Chemical Structure of a Novel Glycolipid from an Extreme Thermophile, *Flavobacterium thermophilum*†

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ABSTRACT: The lipid (12.2% wt/dry cell) of an extreme thermophile, *Flavobacterium thermophilum HB-8* comprised 8% of acetone-soluble lipid (including yellow pigments), 21% of phospholipids, and 71% of glycolipids. The purified major glycolipid was found to contain galactose, glucose, glucosamine, glycerol, fatty acid esters, and fatty acid amide in the ratio of 2:1:1:1:2:1. The esterified fatty acids were mainly 13-methyltetradecanoic and 15-methylhexadecanoic acid. The fatty acid amide was in the form of 15-methylhexadecanoylglucosaminide. Gas liquid chromatography after permethylation indicated the presence of 2,3,5,6-tetra-*O*-methylgalactofuranoside, 3,4,6-tri-*O*-methylglucopyranoside, 3,4,6-tri-*O*-methylgalactopyranoside, and 3,4-di-*O*-methylglucosaminide. The identification of terminal galactofuranose was also confirmed by gas liquid chromatography–mass spectrometry analysis. The sugar sequences of this glycolipid was determined chemical and enzymic hydrolysis. The structure of this glycolipid is proposed to be: Gal*f*-(1→2)-Gal*p*-(1→6)-GlcN (15-methylhexadecanoyl)-(1→2)-Glc*p*-diglyceride.

The extreme thermophiles, recently isolated from hot springs, are microorganisms that grow at temperatures above 70°. They are resistant to high temperatures (thermotolerant) and even glow better at high temperatures (thermophile).

Such bacteria are of particular interest to biologists, because the mystery of thermophilicity is not yet fully explained on a molecular basis. They are also of interest to space biologists, because such organisms might occur on other planets, either restricted ecologically or more widely distributed as a consequence of some early high-temperature stage of planetary evolution.

Oshima and Imahori (1971) isolated an extreme thermophile, *Flavobacterium thermophilum* HB-8 (sp. nov.) from Mine hot springs, Japan. Attempts to explain its thermophilicity have been centered so far around the enzymes, nucleic acids, and ribosomes of the organism (Yoshida and Oshima, 1971; Yoshizaki *et al.*, 1971; Yoshida, 1972; Oshima, 1972). Studies on the stability of the spheroplasts obtained by digestion of the cell walls of the extreme thermophiles suggest that the molecular mechanism of thermophilicity is more closely related to the thermostability of the cell membrane than of to specific macromolecules. The spheroplasts of an extreme thermophile, *Thermus aquaticus*, were found to be stable in distilled water and to maintain their shape even in boiling water (Brock, 1969). Those of *F. thermophilum* also showed remarkable stability during osmotic shock at temperatures as high as 70° and as low as 4° (Oshima, 1972).

This stability may depend upon the strong binding or association of cell membrane components, such as complex lipids, proteins and other membrane constituents (Oshima, 1971). We are interested in the molecular explanation of the stability of the thermophile cell membrane, and have initiated studies on this by analysis of the individual lipid components of the bacterium.

In a previous report (Oshima and Yamakawa, 1972), two possible structures of a novel glycolipid isolated from this bacterium were proposed.

The present paper describes determination of the structure of this glycolipid.

Materials and Methods

An extreme thermophile, *F. thermophilum* HB-8 (sp. nov.), was cultivated in a 50-l. container, at 75°, with vigorous aeration. The cells were harvested after three repeated cultivations in medium consisting of 0.8% (w/v) polypeptone, 0.4% yeast extract, and 0.2% NaCl. Materials for packings of columns and for liquid phases in gas chromatography were obtained from Applied Science Laboratory (State College, Pa.) or from Gaschro-Kogyo Co. (Tokyo). Silylating reagents and methyl palmitate, used as an internal standard, were products of Sigma Chemicals Co. (St. Louis, Mo.). The standard 3,4,6-tri-*O*-methylgalactose was kindly provided by Dr. P. Stoffyn of McLean Hospital, Belmont, Mass., and other methylated sugar derivatives were prepared in our laboratory.

Streptococcal kojibiosyl-(1-1)-glycerol was prepared by Dr. Ishizuka in this laboratory. Bovine brain ganglio-*N*-tetraose used as a standard in Avicel tlc[1] was a generaous gift from Dr. H. Wiegandt (Univ. of Marburg, Germany).

Hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glycerokinase (EC 2.7.1.30), glycerolphosphate dehydrogenase (EC 1.1.1.8), galactose dehydrogenase (EC 1.1.1.48), and the coenzymes used were obtained from Boehringer Mannheim (Germany). Purified fig α-galactosidase (EC 3.2.1.22) and jack bean β-galactosidase (EC 3.2.1.23) were kindly supplied by Dr. Yu-Teh Li of Tulane University.

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[1] Abbreviations used are: Gal*f*, galactofuranoside; Glc*p*, glucopyranoside; GlcN(15-methylhexadecanoyl), 15-methylhexadecanoylglucosaminide; tlc, thin-layer chromatography; glc, gas liquid chromatography; pmr, proton magnetic resonance spectra; gc-ms, gas liquid chromatography–mass spectrometry.

