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Structural Studies on the Glycolipids from the Envelope of the Heterocyst of *Anabaena cylindrica*†

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ABSTRACT: Four glycolipids were isolated and purified from heterocysts of *Anabaena cylindrica*. The structures of these lipids have been studied by mass spectrometry, infrared spectroscopy, and nuclear magnetic resonance spectrometry. The position of the bond between the sugar and the aglycone was determined by permethylation of the intact lipid, followed by hydrolysis, trimethylsilylation, and mass spectrometry. A C-26 and a C-28 polyhydroxy alcohol are glycosylated at their terminal hydroxyl. A C-26 and probably a C-28 hydroxy fatty acid are glycosylated at their carboxylic group.

Certain blue-green algae contain unusual lipids (Nichols and Wood, 1968) which are localized largely or wholly in a laminated layer of the envelope that surrounds the wall of differentiated cells called heterocysts (Walsby and Nichols, 1969; Wolk and Simon, 1969; Winkenbach *et al.*, 1972). The more polar of these lipids are nonsaponifiable glycolipids (Nichols and Wood, 1968; Walsby and Nichols, 1969), the aglycone moieties of which, from *Anabaena cylindrica*, were identified by Bryce *et al.* (1972) as 1,3,25-trihydroxyhexacosane and probably 1,3,25,27-tetrahydroxyoctacosane.

We have fractionated the "heterocyst lipids" from the same alga into four distinct glycolipids. Lipids III and IV consist of a hexose bound by a glycosidic linkage to the terminal hydroxyls of the long-chain polyhydroxy alcohols described by Bryce *et al.* (1972). Lipid I consists of hexose bound to the carboxyl group of 25-hydroxyhexacosanoic acid. Lipid II appears to be similar structurally to lipid I.

Material and Methods

Centrifugal pellets of heterocysts isolated from *A. cylindrica* Lemm. (Wolk, 1968) were extracted three times with five volumes of chloroform–methanol (2:1, v/v) at room temperature. The pooled extracts, concentrated under vacuum, were shaken with chloroform and water in a separatory funnel. The lipids of the heterocyst envelope stayed largely at the interface. The aqueous and interface layers were extracted five times with 50 ml of chloroform. The lipid material at the interface was separated from the water layer by filtration through sintered glass and was then dissolved with chloroform–methanol (2:1) or with hot methanol.

The interface lipids were separated by repeated chromatography on a 21 × 0.5 in. column of a mixture of silicic acid and Celite (1:2, w/w). The lipids were applied to the column in a minimum volume of chloroform–methanol, washed with 200 ml of chloroform, and eluted with 100-ml volumes of chloroform–methanol mixtures containing increasing concentrations (2, 4, 5, 6, 8, and 10%) of methanol. The eluate

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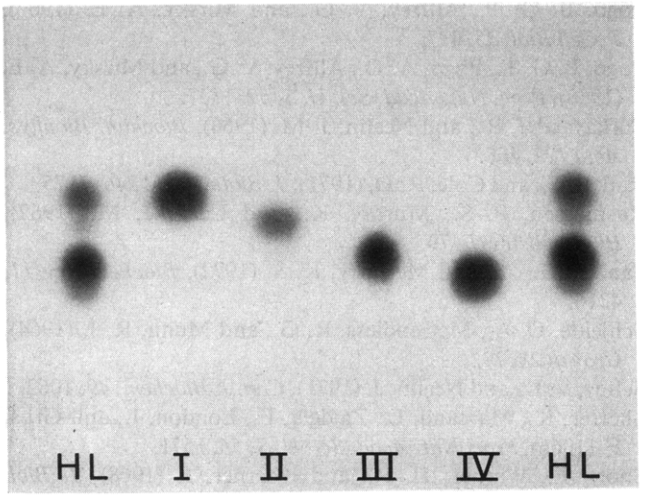

FIGURE 1: Thin-layer chromatogram of envelope lipids from heterocysts (HL) and of the purified lipids I–IV.

fractions (10 ml) from the column were monitored by thin-layer chromatography on silica gel G (Merck), with chloroform–methanol–acetic acid–water (170:30:20:7.4, v/v; Nichols and Wood, 1968) as developing solvent.

Fractions containing mixtures of lipids were rechromatographed as before. The lipids from the pure fractions were collected, dissolved in hot methanol, and crystallized from methanol after addition of water. Each precipitated lipid was filtered on a sintered glass filter, washed with water and with chloroform or hexane, washed through the filter with hot methanol, and dried under vacuum (Figure 1).

Hydrolysis and Derivatizing. Lipids were hydrolyzed in 3% dry methanolic HCl (16 hr, 80°). Lipids I and II were saponified in ammonium hydroxide–methanol (1:5, v/v) in a sealed test tube by heating for 1 hr at 100°, or in 0.5 N KOH in methanol in an ice bath (30 min or 2.5 hr), and were immediately neutralized with acetic acid.

N,O-Bis(trimethylsilyl)acetamide or *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co.) were used to prepare trimethylsilyl (Me_3Si) derivatives. To dried aliquots of pure lipids or of their hydrolysis products were added 20 μl of *N,O*-bis(trimethylsilyl)acetamide or *N,O*-bis(trimethylsilyl)trifluoroacetamide and 20 μl of pyridine. The reaction mixtures were incubated for 30 min at 50°.

Lipids were permethylated with CH_3I in the presence of methylsulfinyl carbanion (Hakomori, 1964).

