(Linnane *et al.*, 1962; Schatz, 1963), and to determine whether the latter also contains a similar CRM.

The data of Table II suggest that the R_2L_w particles from mutants possess approximately one-third the combining ability for antibody (on a protein basis) as do the corresponding antigenic particles from the wild type. If we make the highly questionable assumption that the association constant for the formation of the complex is the same for particles regardless of genetic constitution, it follows that the level of CRM is about one-third that of the wild-type antigen in commercial yeast. It is perhaps not inappropriate at this point to recall the observation that the actual level of the DPNH-cytochrome c reductase in the parent wild type (59 R) is also only one-third of that of commercial yeast (Table VI of the previous paper). Thus the amount of CRM in the mutant may be quite comparable to that of the antigen in the corresponding wild-type strain.

Finally we may make some suggestions concerning the probable nature of the cross-reacting protein. We know the mutant contains mitochondrialike structures (Yatsuayanagi, 1963) and a particulate fraction not too dissimilar from that of the wild type; the latter can be used as a source of a respiratory subparticle having a content of proteins in general and of primary dehydrogenase in particular, again quantitatively similar to that observed in the analogous structure from the wild type (Mahler *et al.*, 1964a). The mutant cell shows a lipid pattern qualitatively and quantitatively similar to that of the wild-type (Mahler *et al.*, 1964b), although we do not yet know whether these lipids are integrated in the normal manner into the matrix of the respiratory particles. The data on the distribution of protein and primary dehydrogenase between R_2H and R_2L particles from wild-type and mutant cells, taken in conjunction with the small extent of inhibition of the dehydrogenase as compared to the reductase and the virtual absence of CRM in the R_2H particles from the mutant, appear to exclude either the primary dehydrogenase or the structural protein(s) of the particles as a likely candidate for the CRM. Thus antigen and CRM appear to be localized in the cytochrome b–cytochrome c_1 region of the respiratory chain. Their exact nature is currently under investigation.

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The Structure of a Myoinositol Mannoside from *Mycobacterium tuberculosis* Glycolipid*

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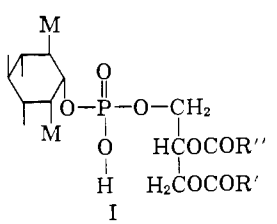
The retention times on gas chromatography of the four possible isomeric pentamethylmyoinositol ethers have been determined. Using these reference values, it has been possible to identify the pentamethylmyoinositol obtained by methanolysis of permethylated myoinositol monomannoside which had been prepared from the glycolipid of *Mycobacterium tuberculosis*. It is established that the mannose is attached to the 2-hydroxyl group of the myoinositol ring.

It has been established (Lee and Ballou, 1964) that "manninositose," the myoinositol dimannoside obtained from a *Mycobacterium* glycophospholipid by alkaline degradation (Anderson *et al.*, 1938), has the mannoses attached to the 2- and 6- positions of the myoinositol ring. In combination with other work (Ballou *et al.*, 1963), this makes it possible to write structure I for the intact lipid:



I

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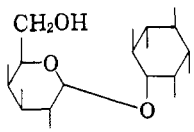
to which may be assigned the definitive name 1-phosphatidyl-L-myoinositol-2,6-bis-α-D-mannopyranoside. There is a discrepancy between this structure and one suggested by others (Vilkas and Lederer, 1960; Angyal and Shelton, 1963), but the reasons for accepting structure I are well documented.

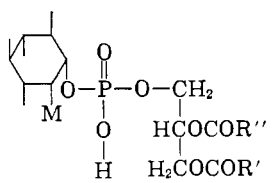
Among the products of alkaline degradation of the crude *Mycobacterium* phospholipid, one also finds a myoinositol monomannoside (Vilkas, 1960). This substance forms a crystalline acetate, mp 178–180°, with a specific rotation in chloroform of $[\alpha]_D$ + 20.5°. The existence of galactinol (Brown and Serro, 1953), a galactoside of myoinositol which has the galactose on the D-1- position of the myoinositol ring (II) (Kabat *et al.*, 1953), suggests that the myoinositol mannoside might have the analogous structure. In fact Angyal and Shelton (1963) report the synthesis of such a compound by coupling tetra-*O*-acetyl-D-mannosyl bromide:



II

with 1,4,5,6-tetra-*O*-acetyl-myoinositol. From the mixture of products was obtained an acetate with mp 178–180° and a specific rotation of $[\alpha]_D + 20.5°$ in chloroform. Since two isomeric products were possible, the position of substitution was established by methylation, which yielded the same pentamethylmyoinositol one gets from galactinol (II). By comparison of the synthetic mannoside with the compound from *M. tuberculosis* phospholipid, it was concluded that they were identical.

