the hydroxyl group of the other fatty acid.

Work is in progress to identify the position of the double bond in the unsaturated fatty acid and the hydroxyl groups which are esterified in the trehalose molecule.

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