Technical grade organic solvents were distilled before use. Pyridine and dimethylformamide were distilled and stored over molecular sieve 3A (Gaschro-Kogyo Co. Tokyo).

Gc-ms spectra of methylated sugar derivatives were kindly determined by Mr. Ariga of Sankyo Co. using a Shimadzu-LKB Model 9000 gc-ms spectrometer. For gas chromatography the column was packed with Anachrom SD (Analabs Inc., 90–100 mesh) coated with 1% silicone OV-17 (Applied Science Laboratory, Inc.).

The crude lipids of the organism were extracted with 20 volumes (2/v) of chloroform–methanol (2:1, v/v). Details of the extraction procedure were reported previously (Oshima and Yamakawa, 1972).

Acetone Fractionation of Total Lipids. The crude lipid extracted from the bacteria was dissolved in 1 volume of chloroform–methanol (2:1, v/v) and lipids were precipitated by addition of 9 volumes of acetone at 4°. The acetone-soluble neutral lipids (mainly pigments) were discarded and the acetone-insoluble precipitate was subjected to column chromatography.

Column Chromatography. Material precipitated with acetone was dissolved in chloroform and applied to a column of silica gel (100 mesh, Kanto Kagaku Co., Tokyo). After elution with 2 column volumes of chloroform, 4 column volumes of chloroform–methanol (C–M) 90:10 (v/v), and 2 volumes of C–M 80:20 (v/v), the lipids were fractionated by gradient elution with from C–M 70:30 (v/v) to C–M 60:40 (v/v). Rechromatography was carried out on columns of silicic acid, Silic AR CC-7 (Mallinckrodt Co., 200–325 mesh) using a gradient of C–M 85:15 (v/v) to C–M 75:25 (v/v) followed by a gradient of C–M 75:25 (v/v) to C–M 55:45 (v/v). In both cases 10–15 mg of lipid/g of silicic acid was applied on the column.

DEAE-cellulose (Seikagaku-kogyo, Japan) was treated with glacial acetic acid and then washed thoroughly with methanol, following the method of Rouser *et al.* (1967). The solvent in the column was gradually replaced by chloroform and the lipids were grossly fractionated using 7 column volumes each of the solvent mixtures C–M 90:10, C–M 70:30, methanol, and C–M–ammonia–salt (Rouser *et al.*, 1967) mixture. The effluent with each solvent mixture was collected. Elution was monitored by analyzing aliquots of effluent for hexose or by tlc using solvent system A or B.

Thin-Layer Chromatography. A glass plate 20 × 20 cm was coated with a 0.25-mm thick layer of silica gal (Wako-gel B-5) and activated by heating at 110° for 3 hr. Samples were applied in streaks 7-mm wide and separated with the following solvent systems: (A) chloroform–methanol–water (65:35:8, v/v); (B) chloroform–methanol–acetic acid–water (65:25:10:8, v/v); (C) chloroform–methanol–28% ammonia–water (65:35:2:6, v/v); (D) chloroform–methanol–acetone (85:25:15, v/v). Lipids were located by exposing the plate to iodine vapor or spraying it with 0.5% ninhydrin in 1-butanol, acid molybdate (Dittmer and Lester, 1964), and periodate–Schiff (Shaw, 1968) reagents. A plate coated with a 0.8-mm thick layer of Wako-gel (B-O) was used for preparative tlc.

A precoated cellulose plate (Avicel SF, Funakoshi Chemicals Co.), 10 × 10 cm, was used for deacylated glycolipid or free sugars, using a solvent mixture of ethyl acetate–pyridine–acetic acid–water (5:5:1:3, v/v). The spots were visualized with periodate–Schiff or alkaline–$AgNO_3$ reagent (Trevelyan *et al.*, 1950).

Gas Chromatography. A Shimadzu GC-5A (Shimadzu Ind. Co., Kyoto) or F&M Model 402 (Hewlett-Packard Ind., Palo Alto, Calif.) apparatus with flame ionization detectors were used for glc. Glass columns of 3 mm (i.d.) × 4 m packed with 25% ethylene glycol succinate polyester on Gas-Chrom CLZ were used for analyses of fatty acid methyl esters. Me_3Si derivatives of carbohydrate were analyzed using columns packed with 3.8% methyl vinyl silicone polymer UC W-98 on Diatoport S, 8% tripalmitin (Ando *et al.*, 1973) and 7% Ucon 550X grease on Celite 545 (silanized with $Me_3SiOSiMe_3$).

The methylated methyl glycosides of neutral sugars were analyzed with a 3 mm × 2 m column of 5% poly(neopentyl glycol succinate) on GasChrom CL7 (100 mesh). 6-*O*-Trimethylsilyldi-*O*-methyl-*N*-acetylglucosaminide was analyzed with a 3 mm × 1 m column of 3% OV-1 on Celite 545 (silanized with $Me_3SiOSiMe_3$).

