Biochem. Biophys. Res. Commun. 118, 1–7.
Parodi, A. J., Mendelzon, D. H., Lederkremer, G. Z., & Martin-Barrientos, J. (1984b) *J. Biol. Chem. 259*, 6351–6357.
Previato, J. O., Mendelzon, D. H., & Parodi, A. J. (1986) *Mol. Biochem. Parasitol. 18*, 343–353.
Wright, A., & Robbins, P. W. (1965) *Biochim. Biophys. Acta 104*, 594–596.

Trehalose-Containing Lipooligosaccharide Antigens of *Mycobacterium* sp.: Presence of a Mono-*O*-methyltri-*O*-acyltrehalose "Core"†

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ABSTRACT: We have described the surface antigens of *Mycobacterium kansasii* as trehalose-containing lipooligosaccharides (LOS) which at the nonreducing "epitope" end bear a unique amino sugar containing diglycosyl unit, whereas the putative reducing end consists of an acylated α,α-trehalose-containing tetraglucosyl "core" [Hunter, S. W., Jardine, I., Yanagihara, D. L., & Brennan, P. J. (1985) *Biochemistry 24*, 2798–2805]. The presence of a new variation on this core, in *Mycobacterium szulgai*, is now reported, →3)β-D-Glc*p*-(1→6)α-D-Glc*p*(1↔1)3,4,6-tri-*O*-acyl-2-*O*-Me-α-D-Glc*p*, representing the first example of an *O*-methyltrehalose unit in nature. The simplest of the LOS class of glycolipids in *M. szulgai* was defined as α-L-2-*O*-Me-Fuc*p*(1→3)α-L-Rha*p*(1→3)α-L-Rha*p*(1→3)β-D-Glc*p*(1→6)α-D-Glc*p*(1↔1)3,4,6-tri-*O*-acyl-2-*O*-Me-α-D-Glc*p*. Further glycosylation of this nonantigen, by an incompletely defined 6-deoxyhexosyl residue, confers specific antigenicity on the organism. Thus, these extraordinary structures, in a manner analogous to the better known lipopolysaccharides from rough variants of *Enterobactericiae*, are highly amphipathic and display variability not only in the immunogenic, distal region but also in the "invariant" lipophilic core. The contribution of these glycolipids to the hydrophobic barrier, the pseudo outer membrane of mycobacteria, is discussed.

Serological differentiation of most individual species and subspecies within the *Mycobacterium* genus is based on one of several classes of glycolipid antigens, vastly different in elemental structure but each endowed with novel, sometimes exotic, non-reducing-end epitopes (Brennan, 1984). The glycopeptidolipids, notably those from members of the *Mycobacterium avium* complex isolated from patients with acquired immunodeficiency syndrome, have been examined in considerable detail; the structures of the oligosaccharide haptens from the predominant 10 serovariants of the 31-member *M. avium* serocomplex have now been fully elucidated (McNeil et al., 1987; Chatterjee et al., 1987). Another group of surface antigens, the so-called lipooligosaccharides of mycobacteria (Hunter et al., 1983), are examples of a rare principle in carbohydrate chemistry, the existence of glycosidically linked trehalose. Although clearly ubiquitous, only the trehalose-containing, pyruvylated glycolipids of *Mycobacterium smegmatis* (Saadat & Ballou, 1983) and the more glycosylated, antigenic variety in *Mycobacterium kansasii* (Hunter et al., 1983, 1985) have been described in detail. Work on those from other important mycobacterial opportunistic pathogens has been hampered by the difficulty encountered in grasping the structural details of the products from *Mycobacterium szulgai* and others. Although precedent dictated that all such products were anchored on acyltrehalose (Saadat & Ballou, 1983; Hunter et al., 1983; Kamisango et al., 1985), we now report that, while the antigenic lipooligosaccharides of *M. szulgai*, like the others, are endowed with a distinctive non-reducing-end epitope, thereby conferring serological specificity, the other end of the oligosaccharide chain is unique in that it consists of a glycosidically linked mono-*O*-methyltri-*O*-acyltrehalose unit.

Experimental Procedures

Extractions of Antigens. The identity of *Mycobacterium szulgai* (strain Johnson; No. 1878 from the collection maintained at the National Jewish Center) was confirmed by agglutination against rabbit antiserum raised against the homologous strain (Schaefer, 1965). Cultures were grown in Fernbach flasks for 4–5 weeks in 7H11 medium without agar (Hunter et al., 1983) or in a glycerol–alanine–salt medium (Takayama et al., 1975) and then autoclaved, and both cells and medium was evaporated to dryness at 50 °C. The resulting solid was extracted with $CHCl_3/CH_3OH$ (Hunter et al., 1983). Dried extracts were dissolved in the biphasic mixture $CHCl_3/CH_3OH/H_2O$ (8:4:2) (Folch et al., 1957); the contents of the lower organic phase were used. Approximately 6 g of washed lipid were obtained from 100 g of dried cells.

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Purification of Lipooligosaccharides. Washed lipids (3–4 g) were applied to a column (2.5 × 30 cm) of Florisil (100–200 mesh) which was irrigated with 200 mL each of $CHCl_3$ followed by 10%, 15%, 20%, 25%, and 50% CH_3OH in $CHCl_3$. LOS-1[1] and LOS-2 were largely concentrated in the 15% and 20% CH_3OH eluates, whereas LOS-3–LOS-6 appeared almost exclusively in the 50% CH_3OH fraction. Alternatively, about 6 g of lipid was applied to a column (2.5 × 45 cm) of DEAE-cellulose (acetate) which was eluted with the same series of solvents; LOS-1 and LOS-2 were confined to the 10% CH_3OH eluate, LOS-3 and LOS-4 were in the 20% eluate, and LOS-5 and LOS-6 appeared in both the 25% and 50% CH_3OH eluates; such anion-exchange chromatography had the advantage of assuring the absence of phospholipids in eluates. Further purification of LOS-1 and LOS-4 employed smaller Florisil columns with more gradual gradations of solvents. Typically, 286 mg of impure LOS-1, when applied to a 6-g column of Florisil, yielded 72 mg of a product suitable for preparative TLC. For final purification, centrifugally accelerated, preparative TLC (Chromatotron, Model 7924, Harrison Research, Palo Alto, CA) was used with homemade radial plates (rotors) of silica gel PF-254 containing $CaSO_4$ (type 60; 1 mm thick; E. Merck, Darmstadt, Federal Republic of Germany) and the solvents $CHCl_3/CH_3OH/H_2O$ (30:8:1) in the case of LOS-1 or $CHCl_3/CH_3OH/H_2O$ (65:25:4) for LOS-4.

Derivation of Oligosaccharides. Pure LOS was used as the source of the corresponding oligosaccharides. Typically, 30 mg of LOS was dissolved in $CHCl_3/CH_3OH$ (2:1, 1 mL) and reacted with an equal volume of 0.2 N $NaOH/CH_3OH$ at 37 °C for 1 h, followed by an additional 1 h at room temperature. The reaction mixture was neutralized with glacial acetic acid and dried under N_2. The products were partitioned between $CHCl_3$ and water, and the $CHCl_3$ phase was backwashed twice with water. The combined aqueous phases were desalted on mixed-bed ion-exchange resin (MB-3), and the neutral oligosaccharide was further purified by gel filtration on a column (1 × 175 cm) of Bio-Gel P-2 in H_2O.