Our studies on the dimannoside, which resulted in assignment of structure I, led us to believe that the monomannoside should have a related structure (i.e., with the mannose on either the 2- or 6-position), since we assumed there must be a biosynthetic relationship between the two compounds. Therefore we undertook an investigation of the structure of the monomannoside by methylation. In summary, methylation of a sample of the substance obtained from *M. tuberculosis* phospholipid, followed by methanolysis, yielded a pentamethylmyoinositol with the retention time on gas chromatography identical to that of 1,3,4,5,6-penta-*O*-methylmyoinositol, and different from the other three possible isomers. This establishes that the mannose is attached to the 2- position of the inositol ring, and, assuming that the myoinositol mannoside is derived from a phospholipid, the intact lipid must have structure III.



III

Materials

Penta-O-methylmyoinositol Reference Compounds.—1,2,4,5,6-Penta-*O*-methylmyoinositol has been prepared previously by methylation of galactinol (Kabat *et al.*, 1953).

The other isomers were obtained by partial methylation of known tetra-*O*-methylmyoinositols. A typical reaction was as follows. About 10 mg of 1,4,5,6-tetra-*O*-methylmyoinositol (Lee and Ballou, 1964) and 50 mg of dry silver oxide were mixed with 100 μl of dry dimethylformamide containing 2.5 μl of methyl iodide (about 1 mole equivalent of methyl iodide to the inositol ether). The mixture was shaken overnight, then 2 ml of absolute ethanol was added and the solids were removed by centrifugation. The supernatant was decanted and concentrated to dryness, giving a residue that was dissolved in a small amount of acetone and analyzed by gas chromatography. Four peaks were obtained corresponding to hexamethylmyoinositol, 1,2,4,5,6-pentamethylmyoinositol, 1,3,4,5,6-pentamethylmyoinositol, and unmethylated tetramethylmyoinositol. Figure 1 shows a tracing of such a chromatogram, while Table I lists the tetramethylmyoinositols which were methylated in this manner and the retention times of the pentamethylmyoinositol(s) produced. By a procedure of cross comparisons described under Results and Discussion it was possible to assign a structure to each of the products, thereby identifying each isomer by retention time (R_t).

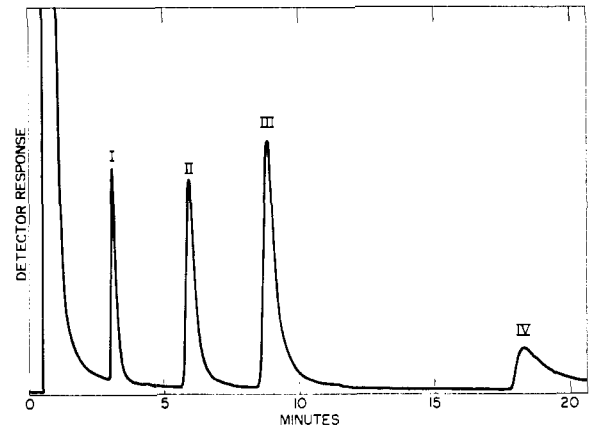


FIG. 1.—A reproduction of the gas-chromatography tracing of partially methylated 1,4,5,6-tetramethylmyoinositol. Peak I is hexamethylmyoinositol, II is 1,2,4,5,6-pentamethylmyoinositol, III is 1,3,4,5,6-pentamethylmyoinositol, and IV is unreacted starting material. The column temperature was 158°.

TABLE I

GAS CHROMATOGRAPHIC RETENTION TIMES OF PENTAMETHYLMYOINOSITOLS OBTAINED BY METHYLATION OF KNOWN COMPOUNDS

Compound Methylated[a]	Pentamethyl-myoinositol Isomer(s) Obtained[b]	Retention Times (R_t, min) 160°	125°
Galactinol	1,2,4,5,6-	6.0	25.0
1,4,5,6-Tetramethyl-myoinositol	1,2,4,5,6- (1)	6.0	25.0
	1,3,4,5,6- (1.3)	8.8	41.5
1,3,4,5-Tetramethyl-myoinositol	1,3,4,5,6- (1)	8.8	41.5
	1,2,3,4,5- (1)	8.9	44.5
1,2,3,5-Tetramethyl-myoinositol	1,2,3,4,5-	8.9	44.5
1,3,4,6-Tetramethyl-myoinositol	1,3,4,5,6- (1)	8.8	41.5
	1,2,3,4,6- (2)	7.9	38.6

[a] The galactinol was completely methylated, while the other compounds were partially methylated according to the procedure in the experimental part. [b] Relative amounts formed, as assessed from peak areas, are given in parentheses.