For gas chromatographic separation of carbohydrate and fatty acid methyl esters, glycolipid (*ca.* 1–2 mg) was dissolved in 1 ml of 0.8 N HCl in dry methanol and heated in a Pyrex glass tube with a Teflon-lined screw cap for 24 h at 80°. The nonpolar compounds released were extracted three times with 1-ml portions of petroleum ether (bp 30–60°). The extracts were combined and washed with water, dehydrated over anhydrous Na_2SO_4, concentrated to a small volume, and analyzed for fatty acid methyl esters. The methanolic phase was neutralized by addition of a small excess of Ag_2CO_3 and kept overnight with 0.2 ml of acetic anhydride to allow N-acetylation of hexosamine. Trimethylsilylation was performed by the method of Sweeley *et al.* (1963) modified by Yamakawa and Ueta (1964). For determination of amide-bound fatty acid, glycolipid (1.2 mg) which had been treated with weak alkali was mixed with methyl palmitate (460 μg) as internal standard and heated with 0.8 N HCl in dry methanol at 100° for 4 hr. The released fatty acid methyl ester was extracted with petroleum ether and analyzed by glc. The content of amide-bound fatty acid was calculated from the peak area by comparison with that of the internal standard.

Analytical Methods. For infrared spectrometry, *ca.* 2 mg of samples was pressed in KBr and scanned with a Hitachi EPI-2 spectrometer.

For pmr studies, 10–50 mg of samples was treated repeatedly with D_2O or methanol-d_4 (Merck, Darmstadt, Germany) to allow exchange of labile protons with deuterium. The final residue was dissolved in 0.4 ml of water-d_2, methanol-d_4, or chloroform-*d*–methanol-d_4 (2:1, v/v). The spectra were recorded with tetramethylsilane or acetone as internal standard at 35° or 80° with a Hitachi Perkin-Elmer-R20A spectrometer (90 MHz).

The hexose content of intact glycolipid was determined using anthrone–sulfuric acid (Yamakawa *et al.*, 1960) with a mixture of galactose and glucose (2:1) as a standard. Esters were determined by the alkaline hydroxamate method of Snyder and Stephens (1959) with methyl palmitate (Sigma Chemicals Co.) or tripalmitin (Tokyo-kasei Co.) as standard.

Glucosamine was determined by the Elson–Morgan reaction (Gatt and Berman, 1966), or analyzed in a Hitachi KLA-3B amino acid analyzer after hydrolysis with 2 N HCl or 100° for 12 hr.

Enzymic Determinations. A mixture of 0.6 ml of 1 N HCl and 225 μg of glycolipid dissolved in methanol (0.2 ml) was heated at 100° in a screw-capped Pyrex tube for 3 hr. The liberated free fatty acids were extracted with petroleum ether and then the hydrolysate was neutralized with dilute NaOH and adjusted to 5.0 ml. About 100–300 μl of the hydrolysate was used for each enzymic determination.

Galactose was determined by measuring reduction of NAD at 340 nm with added galactose dehydrogenase, following the method of Slein (1963). Glycerol was phosphorylated with

glycerokinase and the reduction of NAD with glycerolphosphate dehydrogenase was measured at 340 nm by the method of Wieland (1963). Glucose was determined by the reduction of NADP with Glucose-6-phosphate dehydrogenase after phosphorylation with hexokinase (Finch *et al.*, 1969).

Permethylation. About 2 mg of glycolipid or deacylated glycolipid were dissolved in 0.25 ml of dimethylformamide. Barium oxide (50 mg), barium hydroxide (20 mg), and methyl iodide (0.25 ml) were added in this order and the mixture was stirred at 40° for 2 hr, and then stood at room temperature for 12 hr, following the method of Kuhn and Egge (1973) as modified by Adams and Gray (1968). The permethylated sample was subjected to methanolysis with 3% HCl in dry methanol at 100° for 3 hr. The fatty acid methyl esters were extracted with petroleum ether and the lower methanol phase was subjected to glc.

Periodate Oxidation. The glycolipid (5–10 mg) was suspended in a mixture of 0.2 ml of chloroform and 0.7 ml of ethanol, and oxidation was initiated by addition of a measured amount of 0.2 M aqueous periodic acid, following the method of Carter *et al.* (1961). The mixture was shaken vigorously and then allowed to stand overnight at room temperature in the dark. One drop of ethylene glycol was added to reduce the excess reagent and the mixture was evaporated to dryness. The glycolipid oxidized with periodate was subjected to methanolysis and then to glc for determination of unoxidized sugar residue.

Deacylation of Glycolipid. MILD ALKALINE HYDROLYSIS. Glycolipid (10–50 mg) was dissolved in chloroform (3 ml) and hydrolyzed with 1 ml of 0.4 N methanolic NaOH at 37° for 15 min, following the method of Ballou *et al.* (1965). The hydrolysate was adjusted to pH 2 with Dowex 50-X8 (H^+) and partitioned with 6 ml of water. The lower organic phase was washed with water and the water phase and washing fluid were combined and evaporated to dryness *in vacuo*.

STRONG ALKALINE HYDROLYSIS. The glycolipid (10 mg) was hydrolyzed with 1 N KOH in 90% 1-butanol (1.5 ml) in a screw capped Pyrex tube at 120–125° for 2 hr, following the method of Taketomi and Yamakawa (1963). The hydrolysate was washed with an equal volume of water. The water phase and washing fluid were combined and neutralized with $HClO_4$. The precipitated $KClO_4$ was discarded, and the water phase was evaporated to dryness. A small amount of water was added to the residue and the insoluble material was filtered off. The clear aqueous solution contained the completely deacylated glycolipid with free amino groups, as indicated by the ninhydrin reaction.