Permethylations. In order to establish the location of acyl functions on the oligosaccharide backbone, the native LOS was subjected to the neutral alkylating conditions of Prehm (1980), as follows. To the pure LOS-1 (2 mg), under N_2, was added 30 μL of 2,6-di-*tert*-butylpyridine, 20 μL of methyl trifluoromethanesulfonate, and 200 μL of trimethyl phosphate. The reaction mixture was stirred at 25 °C for 5 h, following which 1 mL of water was added. The mixture was applied to a Sep-Pak C_{18} cartridge (Waters Associates, Inc., Milford, MA), which was eluted successively with water, acetonitrile, and ethanol. The ethanol washes were dried to yield the naturally acylated, per-O-methylated LOS-1. In order to establish glycosyl linkage pattern, pure Ose (15 mg) was dissolved in 1 mL of dry dimethyl sulfoxide, flushed with N_2, and stirred under N_2 with 1 mL of dimethylsufinyl carbanion for 1 h (Hakomori, 1964). CH_3I or C^2H_3I (1 mL) was added slowly and the mixture stirred at room temperature for 45 min. Water (2 mL) was added followed by $CHCl_3$ (3 mL). The organic phase was backwashed 5 times and dried under N_2, and the permethylated oligosaccharide was purified on a Sep-Pak C_{18} cartridge, elution being as described above. The permethylated oligosaccharide was recovered in the acetonitrile fraction. Per-CH_3-Ose or per-C^2H_3-Ose (10 mg) was subjected to graded hydrolysis in 0.3 N HCl at 100 °C for 3 h. The products were extracted into $CHCl_3$ and separated by TLC by being developed twice in ether/acetone (8:1). Complete acid hydrolysis of the per-CH_3-Ose was accomplished with 2 M CF_3COOH (100 °C, 3 h).

Other Analytical Procedures. Hydrolysis of LOS and oligosaccharides for sugar content was conducted with 2 M CF_3COOH at 120 °C for 1 h. Hydrolysates were extracted with hexane, reduced in 0.13 M $NaBH_4$ in 10^{-4} M NaOH overnight at room temperature, and acetylated in acetic anhydride/pyridine (1:1). Details of TLC of intact glycolipids and reducing sugars and paper chromatography of oligosaccharides have been described (Hunter et al., 1983). For analysis of fatty acids, LOS was transesterified in 2 M methanolic HCl at 80 °C overnight. Details of TLC, GC, and GC/MS of fatty acid methyl esters and interpretation of mass spectra have been described (Hunter et al., 1983). Eluates from gel filtration columns were assayed for carbohydrate with phenol–H_2SO_4 (Dubois et al., 1966). Reducing activity was assayed with ferricyanide (Park & Johnson, 1949). 2-*O*-Methylfucose was synthesized or obtained from hydrolysates of the glycopeptidolipid antigen from *M. avium* serovar 25 as described (Brennan et al., 1981).

Gas Chromatography, Mass Spectroscopy, and Nuclear Magnetic Resonance. GC analyses were conducted on a Hewlett-Packard 5710 A gas chromatogram equipped with a dropping-needle injector and linked to a HP 3380 integrator. Unlike in earlier work in which packed columns were used, GC was routinely conducted on a fused silica capillary column of Durabond-1 (J & W Scientific, Rancho Cordova, CA) as described (McNeil et al., 1987). GC/MS was conducted with an all-glass interface coupled to a Teknivent 48K data system or with a VG Instruments MM-16F low-resolution mass spectrometer and data system with a capillary GC inlet, direct probe, and both CI and EI capabilities. Interpretation of mass spectra of alditol acetates has been discussed in detail (Hunter et al., 1985); fragmentation values for products not previously encountered are given in the text. FAB/MS was conducted as described before (Hunter et al., 1985; McNeil et al., 1987). Plasma desorption mass spectra were recorded with a BIO-ION Nordic (Uppsala, Sweden) BIN-10K ^{252}Cf time-of-flight mass spectrometer, using an accelerating voltage of 20 kV and a flight tube length of 15 cm; this instrument is described by Sundqvist et al. (1984). A sample of the LOS was dissolved in $CHCl_3/CH_3OH$/2-propanol (10:45:45) and applied to a nitrocellulose-coated aluminum target foil (Jonsson et al., 1986). The sample was dried and inserted in the mass spectrometer. A sample of the lipooligosaccharide was peracetylated with a 2:1 mixture of trifluoroacetic anhydride and acetic acid (Dell & Tiller, 1986). The peracetylated sample was then deposted on a nitrocellulose PDMS target, dried, and analyzed. Approximately 10 μg of the lipooligosaccharide was used for each analysis. ^1H NMR spectra were recorded at room temperature with a Nicolet NT-360 spectrometer and Nicolet 1180 computer operating in the Fourier transform mode. Chemical shifts were recorded relative to that of HO^2H, which was set at 4.74. Because the chemical shift of HO^2H varies with temperature and pH, an error of ±0.05 ppm is expected. Nevertheless, assignment of each anomeric signal to the different sugars was possible.

Immunological Procedures. Rabbit antiserum was obtained by injecting 1 mL of a suspension of whole *M. szulgai* in 0.5%

[1] Abbreviations: LOS, lipooligosaccharide; Ose, nonreducing oligosaccharide; FAB, fast atom bombardment; MS, mass spectrometry; PDMS, plasma desorption mass spectrometry; GC, gas chromatography; TLC, thin-layer chromatography; Glc*p*, glucopyranose; Rha*p*, rhamnopyranose; Fuc*p*, fucopyranose; Treh, trehalose; Me-Treh, hexa-*O*-methyltrehalose; Me, methyl *or O*-methyl; PBS, phosphate-buffered saline; amu, atomic mass units.

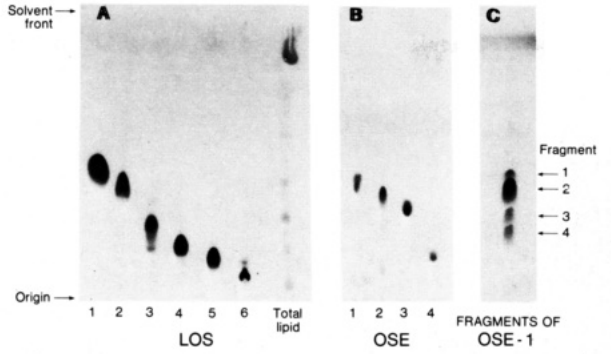

FIGURE 1: Application of TLC to analysis of the native LOSs of *M. szulgai* and fragments derived from them. (A) TLC in $CHCl_3$/CH_3OH/H_2O (65:25:4) of purified LOS-1–LOS-6. Glycolipids were located with a spray composed of 10% H_2SO_4 in ethanol followed by heating at 120 °C. (B) TLC in 1-butanol/pyridine/water (6:4:3) of the oligosaccharides obtained after deacylation of LOS-1–LOS-4. (C) TLC of the partial acid hydrolysate of per-C^2H_3-Ose-1. Partial hydrolysis was conducted in 0.3 N HCl at 100 °C for 3 h; the $CHCl_3$-soluble products were applied to a silica gel G TLC plate and developed in ether/acetone (8:1).