Mass Spectrometry. Mass spectra were recorded on an LKB 9000 combined gas–liquid chromatograph–mass spectrometer. A 4-ft column packed with 3% SE-30 on 100–200 mesh Gas-Chrom Q was used with temperature programming. Instrumental conditions of the mass spectrometer were, unless otherwise stated, as follows: temperature of molecular separator, 250°; temperature of ion source, 290°; accelerating voltage, 3.5 keV; ionizing electron energy, 70 eV. A direct probe inlet system was used for the complete lipids. Backgrounds were subtracted and data prepared in tabular or bar graph form by means of a computerized data system (Sweeley *et al.*, 1970).

Infrared Spectroscopy. Infrared spectra were recorded on a Perkin-Elmer Model 621 spectrophotometer using a KBr pellet.

Nuclear Magnetic Resonance Spectra. Proton magnetic resonance spectra were recorded on a Varian HA-100 spectrometer or a Varian HA-60-IL spectrometer. Tetramethylsilane was used as internal standard. The lipids were dissolved in deuterated dimethyl sulfoxide. The tube containing lipid III had to be heated to 50° to keep the lipid in solution.

Sugar Analysis. Reducing sugar contents were estimated quantitatively by the method of Park and Johnson (1949), and total hexose content by the anthrone method (Roe, 1955). Sugars were identified preliminarily by means of paper chromatography. D-Glucose and D-galactose were estimated quantitatively with commercial enzymatic assay kits (Worthington Biochemical Corp.).

Results

Lipid I

The sugar moiety of lipid I can be isolated either after acid-catalyzed methanolysis and hydrolysis of the methyl hexoside in 1 N trifluoroacetic acid, or after hydrolysis in alkaline conditions. Of the isolated reducing sugar, 92.8% was estimated as D-glucose and 9.5% was estimated as D-galactose. The hexose content was quantitatively estimated by the anthrone reagent as 31.1%, using both glucose and galactose as standards.

The infrared spectrum of lipid I contains strong hydroxyl absorption at wave number 3400 cm^{-1} and strong CH_2 absorption at 2920 and 2850 cm^{-1}. A strong absorption at 1710 cm^{-1} shows the presence of a carboxyl ester. Some lesser absorption peaks in the spectrum of lipid I are also found in the infrared spectrum of acylated glucopyranose (Smith and Mayberry, 1968). An absorption at 925 cm^{-1} indicates the presence of a pyranose ring, an absorption at 840 cm^{-1} and absence of absorption around 895 cm^{-1} suggests the α configuration for the sugar. No absorption specific for unsaturation is observed.

In the 100-MHz nuclear magnetic resonance (nmr) spectrum (given in parts per million) of a saturated solution of lipid I, a peak at δ 1.24 establishes the presence of a long, saturated hydrocarbon chain. A doublet at δ 1.01 (J = 6 cps) can be attributed to a terminal methyl group, probably adjacent to a hydroxyl group. No other terminal methyl groups are present, which is evidence for an unbranched chain. A triplet at δ 2.64 (J = 6 cps) probably can be assigned to a methylene group adjacent to the carboxyl ester. No peaks were observed between δ 5 and 12, which is evidence that there are no free carboxyl or aldehyde groups and that the hydroxyl group on C-1 of the sugar is not free. After addition of CF_3-COOH to the sample, the peaks due to hydroxyl groups were shifted downfield to δ 8; between δ 4 and 5, only a small doublet peak remained. This doublet (δ 4.63, J = 3.6 cps) can be assigned to the proton on C-1 of the sugar (Capon and Thacker, 1964). The chemical shift and coupling constant are in agreement with an α-D-glucoside (Casu *et al.*, 1966).

Gas–Liquid Chromatographic and Mass Spectral Studies on Lipid I. Lipid I was reacted with *N,O*-bis(trimethylsilyl)-acetamide, dried, and introduced into the mass spectrometer by a direct inlet system heated to 105°. Peaks characteristic of trimethylsilyl hexosides (*m/e* 467, 451, 377, 361, 305, 217, 204, 147, and 103) are present (Figure 2, I). The dominant peak at *m/e* 117 can be assigned to an $\omega - 1$ hydroxyl group which is cleaved off as $CH_3CH{=}O^+Si(CH_3)_3$. An alternative interpretation of the peak at *m/e* 117, $(CH_3)_3SiO^+{=}C{=}O$, is not in agreement with the infrared and nmr spectra, which suggest the absence of a free carboxyl group.

When lipid I was subjected to acid-catalyzed methanolysis and then reacted with *N,O*-bis(trimethylsilyl)trifluoroacetam-

ide and subjected to gas–liquid chromatography, peaks were eluted at 169, 173, and 269°. Mass spectrometry showed that the peaks eluted at the lower oven temperatures contained hexose. Comparison of the mass spectrum of the compound appearing at 269° with the mass spectra of hydroxy fatty acids from cutin (Brieskorn and Kabelitz, 1971) suggests that the compound is a trimethylsilyloxyhexacosanoic acid methyl ester. An approximate calculation based on the peak areas from the gas–liquid chromatogram gave a ratio of 1.16 sugar molecules to 1 molecule of aglycone.