Determination of Sugar Sequences. The hydrolysate with strong alkali was further hydrolyzed with 1 ml of 1 N HCl at 100° for 3 hr. The product was applied to a column (8 mm × 10 cm) of Dowex 50-X8 (H^+) (*ca.* 5 ml). The neutral sugars in the hydrolysate were eluted with 10 volumes of distilled water and the fraction containing amino sugar was eluted with 5 volumes of 0.3 N HCl. The effluent was evaporated to dryness and its sugar content was analyzed by glc.

Mild Acid Deglycosylation of Galactofuranoside. The glycolipid or glycolipid after mild alkali treatment (10 mg) was hydrolyzed with 2 ml of 0.1 N HCl in 50% methanol, at 80° for 30 min. The hydrolysate was partitioned with 4 ml of chloroform. The lower chloroform layer was evaporated to dryness and subjected to silica gel preparative tlc. The bands on tlc visualized with iodine vapor were scraped off and the sugar content of each was analyzed by glc. The band containing galactose, glucose, and glucosamine in a ratio of 1:1:1 was subjected to enzymic degradation or to permethylation.

Enzymic Degradation. The intact glycolipid, alkali-deacylated glycolipid, and the sample obtained by mild acid deglycosylation (1–2 mg) were each emulsified with 1 ml of 1% (w/v) sodium taurocholate in sodium citrate buffer (pH 4.5 or 5.5). The solutions were incubated with α- or β-galactosidase for 48 hr, and the galactose residue released was determined enzymatically.

Results

The behavior of the new glycolipid on a silicic acid column differed from that of the diglycosyl diglyceride observed by Vorbeck and Marinetti (1965) in that it was not eluted with pure acetone, although it could be eluted with over 25% (v/v) methanol in chloroform.

The glycolipid eluted from a DEAE-cellulose column with C–M (70–30, v/v) was free from phospholipids and its characterization is now undertaken. However, phospholipids could not be removed from the glycolipid using a Florisil (Radin *et al.*, 1956) or hydroxylapatite column (Slominary and Horowitz, 1970). The white glycolipid recovered was purified by repeated precipitation in methanol with cooling. The pure glycolipid was obtained in a final yield of 460 mg from 63 g of dried cells.

On tlc, the spot of the glycolipid gave a positive reaction with anthrone–sulfuric acid (an unusually brownish spot) and periodate–Schiff's reagent. It gave a negative reaction for phosphorus with acid molybdate and for free amino groups with ninhydrin reagent. The R_F values of this glycolipid have been reported elsewhere (Oshima and Yamakawa, 1972). The glycolipid showed $[\alpha]_D^{22}$ +38.5 ± 0.5 (*c* 0.4, chloroform–methanol, 1:1, v/v). *Anal.* Calcd for $C_{76}H_{140}NO_{25}$ (mol wt 1461): C,62.5; H, 9.59; N, 0.96. Found: C, 61.6; H, 9.45; N, 1.02.

Enzymic and gas chromatographic analyses indicated the presence of galactose, glucose, and glucosamine in a molar ratio of 2:1:1, as shown in Table I.

TABLE I: Analysis of the Sugar Components of Glycolipid.

Method	Molar Ratio		
	Gal	Glc	GlcN
Enzymic determination[b]	2.00 (22.4%)[a]	1 (11.2%)[a]	
Gas chromatographic determination[c]			
8% tripalmitin on Celite 545 (190° (2°/min) to 210°)	2.02	1	0.82
3.8% UC-W 98 on Diatoport S (166° (4°/min) to 245°)	1.98	1	0.80
7% Ucon 550X on Celite 545 (isothermal)	1.95	1	

[a] Percentage weight of the glycolipid. [b] See text for details. [c] Determined as trimethylsilyl derivatives.

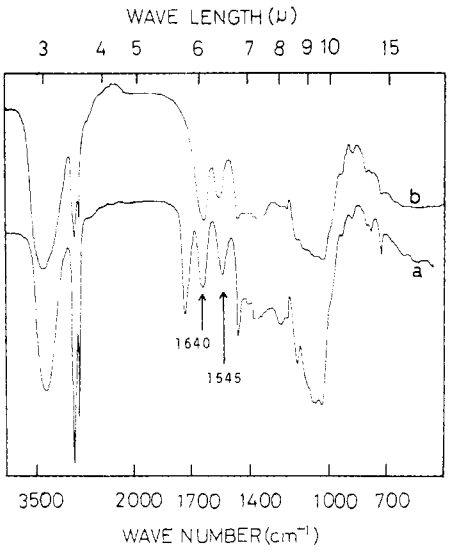

FIGURE 1: Infrared spectra of the new glycolipid before (a) and after mild alkaline treatment (b).

The content of hexose (36.2%) measured with anthrone–sulfuric acid and of glucosamine (13.5%) measured by the Elson–Morgan reaction agreed well with the calculated values of 36.8 and 14.1%, respectively.