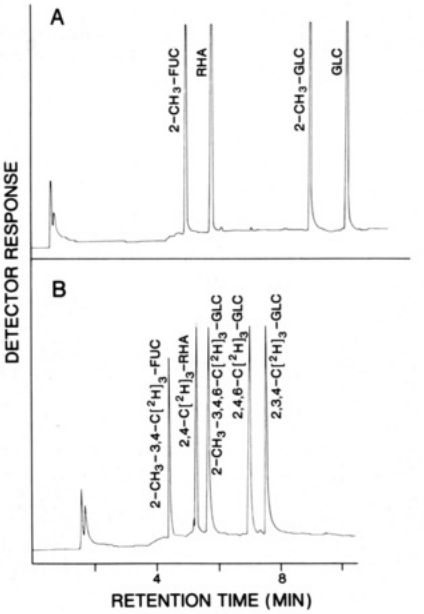

FIGURE 2: Sugar analysis of Ose-1 and per-C^2H_3-Ose-1. (A) The oligosaccharide was hydrolyzed with 2 M TFA for 1 h at 120 °C, reduced, acetylated, and subjected to GC. (B) The per-C^2H_3-Ose-1 was treated in the same way. GC was conducted on a capillary column of DB-1 with a temperature program of 160 °C for 2 min followed by a gradient of 4 deg/min to 240 °C.

phenol in PBS (optical density of 0.3 at a wavelength of 525 nm in a Coleman series spectrometer) into the ear vein of a 3-month-old white New Zealand rabbit twice per week for 2 weeks. Bleedings were begun at the end of 17 days. Sera with a titre of 1:160 or greater in a whole cell seroagglutination assay (Schaefer, 1965) were pooled. Pure LOS was dissolved in absolute ethanol (1 mg/2 mL), and serial dilutions of 1:2 were made. Samples (50 μL) of each dilution were applied to 96-well polystyrene plates (Flow Laboratories, Inc., McLean, VA) and allowed to evaporate to dryness. Plates were washed for 10 min with 0.1% Tween 80 in PBS. Rabbit antiserum, diluted 1:100 (50 μL), was added. Plates were left at room temperature for 30–60 min and then washed 5 times with Tween–PBS, followed by 50 μL of horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, MO) at 1:1000 dilution. Plates were incubated at room temperature for 30–60 min and then washed 5 times with Tween–PBS, followed by the addition of 50 μL of substrate and an additional incubation at room temperature for 30 min as described (Yanagihara et al., 1985). The reaction was terminated with 2.5 M H_2SO_4 and the absorption read at 490 nm on a Biotek ELISA reader.

RESULTS

Previously we had reported that the majority of an assortment of human isolates of *M. szulgai* contain a distinctive spectrum of alkali-labile glycolipids (Brennan et al., 1982). We have since learned that such alkaline lability is indicative of the presence of antigens of the trehalose-containing lipooligosaccharide class (Hunter et al., 1983). These glycolipids represent a major proportion of the lipid population of *M. szulgai*; typically, 50 g of dry cells yields 3 g of lipid, which upon fractionation on Florisil yielded 230 mg of partially purified LOS-1 and LOS-2 and about 180 mg of partially pure LOS-3–LOS-6 (Figure 1A). LOS-1 represents 4–7% of the total bacterial lipid and LOS-3–LOS-6 represents 5–6%; LOS-1 was clearly the major entity. Upon chromatography of 230 mg of impure LOS-1 on a second Florisil column, 55 mg was recovered, of which 28 mg of pure glycolipid was obtained after centrifugal preparative TLC. The reactivities of the individual LOS as antigen against anti-*M. szulgai* rabbit antiserum were tested by ELISA. At several concentrations, LOS-1–LOS-3 were devoid of serological activity; activity first decisively emerged with LOS-4, and highest reactivity was observed in LOS-6. For instance, the mean A_{490} value of LOS-1 was 0.01, that of LOS-4 was 0.37, and that of LOS-6 was 1.24 in which 150 ng of each glycolipid was coated on wells.

Structure of Ose-1. Alkalinolysis of pure LOS-1 (28 mg) yielded 8 mg of a homogeneous-appearing oligosaccharide upon gel filtration. Paper chromatography in ethyl acetate/pyridine/acetic acid/water (5:5:1:3) showed one pure nonreducing oligosaccharide with an R_{Treh} value of 1.14. TLC (Figure 1B) confirmed that the oligosaccharide was pure. LOS-3 and LOS-4 also yielded pure oligosaccharides; only the product from LOS-2 was impure (Figure 1B). Hydrolysis of Ose-1 with 2 M CF_3COOH at 120 °C and capillary GC of the alditol acetates on the DB-1 column showed four sugar components (Figure 2A); GC/MS and cochromatography with authentic standards established the identities. The mass spectrum of the alditol acetate of 2-*O*-Me-Fuc (*m/z* 43, 87, 99, 117, 129, 159, 173, 201, and 275) was identical with that in the oligosaccharide hapten of *M. avium* complex serovariant 25 (Brennan et al., 1981). GC as described (Lindberg, 1972) showed a retention time of 1.35 relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, suggestive of a 2-*O*-Me-Fuc derivative (Lindberg, 1972), and cochromatography with the synthetic sugar (Brennan et al., 1981) showed perfect concordance. The identity of 2-*O*-Me-Glc was established in like fashion; the mass spectrum of the alditol acetate (*m/z* 43, 58, 97, 117, 139, and 259) is that of a 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylhexitol (Janssen et al., 1976).

^1H NMR of Ose-1 (Figure 3) showed six well-resolved anomeric proton signals: δ 4.34 ($J_{1,2}$ = 7.8 Hz), 5.01 ($J_{1,2}$ = <1.5 Hz), 5.09 ($J_{1,2}$ = 3.6 Hz), 5.17 ($J_{1,2}$ = <1.5 Hz), 5.27 ($J_{1,2}$ = 3.6 Hz), and 5.33 ($J_{1,2}$ = 3.3 Hz). Also obvious, between δ 1.2 and δ 1.4 (not shown in Figure 3), were the proton signals of three C–CH_3 from three 6-deoxyhexoses. The anomeric signals at δ 5.01 and 5.17 were assigned to two Rha units because of their small coupling constants; the chemical shifts indicated α configuration (Kasai et al., 1979). The signal at δ 5.09 was assigned to 2-*O*-Me-Fuc, because it disappeared with the loss of 2-*O*-Me-Fuc in fragment 4 (see

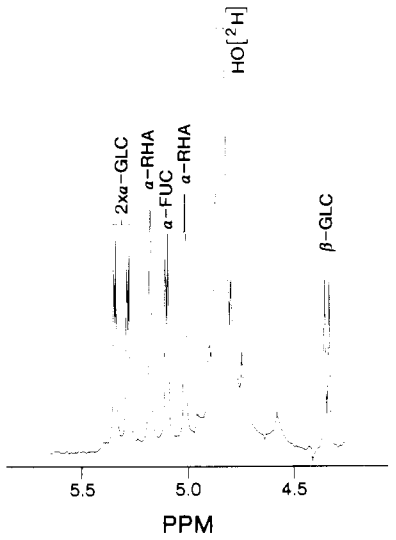

FIGURE 3: The 360-MHz ^1H NMR of Ose-1. The oligosaccharide, derived from LOS-1 by deacylation with NaOH, was dissolved in ^2H_2O. HO^2H was set at 4.74 ppm.

below and Figure 4). An α configuration was assigned to 2-*O*-Me-Fuc on the basis of its coupling constant (Lemieux & Stevens, 1966). The signal at δ 4.31 was assigned to β-hexose, and the two signals at δ 5.27 and 5.33 were assigned to two α-hexoses (Kasai et al., 1979). These assignments were derived in conjunction with results obtained from fragment 2 (see below).