After saponification of lipid I in alcoholic KOH or in a CH_3OH–NH_4OH mixture, reaction with *N,O*-bis(trimethylsilyl)trifluoroacetamide, and gas–liquid chromatography, the major reaction product was eluted at an oven temperature of 255°. The mass spectrum of this compound (Figure 3, I) shows a major peak at *m/e* 117 ($CH_3CH{=}O^+Si(CH_3)_3$) in agreement with an $\omega - 1$ hydroxyl group. Assuming a molecular weight of 556, corresponding to the ditrimethylsilyl derivative of 25-hydroxyhexacosanoic acid, the peaks at *m/e* 466 and 376 may be explained as due to successive losses of trimethylsilanol (M = 90). Further loss of CH_3 gives rise to a peak at *m/e* 451. Loss of CHO gives rise to peaks at *m/e* 422 (451 − 29) and *m/e* 347 (376 − 29).

Lipid I was subjected to methylation according to the method of Hakomori (1964), and then to acid-catalyzed methanolysis. Gas–liquid chromatography, programmed at 5°/min, showed two peaks which eluted at 102 and 108°. Their mass spectra were identical with spectra obtained from pentamethylglucose (Kochetkov and Chizhov, 1966). The different intensities of the peaks at *m/e* 176 and 187 show that the earlier and the later peak contained, respectively, the β and the α anomer.

The mass spectrum of intact lipid I suggests that the sugar moiety has only one linked hydroxyl and that the $\omega - 1$ hydroxyl of the aglycone is free. The nmr spectrum shows that the hydroxyl on C-1 of the sugar is not free and also that the carboxylic acid proton is absent. Mass spectra show that following permethylation or, alternatively, silylation of intact lipid I, the hydroxyl groups on carbons-2, -3, -4, and -6 of the sugar moiety are substituted with, respectively, methyl or trimethylsilyl groups. When permethylation is followed by acid-catalyzed methanolysis, which can introduce a methyl group only at carbon-1 of the sugar, methylation of the sugar is completed to the pentamethyl derivative. Lipid I is in part hydrolyzed under the conditions used for the quantitative estimation of reducing sugars by the Park and Johnson method, and under the conditions used for the formation of trimethylsilyl derivatives, or alternatively in a CH_3OH–NH_4OH mixture, showing that the sugar moiety and the aglycone are linked by a bond which is much less stable than a normal glycosidic bond.

After thin-layer chromatography, none of the lipids gave any reaction with a $AgNO_3$ spray. After reaction with $NaBH_4$, lipid I could no longer be hydrolyzed in a CH_3OH–NH_4OH mixture at 100°, whereas the same material gave rise to a reducing sugar after acid-catalyzed methanolysis and treatment with 1 N CF_3COOH at 100°. Boric acid (0.2%) also stabilized lipid I. This stabilization phenomenon may be compared with the behavior of some gallotannins, in which the "depside"-ester linkage between the carboxyl group of one gallic acid residue and a hydroxyl group of a second such residue is more easily methanolyzed if a vicinal hydroxyl is free (Haworth, 1961). The linkage between the glucose and the hydroxy fatty acid of lipid I should behave similarly to glucogallin (1-*O*-gallo-β-glucoside; Fisher and Bergmann, 1918). As is true of glucogallin, lipid I is hydrolyzed in 0.5 N KOH in MeOH at 0°. The major part of lipid I is hydrolyzed after 30 min, and the hydrolysis is complete after 2.5 hr at 0°. Lipid I is hydrolyzed slowly in 0.5 N acetic acid in MeOH at 90°. As is obvious from the nmr spectrum and the infrared spectrum, lipid I is hygroscopic; Fischer also mentioned the hygroscopicity of glucogallin.

The above results are in agreement with the following proposed structure for lipid I: 25-hydroxyhexacosanoic acid (1-α-D-glucopyranose) ester, together with a lesser amount of D-galactose ester.

Lipid II

The presence of a distinct lipid II was discovered after thin-layer chromatography of some eluate fractions from column chromatography. Because lipid II is present in very small quantities, only incomplete data about it are available. A sample of lipid II, purified by thin-layer chromatography, was hydrolyzed in a MeOH–NH_4OH mixture. Of the total reducing sugar, as determined by the Park and Johnson method, 71% was determined as D-glucose by enzymatic oxidation.

The infrared spectrum of lipid II is very similar to the infrared spectrum of lipid I, with a strong hydroxyl absorption at 3400 cm^{-1}, strong methylene absorptions at 2920 and 2850 cm^{-1}, and carboxyl ester absorption at 1710 cm^{-1}. Weak absorptions at 925 and 840 cm^{-1} are present but less distinct than in the spectrum of lipid I.

A mass spectrum of the Me_3Si derivative of lipid II was obtained with the direct inlet system (Figure 2, II). As in the case of lipid I, peaks characteristic of trimethylsilyl hexosides (*m/e* 467, 451, 377, 361, 305, 217, 204, 147, and 103) are present. This indicates again that the sugar moiety can be silylated at positions 2, 3, 4, and 6 without breaking the link with the aglycone. The dominant peak at *m/e* 117 again can be assigned to an $\omega - 1$ hydroxy group. A number of peaks present in this spectrum are absent from the mass spectrum of lipid I, namely the series of peaks at *m/e* 583, 492, 493, 402, and 403, at *m/e* 615, 525, 434, and 435, and at *m/e* 629, 539, 448, and 449. The occurrence of these peaks indicates that after cleavage of the aglycone from the sugar moiety, two losses of trimethylsilanol can occur from the aglycone. The composition of the cleavage product with *m/e* 402 should be $C_{28}H_{50}O$, and can be compared with the cleavage product with *m/e* 376 ($C_{26}H_{48}O$) present in the mass spectrum of lipid I.