Methods

Gas Chromatography.—Analyses were carried out on an Aerograph "Hy-Fi" Model A-600-B furnished with a hydrogen-flame detector. Anakrom ABS (100–110 mesh), used as the solid support, was coated with 2% neopentylglycol succinate and packed in a stainless steel column 5 feet long and 1/8 inch in outer diameter. The flow rates of nitrogen and hydrogen gas were each about 23 ml/minute. Samples for analysis were dissolved in acetone, and quantitative estimates were based on the peak area of each component.

Methylation of Myoinositol Mannoside.—About 3 mg of myoinositol mannoside nonaacetate, mp 176–178°, isolated and described previously (Ballou *et al.*, 1963), was dissolved in 1 ml of dry methanol and deacetylated by the addition of a drop of barium methoxide solution. After 1 hour a piece of solid carbon dioxide was added to decompose the barium methoxide, and the solution was concentrated to dryness. About 0.5 ml of dry dimethylformamide was added and the tube and contents were heated gently over a Bunsen burner to bring the myoinositol mannoside into solution. The tube was cooled to room temperature and

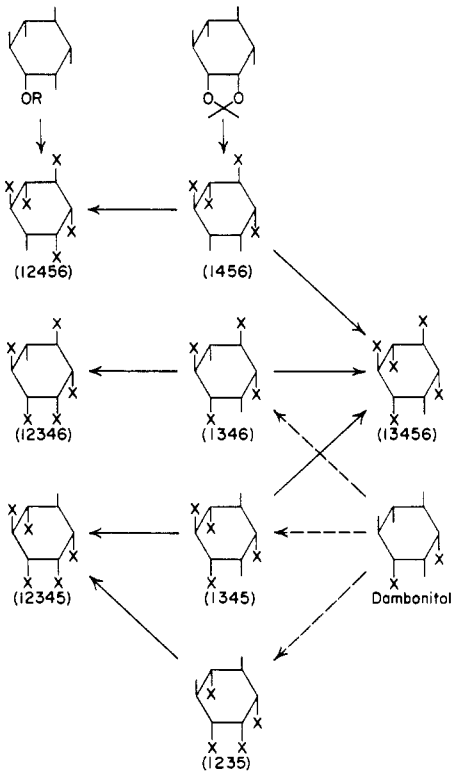


FIG. 2.—A scheme illustrating the reactions and partial methylations by which the pentamethylmyoinositol ethers were obtained and characterized.

200 mg of silver oxide and 5 drops of methyl iodide were added. The mixture was shaken for 18 hours, when a second addition of methyl iodide and silver oxide was made. After the mixture had shaken for another 24 hours, 2 ml of ethanol was added and solids were removed by centrifugation. The supernatant solution was decanted and concentrated to dryness. The residue was dissolved in 0.5 ml of dry methanol containing 5% hydrogen chloride, and the solution was transferred to a vial which was then sealed. The vial was heated at 100° for 18 hours, after which it was cooled and opened and the contents was evaporated to dryness.

The residue was dissolved in a few drops of acetone, and this solution was used for analysis by gas chromatography. The pentamethylmyoinositol component had the retention time recorded in Table II, and corresponded to the 1,3,4,5,6- isomer. The ratio of the area of this peak to that of the methyl tetramethylmannoside was 1:0.92. When the methanolysis mixture was chromatographed with internal standards of each of the four pentamethylmyoinositol references, the pentamethylmyoinositol component differed from all isomers except 1,3,4,5,6-pentamethylmyoinositol, with which it corresponded exactly, giving a peak that was additive with the reference.

RESULTS AND DISCUSSION

Because of the very small amount of myoinositol mannoside available for analysis, the characterization of the pentamethylmyoinositol was based on its gas chromatographic properties. This was sufficient since the myoinositol mannoside itself is a well characterized substance, and the only question to be answered was that of the position of linkage to the inositol ring. For this purpose, it was critical to be able to distinguish between the four possible pentamethylmyoinositol ethers.