The Ose-1 was perdeuteriomethylated and the product purified on a Sep-Pak C_{18} column; TLC in 1-butanol/acetic acid/ether/water (9:6:1:3) showed a homogeneous product of $R_{Me\text{-}Treh}$ = 0.60. Capillary GC of the alditol acetates arising from hydrolysis of the per-C^2H_3-Ose-1 allowed the resolution of five products (Figure 2B). The identities were established by GC/MS (Hunter et al., 1985) as 2-*O*-CH_3-3,4-di-*O*-C^2H_3-Fuc, from a terminal 2-*O*-CH_3-Fuc; 2,4-di-*O*-C^2H_3-Rha, from a 3-linked Rha; 2-*O*-CH_3-3,4,6-tri-*O*-C^2H_3-Glc, from a terminal 2-*O*-CH_3-Glc; 2,4,6-tri-*O*-C^2H_3-Glc, from a 3-linked Glc; and 2,3,4-tri-*O*-C^2H_3-Glc, from a 6-linked Glc unit.

In order to obtain information on the sequence of these sugars, the per-C^2H_3-Ose-1 was subjected to partial acid hydrolysis in 0.3 N HCl. The products were extracted into $CHCl_3$ and subjected to silica gel TLC in ether/acetone (8:1, twice) (Figure 1C). Four clearly identifiable fragments were generated. Fragment 3, on account of mobility similar to that of the starting material (not shown in Figure 1C), was believed to be the unhydrolzed per-C^2H_3-Ose-1. Therefore, analyses were confined to fragments 1, 2, and 4, which clearly represented degradation products of the parent compound. These were isolated in pure form by preparative TLC, and each was examined in turn:

Fragment 1. Analysis of the alditol acetates on the DB-1 column using the same temperature program as described in Figure 2 followed by GC/MS showed but two products, the derivatives of 2-*O*-CH_3-3,4,6-tri-*O*-C^2H_3-Glc and 2,3,4-tri-*O*-C^3H_2-Glc. Upon ethylation with C_2H_5I, hydrolysis and reexamination of the alditol acetates (Figure 4A), the products were recognized as unchanged 2-*O*-CH_3-3,4,6-tri-*O*-C^2H_3-Glc and 2,3,4-tri-*O*-C^2H_3-6-*O*-C_2H_5-Glc. ^1H NMR of the ethylated fragmet 1 showed two superimposed α-anomeric proton signals at δ 5.16 ($J_{1,2}$ = 3.6 Hz). Clearly, glycosylation occurred at the 6′-position of the internal Glc unit of the mono-*O*-methyltrehalose, and O-methylation occurred at the 2-position of the terminal Glc unit; i.e., the structure →6)α-Glc*p*(1↔1)2-*O*-Me-α-Glc*p* was established.

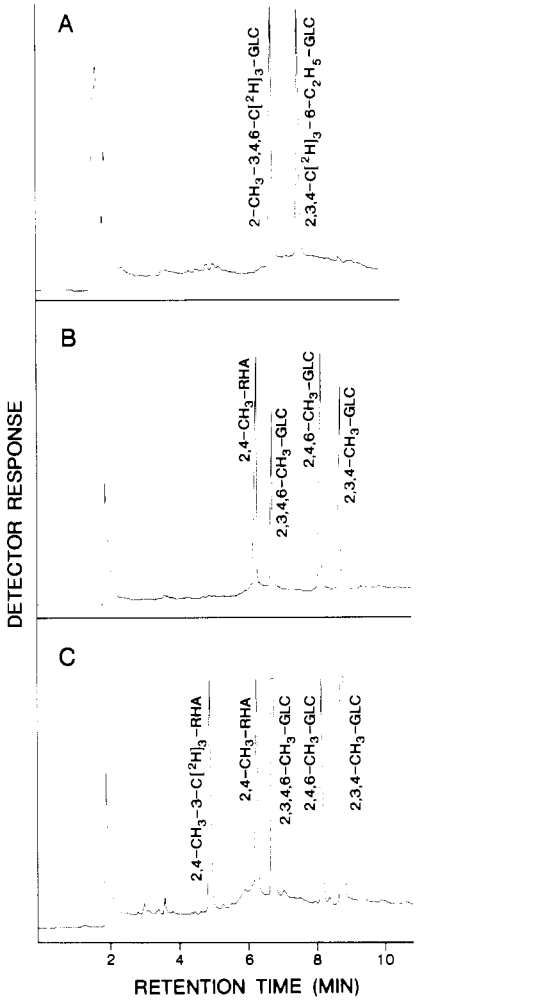

FIGURE 4: Methylation analysis of some of the hydrolysis fragments of per-C^2H_3-Ose-1 shown in Figure 1C. (A) GC of the alditol acetates obtained from ethylated fragment 1. Fragment 1, described in Figure 1C, was isolated by preparative TLC and ethylated. Alditol acetates were prepared and analyzed by GC as described in Figure 2. (B) Sugar analysis of fragment 4 described in Figure 1C. Fragment 4 was isolated by preparative TLC as described in Figure 1C and hydrolyzed, and the alditol acetates were subjected to GC. (C) Fragment 4 was perdeuteriomethylated, and the alditol acetates were again subjected to GC. GC conditions are described in Figure 2.

Fragment 2. Analysis of the alditol acetates as described for fragment 1 showed the presence of but three products: 2-*O*-CH_3-3,4,6-tri-*O*-C^2H_3-Glc, 2,4,6-tri-*O*-C^2H_3-Glc, and 2,3,4-tri-*O*-C^2H_3-Glc (results not shown). ^1H NMR showed one β-Glc signal at δ 4.30 ($J_{1,2}$ = 7.8 Hz) and two α-Glc signals at δ 5.12 and 5.18 ($J_{1,2}$ = ca. 3.6 Hz for each). Thus, the structure →3)β-Glc*p*(1→6)-α-Glc*p*(1↔1)-2-*O*-Me-α-Glc*p* for fragment 2 was established.