After acid-catalyzed methanolysis of lipid II, trimethylsilylation and gas–liquid chromatography programmed at 10°/min, a peak containing silylated hexose was eluted at an oven temperature of 175°. No further peaks were eluted. The hydrolyzed and silylated lipid II was introduced into the mass spectrometer with a direct inlet system that was heated gradually from 50 to 150°. With the data processing system of Sweeley *et al.* (1970), using the repetitive-scanning modification developed by Young (1972), mass spectra were taken every 20 sec (electron energy 22.5 eV) and stored on magnetic tape. After the heating of the sample was completed, scanning of the stored mass spectra by the computer revealed that the sample was not homogeneous. The compound(s) that produced a mass spectrum with a predominant peak at *m/e* 117 was evaporated into the ion source at 100°. The mass spectrum of this compound (Figure 3, II) contains a number of peaks which are present also in the spectrum of the aglycone of lipid I. The peak at *m/e* 117 clearly shows the presence of an $\omega - 1$ hydroxyl. The position of a second hydroxyl is unclear. The peaks at *m/e* 219 and 233, to which can be assigned the

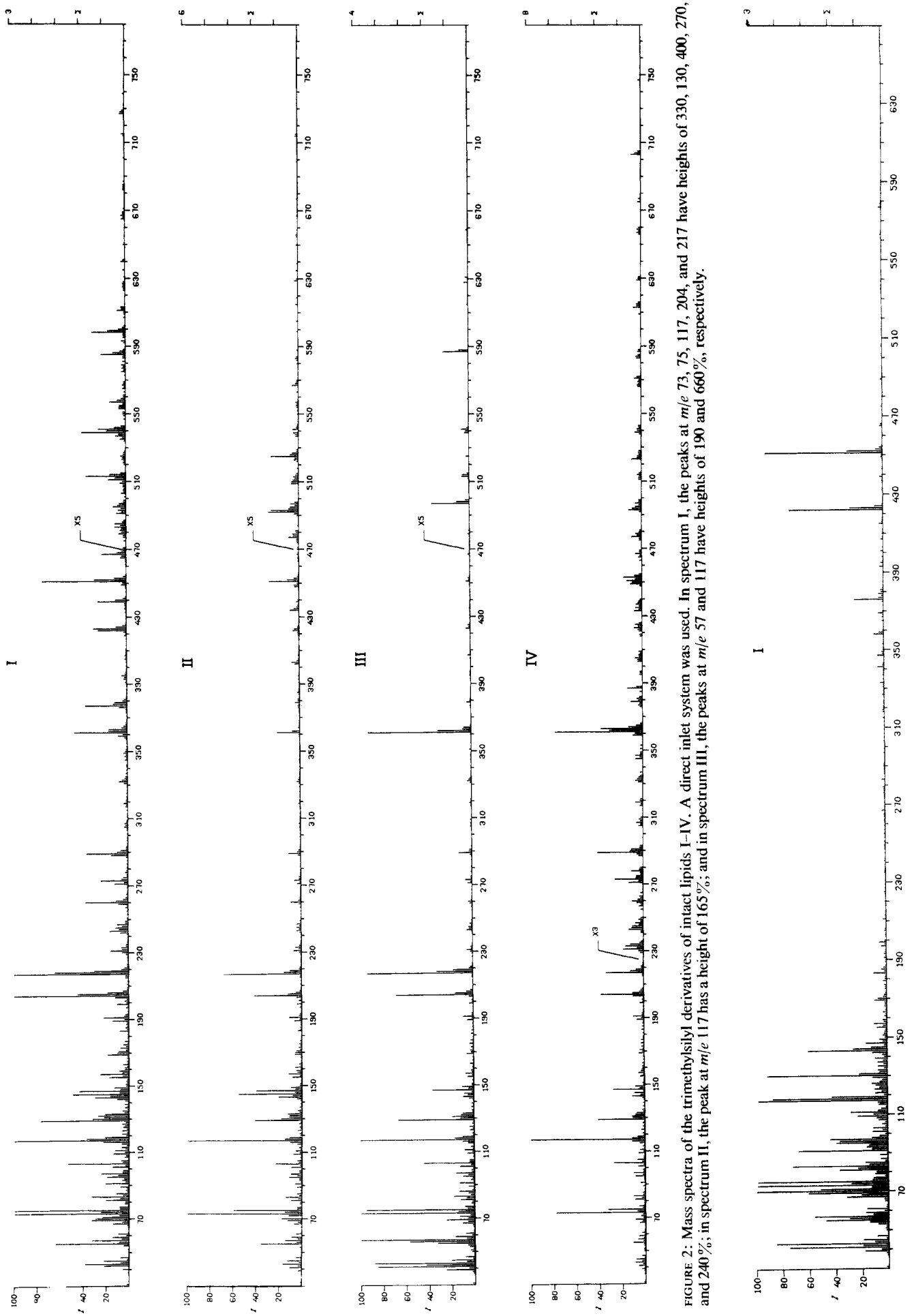

FIGURE 2: Mass spectra of the trimethylsilyl derivatives of intact lipids I–IV. A direct inlet system was used. In spectrum I, the peaks at *m/e* 73, 75, 117, 204, and 217 have heights of 330, 130, 400, 270, and 240%; in spectrum II, the peak at *m/e* 117 has a height of 165%; and in spectrum III, the peaks at *m/e* 57 and 117 have heights of 190 and 660%, respectively.