The ester value of this glycolipid was 1.2 μequiv/mg (1.98 equiv/mol) and its glycerol content, determined enzymatically, was 63 μg/mg (1.09 mol/mol).

Mild alkaline treatment liberated 2 mol of fatty acids of the following compositions/mol of the glycolipid: 15-methylhexadecanoic acid, 58.5%; 13-methyltetradecanoic acid, 33.6%; 14-methylpentadecanoic acid, 4.3%; 17-methyloctadecanoic acid, 2.3%; palmitic acid, 1.2%; 14-methylhexadecanoic acid; trace. These observations suggest that 2 mol of fatty acids was esterified to a glycerol moiety of the glycolipid.

Infrared spectra showed clear bands of amide I (1545 cm^{-1}) and II (1640 cm^{-1}) before and after treatment with weak alkali (Figure 1a,b).

The results of elemental analyses of the glycolipid after treatment with weak alkali were as follows. *Anal.* Calcd for $C_{44}H_{80}NO_{23}$ (mol wt 990): C, 53.3; H, 8.0; N, 1.4. Found: C, 50.2; H, 7.8; N, 1.4.

The amide linkage of the glycolipid was hydrolyzed with strong alkali, and hydrolysis was complete as demonstrated by the ninhydrin reaction and the infrared spectrum. The ninhydrin-positive hydrolysate gave a spot about ganglio-*N*-tetraose composed of Gal-(1→3)-GalNAc-(1→4)-Gal-(1→4)-Glc, on Avicel tlc. On the other hand, after mild alkaline hydrolysis a spot with a very high R_F value and tailing was seen, suggesting the presence of a long chain acyl group.

The pmr spectrum of the intact glycolipid indicated the presence of an isomethyl proton (0.85 ppm) and methylene proton (1.22 ppm) and the absence of an *N*-acetyl proton (2.1 ppm) (Figure 2a,b). The spectra of the glycolipid after hydrolysis with mild alkali also showed isomethyl protons. These observations, together with the result of tlc and infrared analysis, suggest that the amino group of glucosamine is not N-acetylated but probably substituted by a long chain iso-branched acid.

After treatment with weak alkali the glycolipid (1.2 mg) still contained 269 μg (0.83 mol/mol of glycolipid) of 15-methylhexadecanoic acid, as detected by glc. This observation, together with the infrared spectrum pmr analysis and the behavior on tlc, proved that one mole of 15-methylhexadecanoic acid was linked to the amino group of the glucosamine residue.

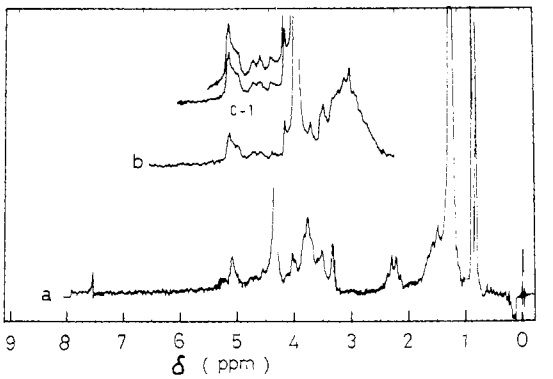

FIGURE 2: (a) Pmr spectrum of the intact glycolipid taken with chloroform-*d*–methanol-d_4 (2:1, v/v) as solvent at 35°. Tetramethylsilane was used as an internal standard. (b) Anomeric proton region of the pmr of partially deacylated glycolipid taken with D_2O at 80°.

Figure 3 shows results on permethylation of the glycolipid. The presence of methyl 2,3,5,6-tetra-*O*-methylgalactofuranoside indicates the presence of a terminal galactofuranosyl residue. Substitution at C-2 of galactopyranose and of glucopyranose were demonstrated by the presence of glc peaks of methyl 3,4,6-tri-*O*-methylgalactopyranoside and methyl 3,4,6-tri-*O*-methylglucopyranoside. Methyl 3,4-di-*O*-methylglucosaminide was demonstrated by glc after trimethylsilylation, which indicated the presence of C-6 substitution of the glucosamine residue. The terminal galactofuranosyl residue was further confirmed by gc-ms spectromethy (Oshima and Yamakawa, 1972).

All the sugar residues in the glycolipid were susceptible to periodate oxidation and no sugar component of the glycolipid remaining unoxidized was detected by glc. This confirms that the glycosidically linked sugar chain was straight, not branched, and also confirms the C-6 substitution of the N-acylated glucosamine, since C-3 or C-4 substitution of glucosamine results in resistance to oxidation.