Fragment 4. This fragment was obtained in an experiment similar to that described in Figure 1C, but in which per-*O*-CH_3-Ose, rather than the per-*O*-C^2H_3-Ose, was the object of partial acid hydrolysis. The partially methylated alditol acetates arising from purified fragment 4 are shown in Figure 4B; these were recognized as 2,4-di-*O*-CH_3-Rha, 2,3,4,6-tetra-*O*-CH_3-Glc, 2,4,6-tri-*O*-CH_3-Glc, and 2,3,4-tri-*O*-CH_3-Glc. This result indicates that fragment 4 contains a 3-linked rhamnose(s) in addition to fragment 2. In order to confirm the point at which further glycosylation takes place, fragment 4 was deuteriomethylated, and the partially methylated, partially deuteriomethylated alditol acetates were analyzed (Figure 4C). A new product, 2,4-di-*O*-CH_3-3-*O*-C^2H_3-Rha, was identified. Thus, the point of further glycosylation on the

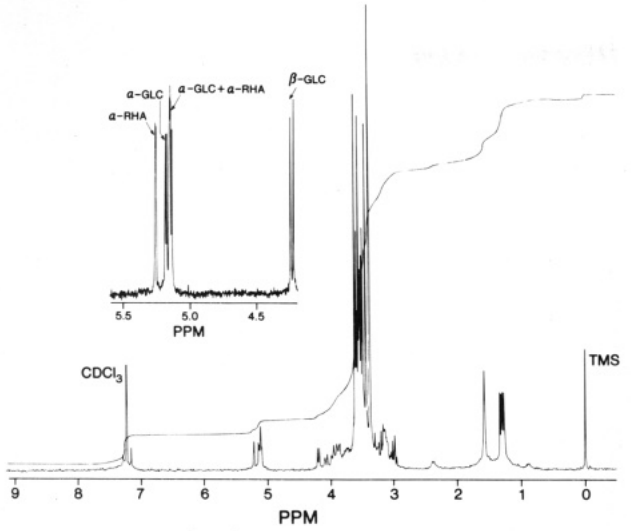

FIGURE 5: The 360-MHz ^1H NMR of fragment 4. Purified fragment 4, isolated by preparative TLC as described in Figure 1C, was dissolved in C^2HCl_3 for analysis.

linear oligosaccharide is the 3-position of the terminal Rha of fragment 4, and the presence of 2,4-di-*O*-CH_3-Rha after deuteriomethylation indicates that there are two such rhamnose residues present in fragment 4. Indeed, ^1H NMR (Figure 5) supports this conclusion. The results were interpreted as follows: one β-Glc (δ 4.22, $J_{1,2}$ = 7.8 Hz); one α-Glc and one α-Rha overlapping at ca. δ 5.15; one further α-Glc at δ 5.18 ($J_{1,2}$ = 3.6 Hz); one α-Rha at δ 5.25 ($J_{1,2}$ = <1.5 Hz). Further evidence for the existence of two Rha units was provided by the presence of two C–CH_3 proton signals at δ 1.2–1.4. Thus, the combined evidence points to the following structure for fragment 4:

→3)α-Rha(1→3)-α-Rha(1→3)-β-Glc(1→
6)-α-Glc(1↔1)α-Glc

Positive ion FAB/MS of the C^2H_3 fragment 4 revealed an intense $(M + NH_4)^+$ ion at m/z 1027 and a distinct $(M + Na)^+$ ion at m/z 1032, pointing to a M_r of 1009 for C^2H_3 fragment 4, in accord with the proposed structure.

The original Ose-1, that arising from LOS-1, was also examined by positive ion FAB/MS. The nonderivatized Ose showed a prominent $(M + Na)^+$ ion at m/z 993 and an $(M + H)^+$ ion at m/z 971, for a M_r of 970. Positive ion FAB/MS of the per-C^2H_3-Ose-1 showed a strong $(M + NH_4)^+$ ion at m/z 1243 (Figure 6) for a M_r of 1225. This and all of the aforementioned evidence is in accord with the structure

2-*O*-Me-Fuc(1→3)-α-Rha(1→3)-α-Rha(1→3)-β-Glc(1→
6)-α-Glc(1↔1)α-Glc

The procedure of Gerwig et al. (1978) with our own adaptations (McNeil et al., 1987) was applied to the question of the ring form of the sugars of LOS-1. Ose-1 was demethylated with Li^+ in ethylenediamine as described (Mort & Bauer, 1982; McNeil et al., 1987). The (*R*)-(−)-butyl glycosides were prepared with (*R*)-(−)-butanolic hydrochloride and the $(CH_3)_3Si$ derivatives analyzed by GC on the fused silica capillary DB-1 column with the authentic D and L sugar derivates as standards. The ring form of the sugars in Ose-1 were identified as L-Fuc, L-Rha, and D-Glc. Results of analogous analyses and interpretation of the GC data have been described in detail (McNeil et al., 1987).

Identification and Location of Acyl Groups on LOS-1. The fatty acids of LOS-1 as the methyl esters were identified by GC/MS (Hunter et al., 1983) as 2,4,6-trimethyldocosanoic acid (m/z 88, 101, 129, 143, 171, and 396 for the methyl ester), 2,4-dimethyleicosanoic acid (m/z 88, 101, 129, and