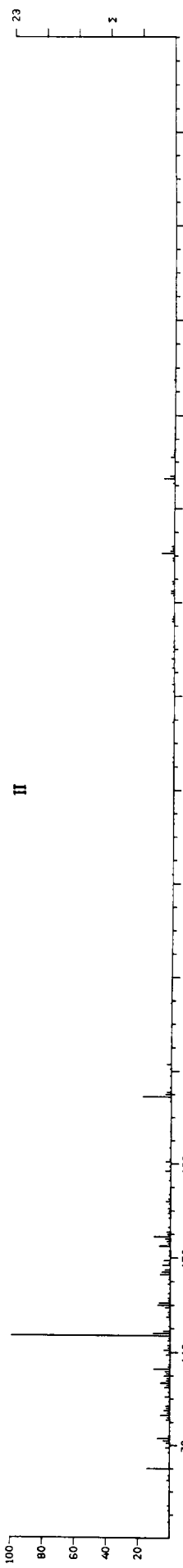

FIGURE 3: Mass spectra of the trimethylsilyl derivatives of the aglycones of lipids I and II. A combined gas–liquid chromatograph–mass spectrometer (70 eV) was used for lipid I, and a direct inlet system (22.5 eV) for lipid II. In spectrum I, the peak at *m/e* 117 has a height of 565%.

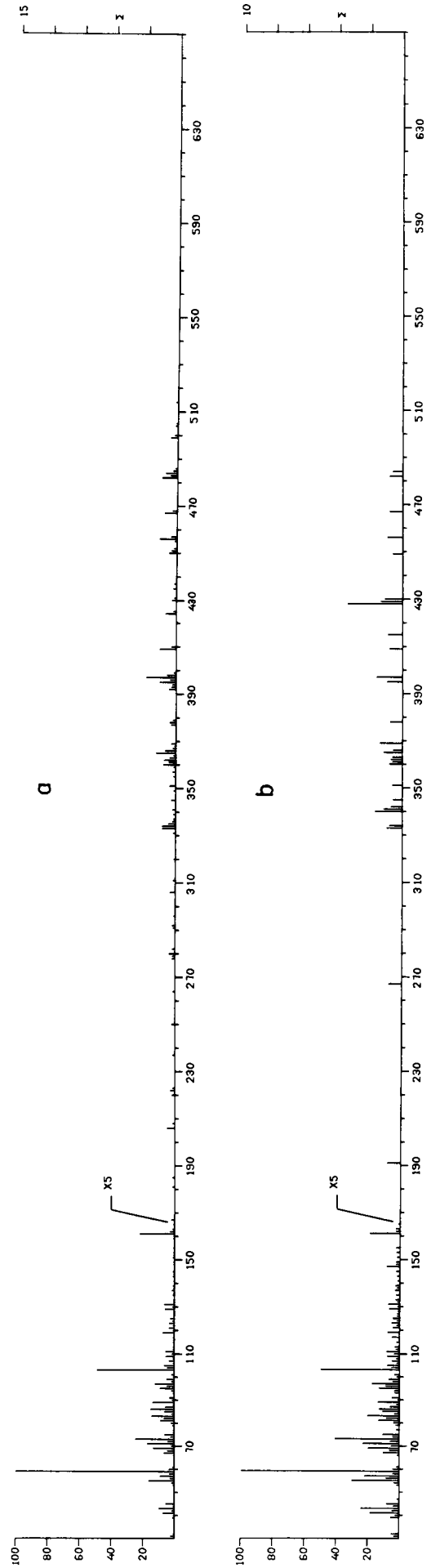

FIGURE 4: Mass spectra of: (a) the aglycone of permethylated lipid III, trimethylsilylated at the bonding hydroxyl; (b) the aglycone of permethylated lipid IV, trimethylsilylated at the bonding hydroxyl; and (c) the trimethylsilylated aglycone of lipid IV. A combined gas–liquid chromatograph–mass spectrometer was used for all spectra.

structures $CH_3CH(OSi(CH_3)_3)CH{=}O^+Si(CH_3)_3$ and $CH_3CH(OSi(CH_3)_3)CH_2CH{=}O^+Si(CH_3)_3$ respectively, suggest that the second hydroxyl is located at the $\omega - 2$ or at the $\omega - 3$ position.

For an explanation of the low intensity of the *m/e* 233 peak, see the discussion of lipid IV below. If the aglycone of lipid II is a dihydroxyoctacosanoic acid, then the molecular weight of the silylated methyl ester would be 614 daltons. The peak at *m/e* 483 can be due to loss of 131 daltons ($CH_3CH(OSi(CH_3)_3)CH_2$), which supports assignment of the second hydroxyl group to the $\omega - 3$ position. Further loss of CH_3OH would give rise to the peak at *m/e* 451. If the structure of lipid II bears the same relationship to the structure of lipid I that the structure of lipid IV bears to the structure of lipid III, the second hydroxyl of lipid II would be on the $\omega - 3$ position.

As is the case for lipid I, lipid II is hydrolyzed in 0.5 N KOH in CH_3OH at 0° or in a NH_4OH–CH_3OH mixture at 100°.