The glycosidic linkage of the terminal galactofuranosyl residue was hydrolyzed with mild acid, leaving the other three

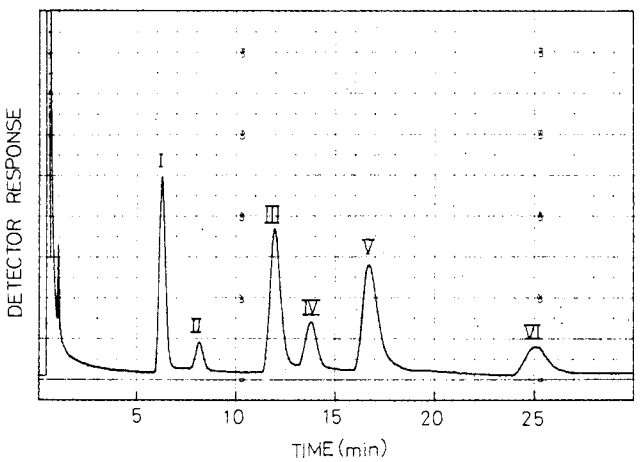

FIGURE 3: Gas liquid chromatogram of permethylated sugar residue of the glycolipid. Analyzed with 5% poly(neopentyl glycol succinate) on Gas Chrom CLZ, 6-ft glass column at 160°. Peak I: methyl 2,3,5,6-tetra-*O*-methyl-β-galactofuranoside; II: methyl 2,3,5,6-tetra-*O*-methyl-α-galactofuaranoside; III, IV: methyl 3,4,6-tri-*O*-methyl-α- and β-glucopyranoside; V, VI: methyl 3,4,6-tri-*O*-methyl-α- and β-galactopyranoside.

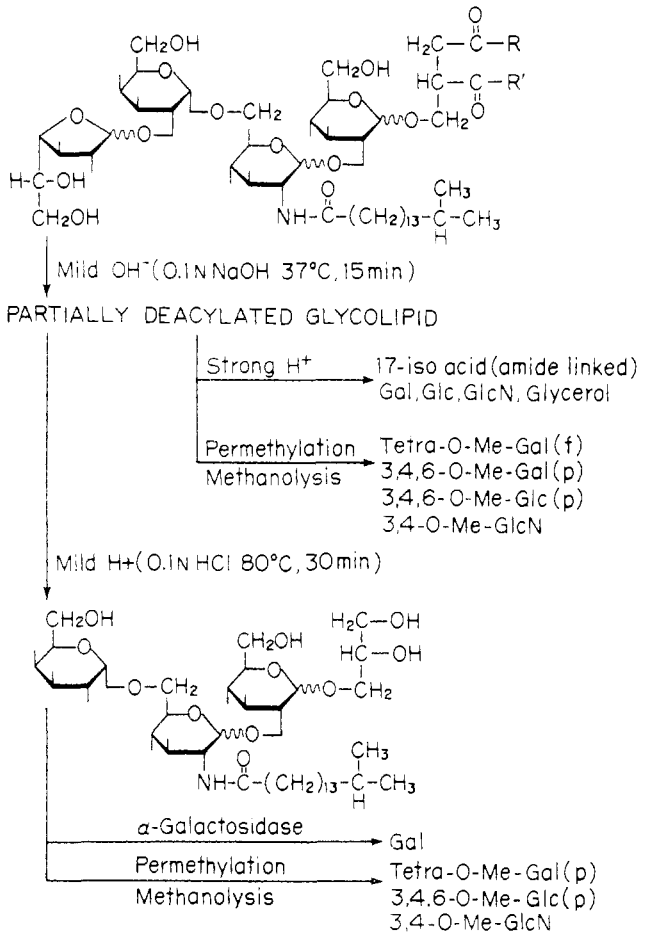

FIGURE 4: Scheme of degradation of the glycolipid demonstrated by glc.

glycosidic linkages of galactose, glucose, and glucosamine intact (see Figure 4).

Permethylation of the glycolipid after mild acid hydrolysis proved the presence of 2,3,4,6-tetra-*O*-methylgalactopyranose, indicating that the penultimate sugar was galactopyranose. α-Galactosidase released this galactose, suggesting that the penultimate galactopyranose is linked by an α-glycosidic bond.

To determine the sequence of the remaining two sugars, the acyl group was removed and then the material was treated with strong acid. When the amino group of glucosamine is free the glycosidic linkage of glucosamine is known to be resistant to acid strong enough to hydrolyze other glycosidic bonds. The presence of a glucose residue in the fraction containing amino sugar eluted from a Dowex 50 (H^+) column with 0.3 N HCl after the above treatment indicated that the glucosamine was glycosidically linked to the glucose residue in the glycolipid. Glycerol and galactose were detected in the fraction eluted from the resin with water.

The chemical degradation of the glycolipid is shown schematically in Figure 4.

From these chemical, physical, and enzymic analyses it is concluded that the structure of this new glycolipid is: Gal*f*-(1→2)-Gal*p*-(1→6)GlcN-(15-methylhexadecanoyl)-(1→2)-Glc*p*-diglyceride.

Discussion

Several strains of extreme thermophiles have been isolated from various hot springs and many attempts have been made to explain the thermophilicity of the bacteria (Brock and Freeze, 1969; Saiki and Arima, 1971; Heinen, 1971; Oshima, 1972).

There have been few studies on the lipids of these extreme thermophiles. The lipids of an extreme thermophile, *Thermus aquaticus* contained carotenoids, glycolipids, and phospholipids, and these were all in the cell membrane (Ray *et al.*, 1971). Cells of *T. aquaticus* responded to an elevation in temperature by increase in their content of total lipids and especially glycolipids. The amount of these glycolipids (mono- and diglycosyl diglycerides) increased fourfold on increasing the temperature from 50 to 70° (Ray *et al.*, 1971). An increase in the glycolipid content of the thermophilic alga, *Cyanidium caldarium* on raising the temperature was also observed by Adams *et al.* (1971).