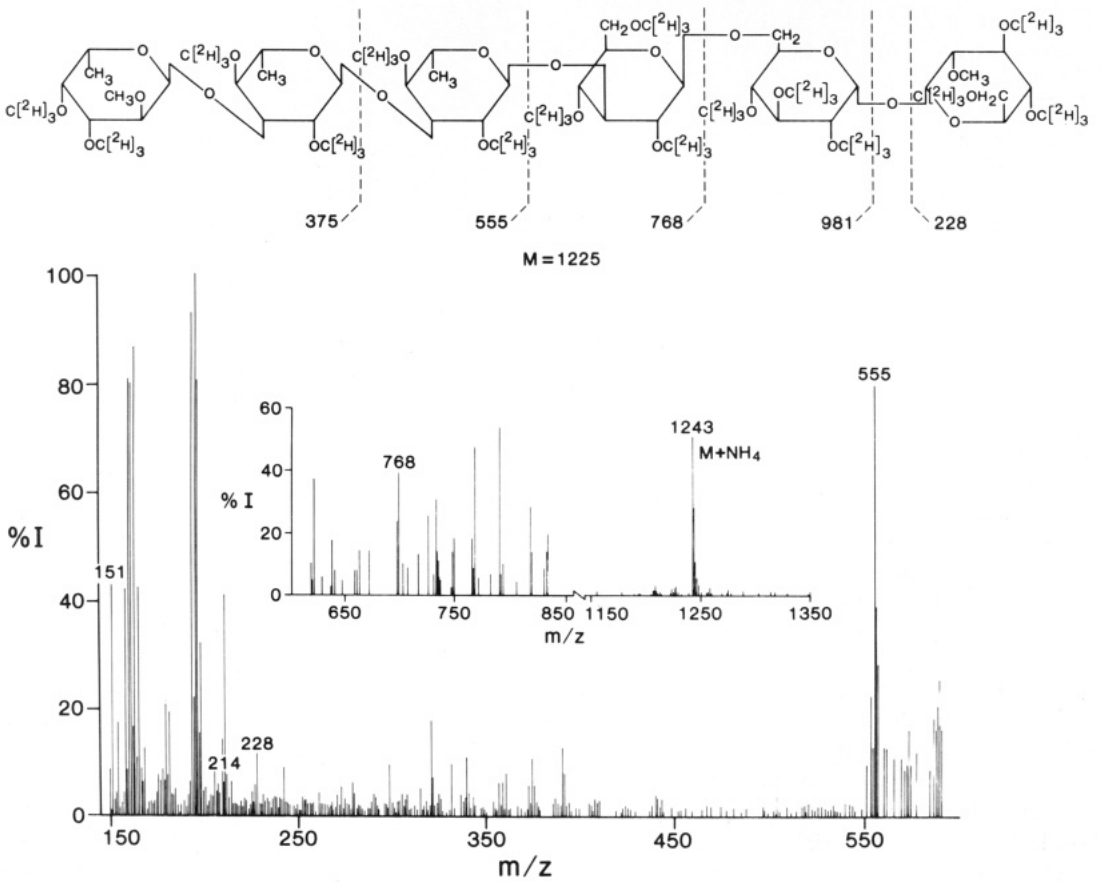

FIGURE 6: Positive ion FAB/MS of perdeuteriomethylated Ose-1.

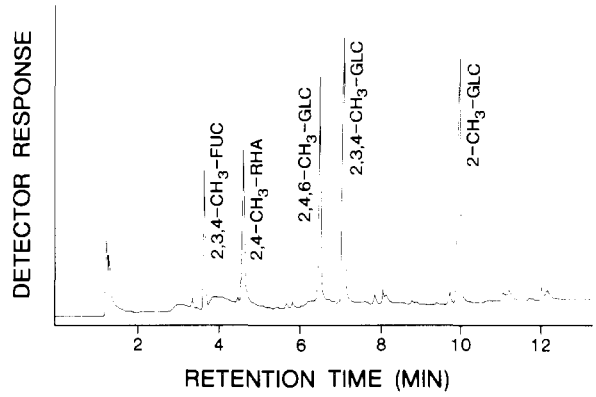

FIGURE 7: GC of the alditol acetates obtained from methylated, and subsequently deacylated, LOS-1. LOS-1 was first permethylated with methyl trifluoromethanesulfonate and then deacylated as described under Experimental Procedures. GC conditions are described in Figure 2.

354), 2-methyl-3-hydroxytetradecanoic acid (m/z 88, 117, 138, 198, 222, 223, 254, and 257), and 2-methyltetradecanoic acid (m/z 88, 101, 143, and 256). These were present in the approximate molar ratio of 1:0.2:1:1. In order to determine the location of these acyl residues on the oligosaccharide backbone of LOS-1, considerable effort was expanded in developing methods that would allow maximum derivatization and minimal de-O-acylation. Our variation on one suitable procedure (Prehm, 1980) is described under Experimental Procedures. The pure glycolipid was methylated with methyl trifluoromethanesulfonate in trimethyl phosphate, deacylated, and hydrolyzed, and the alditol acetates were examined by GC/MS (Figure 7). The terminal 2-*O*-Me-Glc appeared as such and was the only glycosyl unit unaffected by the methylation step. Accordingly, the 2-*O*-Me-Glc unit must be fully substituted by the *O*-acyl residues; i.e., three acyl residues must be esterified at the 3-, 4-, and 6-hydroxyl residues of the 2-*O*-Me-Glc.

PDMS of native LOS-1 showed the $(M + Na)^+$ ion at m/z 1823 (spectrum not shown), indicating the presence of one each of C_{15}, C_{15}:OH, and C_{25} fatty acid per molecule of LOS-1. A weak $(M + Na)^+$ peak at m/z 1781 suggested that a small portion (≈20%) of the LOS-1 population has a C_{22} fatty acid in place of the C_{25} fatty acid. Results of PDMS of the peracetylated LOS-1 are shown in Figure 8. A $(M + Na)^+$ ion at m/z 2369.6 and fragment ions at m/z 245.6, 477.3, 706.9, 993.9, and 1282.4 support the evidence for the structure of LOS-1. Clearly there are three fatty acids present, one each of C_{15}, C_{15}:OH, and C_{25}, on the terminal 2-*O*-Me-Glc. This is in accord with the results obtained from methylation with methyl trifluoromethanesulfonate. Obviously, these three fatty acids are located on O-3, O-4, and O-6 of the terminal 2-*O*-Me-Glc. However, which fatty acid is on which hydroxy function has not been determined.

LOS-4. Proton NMR of LOS-4 showed seven anomeric protons; six of these were identical with those found in LOS-1. The presence of an additional α-6-deoxyhexose in LOS-4 was indicated by the signals of a *C*-methyl at 1.15 ppm and a new anomeric proton at 5.1 ppm ($J_{1,2}$ = 3.7 Hz). However, sugar analysis by GC of the alditol acetates showed only 2-*O*-Me-Fuc, Rha, 2-*O*-Me-Glc, and Glc; no additional new sugar was detected. Methylation analysis showed 3-linked 2-*O*-Me-Fuc, 3-linked rhamnose, 3-linked terminal 2-*O*-Me-Glc, and 6-linked Glc; no terminal sugar other than 2-*O*-Me-Glc was detected. FAB/MS of the per-C^2H_3-Ose-4 suggested an M_r of 1665. All of these data indicate the presence of an unidentified sugar in LOS-4 which is likely to be responsible for