A possible structure for lipid II is 25,27-dihydroxyoctacosanoic acid (1-α-D-glucopyranose) ester or 26,27-dihydroxyoctacosanoic acid (1-α-D-glucopyranose) ester. While the first structure appears to us to be the more probable, it is not excluded that a mixture of both molecules is present. Lesser amounts of the D-galactose ester are perhaps also present.

Lipid III

The sugar content of crystallized lipid III, in comparison with a glucose standard, and corrected for the presence of galactose, was estimated at 30.5% by the anthrone reaction. The sugar moiety was isolated after acid-catalyzed methanolysis, and hydrolysis of the 1-methyl hexoside in 1 N CF_3COOH. Glucose was shown to be the major sugar present by paper chromatography. Of the isolated reducing sugar, 94% was estimated as D-glucose and 13% was estimated as D-galactose. Alkaline hydrolysis of lipid III released only 0.1% reducing sugar as estimated by the method of Park and Johnson.

The infrared spectrum of lipid III shows strong hydroxyl absorption at 3400 cm^{-1} and strong CH_2 absorption at 2920 and 2850 cm^{-1}. No absorption is observed around 1700 cm^{-1}, indicating that the lipid does not contain any carboxylic acid or ester. Absorption bands due to unsaturation were also absent from the infrared spectrum. Smaller absorption bands at 925 and at 840 cm^{-1} indicate that the sugar linkage is in the same configuration as in lipid I.

In the 60-MHz nmr spectrum, the peak at δ 1.23 reveals the presence of an unbranched chain of about 20 methylene groups. The doublet at δ 1.02 (J = 6 cps) shows that only one terminal methyl group, adjacent to a hydroxyl group, is present. The presence of a small doublet at δ 4.63 (J = 3.5 cps) indicates that the sugar is present as an α-pyranoside.

Gas–Liquid Chromatographic and Mass Spectral Studies on Lipid III. Lipid III was reacted with *N,O*-bis(trimethylsilyl)-acetamide and subjected to gas–liquid chromatography programmed at 4°/min to 300°. Lipid III was eluted after 15 min at 300°. To avoid heavy background from the liquid phase of the column at this temperature, the trimethylsilyl derivative was introduced into the mass spectrometer by a direct inlet system. The mass spectrum (Figure 2, III) contains peaks characteristic of a trimethylsilyl glucoside (*m/e* 467, 451, 377, 361, 305, 217, 204, 147, and 103). The Me_3Si derivative of the aglycone was eluted at 245°.

In the spectrum of the intact lipid, the peak at *m/e* 103 can be derived from the terminal CH_2OH group of the sugar moiety. The much greater prominence of that peak in the spectrum of the aglycone (see also Bryce *et al.*, 1972) points to the possibility that the sugar is linked to the terminal hydroxyl of the aglycone. A peak at *m/e* 117 ($CH_3CH{=}O^+Si(CH_3)_3$) is present in both spectra. The aglycone peak at *m/e* 219, which is absent from the spectrum of the complete lipid, can be due to either of two structures, $CH_3CH(OSi(CH_3)_3)CHO^+Si(CH_3)_3$ or $CH_2(OSi(CH_3)_3)CH_2CHO^+Si(CH_3)_3$. Peaks in the mass spectrum of the aglycone at *m/e* 615, 540, 450, and 360 are consistent with the interpretation that the aglycone is a C-26 compound with three alcohol functions. The Me_3Si derivative of such a compound would have a molecular weight of 630 daltons. Loss of a methyl group or loss of one, two or three trimethylsilanol molecules would give rise to the peaks with *m/e* 615, 540, 450, and 360.

Permethylation of lipid III was followed by acid catalyzed methanolysis and formation of the Me_3Si derivative of the partially methylated aglycone. This compound was eluted from the gas–liquid chromatographic column, programmed at 4°/min, at 277°. Only hydroxyl groups involved in a glycosidic linkage and therefore not methylated by the permethylation reaction should have been trimethylsilylated.

The mass spectrum of the partially methylated, partially silylated aglycone (Figure 4a) has prominent peaks at *m/e* 59 ($CH_3{-}CH{=}O^+CH_3$), 103 ($CH_2{=}O^+Si(CH_3)_3$), and 161 ($(CH_3)_3SiOCH_2CH_2CH{=}O^+CH_3$).

Alternative interpretations of the peak at *m/e* 161 are excluded because the mass spectrum shows that the compound contains only one Me_3Si group, while the peak at *m/e* 103 shows that this group—and thus, in the intact lipid, the sugar—is present on the terminal hydroxyl of the aglycone. Peaks at *m/e* 514, 499 (M − 15), and 424 (M − 90) further confirm the structure of the compound as the mono(trimethylsilyl), dimethyl derivative $CH_2(OSi(CH_3)_3)CH_2CH(OCH_3)(CH_2)_{21}CH(OCH_3)CH_3$.

The structure of lipid III is thus 1-(*O*-α-D-glucopyranosyl)-3,25-hexacosanediol, together with about 10% of the α-D-galactoside.

Lipid IV

The sugar moiety of lipid IV was isolated after acid-catalyzed methanolysis, and hydrolysis of the methyl hexoside in 1 N CF_3COOH. Of the isolated reducing sugar, 90.4% was estimated as D-glucose, and 10.4% was estimated as D-galactose. The sugar content of lipid IV, in comparison with a glucose standard and a galactose standard, was estimated at 26.9% by the anthrone method.