These findings suggest that glycolipids might have some function in stabilizing the cell membranes at high temperature. The high content of the new glycolipid in the present organism also suggests its role in growth at high temperature.

Comparative studies on the glycolipid content of *Streptococcus pyogenes* and its derived L-form membrane were performed by Cohen and Panos (1966) and similar studies on *Staphylococcus aureus* were made by Ward and Perkins (1968). L-form membranes were found to contain 2- to 3-fold more diglycosyl diglyceride than protoplast membranes of the wild type. It is believed that the increased glycolipid content of the L-form membranes may reflect a compensation for lack of a rigid cell wall. It was also sggested that the glycolipid might have a role in maintaining the structural integrity of the protoplast membrane.

The content of the new glycolipid (70% of the total lipid) is very high compared to those of other glycolipids found in bacteria. Mono-, di-, and triglycosyl diglycerides are widely distributed in microorganisms, and the content of diglycosyl diglyceride (usual the predominant glycolipid in bacteria) varies from a few per cent of the total lipid in *Lactobacilli* and *Staphylococcus* (Brundish *et al.*, 1966, 1967) to 50% in *Streptococcus hemolyticus* (Ishizuka and Yamakawa, 1968).

The only glycolipid known to have four sugar residues (galactose and glucose) was found in *Lactobacillus* species (Shaw, 1970). Our new glycolipid also contained four sugar residues, but two of these were unusual sugars, namely, terminal galactofuranoside and glucosamine with a branched long-chain fatty acid in an amide linkage.

Brundish *et al.* (1967) studied the molecular shape of diglycosyl diglyceride and proposed its structural significance. They suggested that the glycolipid could adopt a conformation in which all of the hydroxyl groups lay on one side of the molecule and the lipophilic components (*i.e.*, fatty acids, the ring oxygens of sugars and glycosidic oxygen) lay on the other side. The hydrophilic regions of several molecules could come together to form pores in the membrane through which small molecules might pass (Shaw, 1970). These pores might be within the membrane or even on the surface where some involvement in binding or anchoring of intracellular components might be possible. The tetraglycosyl diglyceride studied here may have some function in regulating the size of these pores.

The new glycolipid contained iso- or anteiso-branched fatty acid but no hydroxy or unsaturated fatty acids.

The presence of an amide bound iso-branched acid (15-methylhexadecanoic acid) in the glucosamine residue of the glycolipid is very unusual. Among naturally occurring substances, this structure has only been found in the lipid A fraction of the lipopolysaccharide of *Escherichia coli* (Burton and

Carter, 1964) and *Salmonella* (Kasai, 1966) as *N*-(13-hydroxytetradecanoyl) glucosaminide.

The unusual stability of the glycosidic bond of hexosamine with a free amino group on acid hydrolysis has long been recognized (Moggridge and Neuberger, 1938). When the amide-linked fatty acid is released by strong alkali, the glycoside of the glucosamine residue becomes stable on hydrolysis with strong acid (1 N HCl, 80°, 3 hr). Using these chemical degradation reactions it was clearly shown in the present work that the glucosamine was glycosidically linked to the glucose residue in the glycolipid molecule.

Galactofuranosyl diglyceride was reported by Reeves *et al.* (1964) and Plackett (1967). These authors tentatively identified the galactofuranosyl residue by determination of the velocity constant of its acid hydrolysis and its relatively higher R_F value than those of other glycosyl diglycerides on tlc. In the present study permethylation of the glycosyl residues provided unequivocal proof of terminal galactofuranose.

Furthermore mass spectra of the methylated galactofuranoside residue of the new glycolipid showed the typical C-5 side-chain fragmentation (*m/e* 161) from the furanose ring, as proposed by Heyns and Scharmann (1965). Methylated galactopyranoside shows extensive C-6 side-chain fragmentation (*m/e* 175).

The presence of the galactofuranosyl residue in the new glycolipid was also demonstrated on tlc by the periodate–Schiff's reaction. The intact glycolipid rapidly gave a purple spot, characteristic of formaldehyde, whereas usual glycosyl diglyceride slowly developed a blue color on oxidation of carbohydrate. The rapidly developed purple color of the formaldehyde might be produced by the oxidation of the C-6 side chain of the galactofuranosyl residue of the glycolipid.

Little is known about the biosynthesis of galactofuranoside. Trejo *et al.* (1971) reported that UDP-galactofuranose was incorporated into galactocarolose, the galactofuranose polymer found in *Penicillium charlesii*. UDP-2-ketogalactose has been proposed as an intermediate in the isomerization of UDP-galactopyranose to UDP-galactofuranose (Fobes and Gander, 1972). The biosynthesis of the galactofuranosyl residue in this glycolipid is interesting and requires study.

Problems to be solved on the chemical structure of this glycolipid are determination of: (1) the anomeric configuration of each glycosidic linkage other than that of the galactopyranosyl residue which has been determined as α and (2) the steroisomeric configuration of the glycerol residue of the glycolipid.