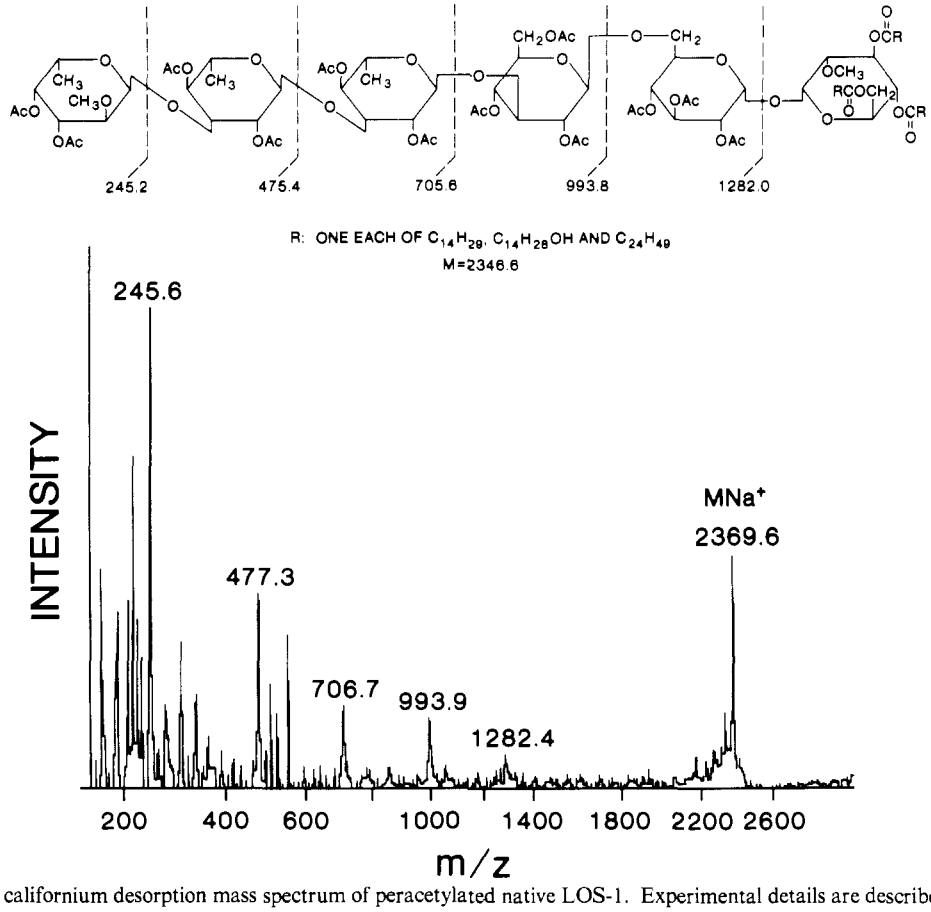

FIGURE 8: Positive ion californium desorption mass spectrum of peracetylated native LOS-1. Experimental details are described under Experimental Procedures.

the serological specificity of *M. szulgai*.

Discussion

Following the initial revelation of the presence of glycosidically linked trehalose in the dominant glycolipids of *M. smegmatis* (Saadat & Ballou, 1983) and *M. kansasii* (Hunter et al., 1983, 1985), we sought to differentiate the "atypical" mycobacteria into those containing such acylated oligosaccharides and those endowed with the glycopeptidolipid antigens (McNeil et al., 1987; Chatterjee et al., 1987). Atypical mycobacteria have assumed a new importance on account of the role of many of them as formidable secondary infectious agents in acquired immunodeficiency syndrome and other disorders (Good, 1985). *M. szulgai*, first recognized as a distinct taxon on the basis of a highly characteristic, but chemically undefined, set of glycolipids (Marks et al., 1972; Schaefer et al., 1973) and considered as an opportunistic pathogen (Good, 1985; Wayne, 1986) implicated mostly in pulmonary infections (Wolinsky, 1979) rather than disseminated disease, was an obvious choice for chemotaxonomy based on glycolipid antigens. However, considerably difficulty was encountered in developing the structures of the dominant antigens to their present state. FAB/MS invariably demonstrated a shortfall of 14 amu between the postulated structures of the oligosaccharides from LOS-1 and LOS-4 (Barr et al., 1984) and the observed molecular weights. Only when capillary gas chromatography columns superceded the earlier packed columns (Hunter et al., 1985) for resolution of alditol acetates and more vigorous acid hydrolysis (2 M CF_3COOH, 120 °C) was implemented did the presence of 2-*O*-methylglucose emerge and, consequently, the existence of a mono-*O*-methyltrehalose. However, success in solving the riddle of the core structure of these lipooligosaccharides is tempered by our inability to identify the *M. szulgai* specific, single sugar epitope present in LOS-4 and LOS-6. Elsewhere (Chatterjee et al., 1987), we have begun to describe the exotic dideoxy, branched, or N-acylated sugars, or sugar acids, now being encountered within the glycopeptidolipid of *M. avium* subspecies. The product from *M. szulgai*, not readily amenable to conventional analysis and to date recognizable only by ^1H NMR, probably belongs to one of these classes. Despite the initial hiatus in extending the structures of these extraordinary surface antigens, we have still conducted sufficient preliminary work to conclude that the majority of the currently listed 54 species within the *Mycobacterium* genus (Skerman et al., 1980; Good, 1985), including opportunistic pathogens and saprophytes, are endowed with the trehalose-containing surface lipooligosaccharides. However, it is not yet possible to predict how many are anchored on acyltrehalose itself as distinct from the *O*-methyl derivative.

In the initial work on the acylated oligosaccharides of *M. kansasii*, we had not attempted to pinpoint the position of the 2,4-dimethyltetradecanoyl residues. Since then, we have developed the requisite methodology.[2] The diazomethane–boron trifluoride etherate methylation procedure (Deferrari et al., 1972), methylation with methyl trifluoromethanesulfonate (Arnarp et al., 1975), and a procedure involving acyl location through use of 1-methoxyethyl protecting groups (Gray, 1976) had all suffered in our hands from the problem of under O-methylation. Modification of Arnarp's procedure, using trimethyl phosphate as a solvent, proved to be highly suitable for present purposes. The surprise of the outcome was the manner in which the fatty acyl residues were singularly located on the terminal 2-*O*-methylglucose unit of the *O*-methyltrehalose. We had speculated, on the basis of the reports of simple acylated trehaloses in some mycobacteria (Vilkas et al., 1968; Minnikin et al., 1985), that the acyl residues would be preferentially located on the trehalose unit of the lipooligosaccharides. Indeed, comprehensive acyl-locating procedures applied to the simpler pyruvylated, trehalose-containing glycolipids of *M. phlei* had demonstrated that two fatty acyl residues were symmetrically divided between the two glucosyl units of the glycosylated trehalose (Kamisango et al., 1985). However, in the present instance and in the two other examples studied to date, the trehalose-containing lipooligosaccharides of *M. kansasii* and of an unknown *Mycobacterium* sp.,[2] the acyl groups are also three in number and are asymmetrically located on the terminal glucosyl residue. Accordingly, the trehalose-containing lipooligosaccharides are unexpectedly amphipathic. Yet, the fatty acyl residues of the trehalose-containing lipooligosaccharides, all methyl branched at C-2 (Hunter et al., 1983; Kamisango et al., 1985) and some additionally hydroxylated at C-3, are distinctly different from the fatty acids—palmitate, stearate, and tuberculostearate—of the phospholipids of the cytoplasmic membrane of *Mycobacterium* sp. (Goren & Brennan, 1979). Seemingly, then, the lipooligosaccharides are not membranous but are clearly surface oriented (Hunter et al., 1985). On the basis of earlier freeze–fracture studies (Kim et al., 1976), Minnikin (1982) had proposed the existence in mycobacteria of a "hydrophobic interaction area", a lipid barrier, a form of pseudo outer membrane. The lipooligosaccharides, copious, highly amphipathic polar lipids endowed with a distinct set of fatty acyl functions, are ideally equipped to contribute to the outer asymmetric half of such a hydrophobic region. In fact, this feature is not the only resemblance between mycobacteria and Gram-negative bacteria; in both, only about 10% of the cell wall mass is composed of peptidoglycan, which, like that of most *Enterobactericae*, is all of the *meso*-diaminopimelic acid containing A1j class (Schleifer & Kandler, 1972). Thus, *M. szulgai* and its peculiar set of lipooligosaccharides present a model by which to test the principle of a pseudo outer membrane in mycobacteria.

[2] Work conducted with Raymond T. Camphausen.

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References

Arnarp, J., Keene, L., Lindberg, B., & Lonngren, J. (1975) *Carbohydr. Res. 44*, C5–C7.

Barr, V. L., Hunter, S. W., & Brennan, P. J. (1984) *Abstr. Annu. Meet. Am. Soc. Microbiol. U10*, p 88.