The infrared spectrum of lipid IV is similar to that of lipid III. As in the spectrum of lipid III, no absorption attributable to unsaturation or to a carboxylic acid or ester is observed. An absorption band at wave number 925 cm^{-1} indicates that the sugar is present as a ring. A weak absorption at 840 cm^{-1} suggests an α configuration for the linkage between the sugar and the lipid.

In the 100-MHz nmr spectrum of 5 mg of lipid IV, a peak at δ 1.23 reveals the presence of a long, saturated hydrocarbon chain. As with the nmr spectrum of lipid III, a doublet at δ 1.02 with a coupling constant of about 6 cps shows the presence of a single terminal methyl group, indicating that the carbon skeleton is unbranched. A further similarity of lipid IV with lipid III is indicated by a small doublet found at δ 4.63, with a coupling constant of 3.7 cps, which confirms that the sugar is present as an α-pyranoside.

Gas–Liquid Chromatographic and Mass Spectral Studies on Lipid IV. The Me_3Si derivative of lipid IV was introduced into the mass spectrometer by a direct inlet system heated to

110°. Peaks characteristic of trimethylsilyl hexosides (*m/e* 467, 451, 377, 361, 305, 217, 204, 147, and 103) are present (Figure 2, IV), although less prominent in this spectrum than in the spectrum of the Me_3Si derivative of lipid III. As in the mass spectrum of lipid III, the dominant peak at *m/e* 117 is clearly derived from an $\omega - 1$ hydroxyl group. The series of peaks with *m/e* 747, 657, 567, 477, and 387 and with *m/e* 703, 613, 523, and 433 can be ascribed to the sequential loss of trimethylsilanol molecules.

When the Me_3Si derivative of the aglycone of lipid IV was subjected to gas–liquid chromatography isothermally at 260°, it was eluted 17.5 min after injection. When the oven temperature was programmed at 5°/min the peak appeared at an oven temperature of 292°.

The peak of highest mass (*m/e* 656) in the mass spectrum of the aglycone (Figure 4c) is most easily interpreted as corresponding to M − 90, where M = 746 is the mass of the fully silylated aglycone, and is also the mass of a C-28 hydrocarbon chain with four silylated hydroxyl groups. The peaks at *m/e* 103 and 117 show that there are hydroxyl groups at an ω and an $\omega - 1$ position at opposite ends of the aglycone, while the peaks at *m/e* 219 and 233 show that an $\omega - 2$ and an $\omega - 3$ hydroxyl are also present. The peak at *m/e* 233 is relatively small because of the preferential abscission of smaller structural subunits. Thus, the two structures for the aglycone of lipid IV which are consistent with this spectrum are (A) $CH_2(OH)CH_2CH_2CH(OH)(CH_2)_{21}CH(OH)CHOHCH_3$ and (B) $CH_2(OH)CH_2CH(OH)(CH_2)_{21}CH(OH)CH_2CHOHCH_3$.

According to this interpretation, the peaks at *m/e* 566, 476, and 386 arise by loss of 2, 3, and 4 trimethylsilanol molecules. In addition, the peaks at *m/e* 629 and 615 can be explained by the loss of fragments of mass 117 ($CH_2CH_2OSi(CH_3)_3$) and 131 ($CH_3CH(OSi(CH_3)_3)CH_2$). Subsequent losses of trimethylsilanol molecules give rise to peaks at *m/e* 539 and 525, 449 and 435, and 359 and 345. These fragments can be derived from both structures A and B.

Permethylated lipid IV was subjected to acid-catalyzed methanolysis and the aglycone thereby liberated was reacted with *N,O*-bis[(trimethylsilyl)trifluoroacetamide]. When the mixture was subjected to combined gas–liquid chromatography–mass spectrometry, with the oven temperature programmed at 5°/min, the main product was eluted at 280°.

Of the prominent peaks in the mass spectrum of the silylated aglycone, only the peak at *m/e* 103 is present in the spectrum of the aglycone of the permethylated lipid (Figure 4b), suggesting that the terminal hydroxyl is involved in the glycosidic linkage. The peak at *m/e* 59 can be assigned to a methylated $\omega - 1$ hydroxyl ($CH(=O^+CH_3)CH_3$). The peak at *m/e* 161 can be assigned to a C-3 fragment containing one methylated hydroxyl and one trimethylsilylated hydroxyl.

Since the peak at *m/e* 233 in the mass spectrum of the trimethylsilyl aglycone is absent from the spectrum of the aglycone of the methylated lipid, one or both of the Me_3Si groups of the corresponding C-4 unit must be replaced by a methyl group. This would give rise to a peak with *m/e* 175 or 117. Since a peak with *m/e* 175 is totally absent, the minor peak at *m/e* 117 should be assigned the structure: $CH_2(OCH_3)CH_2CH_2CH{=}O^+CH_3$ (a) or $CH_3CH(OCH_3)CH_2CH{=}O^+CH_3$ (b). However, since the terminal hydroxyl is trimethylsilylated (peak at *m/e* 103), structure a with a terminal methylated hydroxyl is excluded, and hence structure B for the aglycone must be preferred. A tentative structure for the partially methylated, partially silylated aglycone is therefore: $CH_2(OSi(CH_3)_3)CH_2CH(OCH_3)(CH_2)_{21}CH(OCH_3)CH_2CH(OCH_3)CH_3$.