Acknowledgments

The authors are grateful to Dr. T. Oshima and Dr. K. Imahori for providing the extremely thermophilic bacteria used. They are also indebted to Dr. P. Stoffyn of McLean Hospital for a generous gift of 3,4,6-tri-*O*-methylgalactose, to Dr. Yu-Teh Li of Tulane University for providing purified galactosidases, and to Mr. T. Ariga of Sankyo Co. for taking gc-ms spectra of the methylated sugar derivatives.

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A Comparison of the Kinetics and Stoichiometry of Proton Uptake with Aldehyde Reduction for Liver Alcohol Dehydrogenase under Single Turnover Conditions†

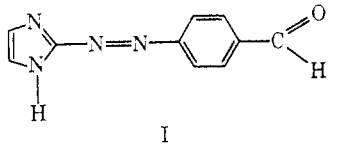
Michael F. Dunn

ABSTRACT: The equine liver alcohol dehydrogenase catalyzed reduction of aldehydes by reduced nicotinamide adenine dinucleotide (NADH) involves the uptake of 1 mol of hydrogen ion/mol of aldehyde reduced (*e.g.*, aldehyde + NADH + $H^+ \rightleftharpoons$ alcohol + NAD^+). A rapid-mixing kinetic technique utilizing a pH indicator–buffer system has been employed in this study to investigate the relationship of proton uptake to the chemical steps involved in the catalytic mechanism. Our previous studies (Bernhard, S. A., Dunn, M. F., Luisi, P. L., and Schack, P. (1970), *Biochemistry 9*, 185) have shown that reduction of aromatic aldehydes in the pH region 8–10 occurs *via* two kinetic processes, a "burst" and a slow step, each of equal concentration change under conditions of excess enzyme. These two processes are remarkably different in rate at pH 8.8 (~200 sec^{-1} and 0.2–10 sec^{-1}, depending on the substrate). Each step involves a net conversion of reactants to products in approximately equal amounts under these single-turnover conditions. In this study, it has been found that under single-turnover conditions the *total* change in hydrogen ion concentration predicted by the chemical reaction occurs in a single first-order process with a rate identical with the rate of the slow step. *No net uptake of protons from solution occurs in the burst process* even though one-half of the net transformation occurs in this step. In a control kinetic study it was found that the displacement of enzyme-bound NAD^+ by NADH is accompanied by the uptake of hydrogen ion from solution by the enzyme. It is proposed that this phenomenon has its origins in a coenzyme oxidation-state-dependent perturbation of the pK_a' of an enzyme-site residue. It also is proposed that the pK_a' perturbation (a) accounts for the absence of a burst uptake of protons during the burst reduction of aldehyde, and (b) is linked to the manifestation of catalytic nonequivalence of the two enzyme catalytic sites.

In our previous studies we have used the stopped-flow, rapid mixing kinetic technique to investigate transient kinetic steps in the horse liver alcohol dehydrogenase (hereafter simply called alcohol dehydrogenase) catalyzed reduction of chromophoric aromatic aldehydes and an aldehyde analog (Bernhard *et al.*, 1970; Dunn and Bernhard, 1971). These studies have shown that reduction occurs in two distinct kinetic steps when the reaction is limited to a single turnover of enzyme sites. Each step corresponds to the net conversion of reactants to products in approximately equal amounts. When NADH[1] and aldehyde are present in large excess of the site concentration, there is a presteady-state burst conversion of reactants to products in an amount equal to one-half of the total site concentration. On the strength of these findings, we concluded that the two alcohol dehydrogenase sites become catalytically nonequivalent during a single-turnover cycle. Additional kinetic evidence in support of this interpretation has been reported by McFarland and Bernhard (1972) and by Luisi and Favilla (1972); and Everse (1973) has reported equilibrium binding studies consistent with this view.

Both the amino acid sequence (Jörnvall, 1973) and the low-resolution X-ray structure work (Brändén *et al.*, 1973) indicate that the crystalline native enzyme is composed of two identical subunits. The crystalline native enzyme exhibits orthorhombic symmetry (space group $C222_1$) with a crystallographic twofold axis of symmetry through the dimeric molecule. The crystalline enzyme–coenzyme complex exhibits a lower crystal symmetry (Brändén *et al.*, 1973). The symmetry loss on complex formation is believed to reflect a loss of symmetry at the molecular level.

The present work examines the relationship between the two-step process for the reduction of 4-(2′-imidazolylazo)-benzaldehyde (azoaldehyde, I) and the process of hydrogen



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ion uptake (eq 1) from solution under single-turnover condi-

$$R{-}\overset{O}{\overset{\|}{C}}{-}H + NADH + H^+ \overset{E}{\rightleftharpoons} R{-}CH_2OH + NAD^+ \quad (1)$$

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[1] Abbreviations used are: NADH and NAD^+, reduced and oxidized nicotinamide adenine dinucleotide, respectively; azoaldehyde and azoalcohol, 4-(2′-imidazolylazo)benzaldehyde and 4-(2′-imidazolylazo)-benzyl alcohol, respectively.