Brennan, P. J. (1984) in *Microbiology 1984* (Leive, L., & Schlessinger, D., Eds.) pp 366–375, American Society for Microbiology, Washington, DC.

Brennan, P. J., Mayer, H., Aspinall, G. O., & Nam Shin, J. E. (1981) *Eur. J. Biochem. 115*, 7–15.

Brennan, P. J., Heifets, M., & Ullom, B. P. (1982) *J. Clin. Microbiol. 15*, 447–455.

Chatterjee, D., Aspinall, G. O., & Brennan, P. J. (1987) *J. Biol. Chem. 262*, 3528–3533.

Deferrari, J. O., Gros, E. G., & Thiel, I. M. E. (1972) *Methods Carbohydr. Chem. 6*, 365–367.

Dell, A., & Tiller, P. R. (1986) *Biochem. Biophys. Res. Commun. 135*, 1126–1134.

Dubois, M., Gilles, K. A., Hamilton, J. K., Robins, P. A., & Smith, F. (1966) *Anal. Chem. 28*, 350–356.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Gerwig, G. J., Kamerling, J. P., & Vliegenthart, J. F. G. (1978) *Carbohydr. Res. 62*, 349-357.
Good, R. C. (1985) *Annu. Rev. Microbiol. 39*, 347-369.
Goren, M. B., & Brennan, P. J. (1979) in *Tuberculosis* (Youmans, G. P., Ed.) pp 69-193, Saunders, Philadelphia.
Gray, G. R. (1976) *Methods Carbohydr. Chem. 7*, 157-159.
Hakomori, S. (1964) *J. Biochem. (Tokyo) 55*, 205-208.
Hunter, S. W., Murphy, R. C., Clay, K., Goren, M. B., & Brennan, P. J. (1983) *J. Biol. Chem. 258*, 10481-10487.
Hunter, S. W., Jardine, I., Yanagihara, D. L., & Brennan, P. J. (1985) *Biochemistry 24*, 2798-2805.
Janssen, P. E., Kenne, L., Liedgren, H., Lindberg, B., & Lonngren, J. (1976) *Chem. Commun., Univ. Stockholm No. 8*, 1-75.
Jonsson, G. P., Hedin, A. B., Hakansson, P. L., Sundqvist, B. U. R., Save, B. G. S., Nielsen, P. F., Roepstorff, P., Johansson, K.-E., Kamensky, I., & Lindberg, M. S. L. (1986) *Anal. Chem. 58*, 1084-1087.
Kamisango, K., Saadat, S., Dell, A., & Ballou, C. E. (1985) *J. Biol. Chem. 260*, 4117-4121.
Kasai, R., Okihara, M., Asakawa, J., Mizutani, K., & Tanaka, O. (1979) *Tetrahedron 35*, 1427-1432.
Kim, K. S., Salton, M. R. J., & Barksdale, L. (1976) *J. Bacteriol. 125*, 739-743.
Lemieux, R. V., & Stevens, J. D. (1966) *Can. J. Chem. 44*, 249-262.
Lindberg, B. (1972) *Methods Enzymol. 28*, 178-195.
Marks, J., Jenkins, P. A., & Tsukamura, M. (1972) *Tubercle 53*, 210-214.
McNeil, M., Tsang, A. Y., & Brennan, P. J. (1987) *J. Biol. Chem. 262*, 2630-2635.
Minnikin, D. E. (1982) in *The Biology of the Mycobacteria* (Ratledge, C., & Stanford, J., Eds.) Vol. 2, pp 95-184, Academic, London.
Minnikin, D. E., Dobson, G., Sesardic, D., & Ridell, M. (1985) *J. Gen. Microbiol. 131*, 1369-1374.
Mort, A. J., & Bauer, W. D. (1982) *J. Biol. Chem. 257*, 1870-1875.
Park, J. T., & Johnson, M. J. (1949) *J. Biol. Chem. 181*, 149-151.
Prehm, P. (1980) *Carbohydr. Res. 78*, 372-374.
Saadat, S., & Ballou, C. E. (1983) *J. Biol. Chem. 258*, 1813-1818.
Schaefer, W. B. (1965) *Am. Rev. Respir. Dis. 92 (Suppl.)*, 85-93.
Schaefer, W. B., Wolinsky, E., Jenkins, P. A., & Marks, J. (1973) *Am. Rev. Respir. Dis. 108*, 1320-1326.
Schleifer, K. H., & Kandler, O. (1972) *Bacteriol. Rev. 36*, 407-477.
Skerman, V. B. D., McGowen, V., & Sneath, P. H. A. (1980) *Int. J. Syst. Bacteriol. 30*, 225-240.
Sundqvist, B., Kamensky, I., Hakansson, J., Kjellberg, J., Salehpour, M., Widdiyasekera, S., Fohlman, J., Peterson, P. A., & Roepstorff, P. (1984) *Biomed. Mass Spectrom. 11*, 242-257.
Takayama, K., Schones, H. K., Armstrong, E. L., & Boyle, R. W. (1975) *J. Lipid Res. 16*, 308-317.
Vilkas, E., Adam, A., & Senn, M. (1968) *Chem. Phys. Lipids 2*, 11-16.
Wayne, L. G. (1986) *CRC Crit. Rev. Microbiol. 12*, 185-222.
Wolinsky, F. (1979) *Am. Rev. Respir. Dis. 119*, 107-159.
Yanagihara, D. L., Barr, V. L., Knisley, C. V., Tsang, A. Y., McClatchy, J. K., & Brennan, P. J. (1985) *J. Clin. Microbiol. 21*, 569-574.

Volume Changes during Enzyme Reactions: Indications of Enzyme Pulsation during Fumarase Catalysis†

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ABSTRACT: Overall activation volumes for multistep reactions are not usually pressure independent. The present investigation gives a quantitative description of this effect under Theory. Simple relations are obtained which can easily be applied to experimental data and which allow more insight into the dynamics of enzyme reactions. This is demonstrated under Experimental Application for the conversion of fumarate to L-malate catalyzed by the enzyme fumarase. The volume profile of this reaction indicates a pulsation of the enzyme molecule during catalysis. The appendix discusses the question whether Eyring's transition-state theory is an appropriate basis for investigations of this kind.

Although pressure is a thermodynamic parameter as important as temperature, it has long been disregarded and has only lately gained importance in biology and biochemistry. High-pressure experiments have revealed the role of membranes in anesthetics (Lodge, 1985) and the action of ethanol (Alkana et al., 1985). Pressure affects the photocycle of purple membranes (Marque & Eisenstein, 1984) probably because the charge transfer through the membrane is affected (Canfield & Macey, 1984). Hydrostatic pressure has been used to kill bacteria, spores, yeasts, and molds in order to sterilize sensitive pharmaceutical products (Butz & Ludwig, 1986; Mentrup et al., 1988). When applied to fertilized eggs, high-pressure produces triploid cells (Vasetskii et al., 1985), probably by inhibiting microtubule assembly and spindle formation, and may become an interesting tool for the study of genetic diseases such as trisomies. Pressure-inactivated lymphocytes have been used in multiple sclerosis research (Cohen, 1985). Another promising application seems to be the use of high pressure for the isolation of certain membrane

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