According to this interpretation, the peak at *m/e* 482 corresponds to M − 90, and no further loss of trimethylsilanol occurs. Since only the terminal hydroxyl is trimethylsilylated, this hydroxyl must be involved in the glycosidic linkage with the hexose. The structure of lipid IV is thus: 1-(*O*-α-D-glucopyranosyl)-3,25,27-octacosanetriol, together with lesser amounts (about 10%) of the α-D-galactoside.

Discussion

The four lipids analyzed are quite different in chemical behavior, but are structurally similar in that all of them contain a long, straight, saturated hydrocarbon chain with a glucose or a galactose at one end and a free $\omega - 1$ hydroxyl at the other end.

Acylated sugars have been isolated from a number of sources, including mycoplasm (Smith and Mayberry, 1968), bacteria (Welsh *et al.*, 1968), yeast, and a fungus (Brennan *et al.*, 1970a). Monoacyl glucoses have also been isolated from corynebacteria and mycobacteria (Brennan *et al.*, 1970b). In those instances in which the positions of the acyl groups on the sugars were determined, they were not linked to C-1 of the sugar. However, a link between the C-1 hydroxyl of glucose and a carboxyl group does occur in one class of natural products, namely the hydrolyzable tannins (Fischer and Bergmann, 1918), so that there appears to be no reason to doubt the validity of the structures proposed for lipids I and II.

The structures of the aglycones of lipids III and IV have been studied by Bryce *et al.* (1972), whose results are confirmed by ours. Because Bryce *et al.* included a saponification step in their purification procedure and purified the aglycones only after acid hydrolysis, the acyl lipids (lipids I and II) were not studied and the positions of the sugar moieties in lipids III and IV could not be determined. Our results from methylation of lipids III and IV clearly show the positions of the sugars in both lipids, and more firmly establish the validity of the structure proposed by Bryce *et al.* for the aglycone of lipid IV.

As has been demonstrated by Winkenbach *et al.* (1972), the glycolipids described in this paper are present in a laminated layer in the envelope of the heterocyst of *A. cylindrica*. Since the thickness of the laminae, 35–40 Å, corresponds approximately to the length (aglycone 33 Å, hexose 5 Å) of lipid III, the major lipid, the hydrophobic hydrocarbon chains may be closely packed while the hydrophilic ends form the top and the bottom of each lamina. Such an assemblage could provide a very efficient barrier to the penetration of lipophobic chemicals, and perhaps oxygen, into the heterocysts.

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Analyses of Oxygen Equilibria of Native and Chemically Modified Human Adult Hemoglobins on the Basis of Adair's Stepwise Oxygenation Theory and the Allosteric Model of Monod, Wyman, and Changeux†

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ABSTRACT: Precise oxygen equilibrium curves of human adult hemoglobin and chemically modified derivatives prepared by the treatment of the hemoglobin with iodoacetamide, *N*-ethylmaleimide, or carboxypeptidase A were determined in the absence and presence of 2,3-diphosphoglycerate. These equilibrium data were analyzed according to Adair's stepwise oxygenation theory and the allosteric model of Monod, Wyman, and Changeux. The parameters involved in the oxygen saturation function of the theory and model were estimated with considerable accuracy by a least-squares method. It has been shown that 2,3-diphosphoglycerate increases the cooperativity of oxygenation of iodoacetamide-treated and *N*-ethylmaleimide-treated hemoglobins by reducing their affinity to the first, second, and third oxygen molecules without affecting the affinity to the fourth molecule as previously observed in native hemoglobin. Both the oxygen affinity and the cooperativity of carboxypeptidase-treated hemoglobin were scarcely affected by the phosphate. The oxygen association constant for R state, K_R, was insensitive to 2,3-diphosphoglycerate whereas the association constant for T state, K_T, was markedly reduced by the phosphate except for carboxypeptidase-treated hemoglobin. Both the chemical modifications of protein and the addition of 2,3-diphosphoglycerate influence not only the allosteric constant, L, but also the ratio c ($=K_T/K_R$), contrary to early assumptions. From estimates of Monod–Wyman–Changeux's parameters, the principal courses of oxygenation in the T- and R-state system were deduced. The degree of ligation at which the allosteric transition takes place and the switching rate of transition at that degree of ligation were also obtained. 2,3-Diphosphoglycerate shifts the switching point toward later stages of oxygenation and increases the switching rate, making the oxygenation course and oxygen equilibrium curves asymmetrical. The phosphate increases cooperativity by reducing c; however, this effect is partially canceled out by the enhancement of the deviation of Lc^2 from unity. All these effects caused by the phosphate can be explained by assuming that the phosphate preferentially combines with the T-state molecules. The dependence of c and K_T on the allosteric effector, 2,3-diphosphoglycerate, was not anticipated in the original model and the present study suggests that the model needs modifications to explain the heterotropic effects in hemoglobin.

Hemoglobin has excited much investigation because of its physiologically and physically interesting action in oxygen binding which results from heme–heme interactions: indirect interactions mediated by conformational changes in the protein moiety between the distinct specific sites for oxygen binding.

In the discussion of oxygen equilibrium characteristics of hemoglobin, the oxygen affinity and the magnitude of cooperativity of oxygenation have usually been expressed by the oxygen pressure for 50% oxygen saturation, P_{50}, and Hill's coefficient, n, respectively (Rossi-Fanelli *et al.*, 1964; Antonini and Brunori, 1971). These measures, however, have no direct physical meaning and are too simple to describe quantitatively the functional behavior of hemoglobin. Roughton and his coworkers (Roughton *et al.*, 1955; Roughton, 1963; Roughton

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