One pentamethylmyoinositol was known at the time this study was begun, that being the 1,2,4,5,6-isomer formed by hydrolysis of exhaustively methylated galactinol. The other three isomers were prepared, in amounts sufficient for gas chromatography, by partial methylation of known tetramethylmyoinositols (Lee and Ballou, 1964). Figure 2 illustrates the reactions involved and demonstrates how the cross comparisons of products formed in each methylation reaction allows the assignment of identity to each of the pentamethylmyoinositol peaks observed in the gas chromatograms on the basis of retention time (R_t). 1,4,5,6-Tetramethylmyoinositol yields two pentamethylmyoinositols; one corresponds to the well-characterized 1,2,4,5,6- isomer (R_t 25.0) obtained from galactinol, and therefore the other must be 1,3,4,5,6-pentamethylmyoinositol (R_t 41.5). The 1,3,4,6-tetramethyl ether gives two pentamethyl ethers. Since one corresponds to 1,3,4,5,6-pentamethylmyoinositol (R_t 41.5), the other must be the 1,2,3,4,6- isomer (R_t 38.6). 1,3,4,5-Tetramethylmyoinositol also yields the 1,3,4,5,6-pentamethyl ether (R_t 41.5), but it gives a second peak which must be the 1,2,3,4,5- isomer (R_t 44.5). This latter compound is the only pentamethyl ether which can be obtained from 1,2,3,5-tetramethylmyoinositol; and, as we see, this is the only pentamethyl peak (R_t 44.5) that was observed on partial methylation.

TABLE II
GAS CHROMATOGRAPHIC RETENTION TIMES OF PENTAMETHYLMYOINOSITOLS

Isomer	Retention Times 160°	125°
1,2,4,5,6-	6.0	25.0
1,2,3,4,6-	7.9	38.6
1,3,4,5,6-	8.8	41.5
1,2,3,4,5-	8.9	44.5
From myoinositol mannoside[a]	8.8	41.5
From myoinositol glucoside[b]	8.9	44.5

[a] Obtained from *M. tuberculosis* glycolipid. [b] Supplied by Professor H. E. Carter and derived from phytoglycolipid.

These partial methylation studies offer an opportunity to compare the relative rates of methylation at different positions on the inositol ring, since the relative amounts of the different isomers formed can be estimated directly from the areas under the curves of the gas-chromatographic tracings. Each of the three tetramethyl ethers has a free axial 2-hydroxyl group and one free equatorial hydroxyl group. One might have predicted preferential methylation of the more accessible equatorial hydroxyls. In fact, the two groups in the 1,3,4,5- and 1,4,5,6-tetramethyl ethers are methylated at about equal rates, while the axial 2-hydroxyl in the 1,3,4,6- isomer is methylated twice as fast as the equatorial 5-hydroxyl group.

Gas chromatography of the methanolyzed methylated myoinositol mannoside from *M. tuberculosis* phospholipid gave peaks corresponding to methyl 2,3,4,6-tetra-*O*-methyl-D-mannoside and 1,3,4,5,6-pentamethylmyoinositol. This establishes that the mannose is attached to the 2- position of the inositol ring. Such a structure for the monomannoside brings it into line with the structure we have already shown to be present in the myoinositol dimannoside, and suggests that the biosynthesis of the *Myobacterium* inositol phospholipids involves the stepwise glycosylation of phosphatidyl-

myoinositol, first at the 2- position and then at the 6-position of the ring.

ACKNOWLEDGMENT

The authors are indebted to Dr. Erna Vilkas for the sample of myoinositol mannoside which was isolated during the work described by Ballou, Vilkas, and Lederer (1963).

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Evidence Favoring the Nonspecificity of 3-Hydroxysteroid Dehydrogenases in Relation to Steroid Conformation*

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The high-speed supernatant of homogenates from male rat liver contained enzymes which, after combination with TPN or DPN, were able to oxidize 3α- or 3β-hydroxysteroids derived from the hormone and cardenolide series. In the presence of a tenfold excess of cofactor the differences in the conformation of the substrates, i.e., A/B *cis*-, C/D *trans*- and A/B *trans*-, C/D *trans*-ring junction on the one hand and A/B *cis*-, C/D *cis*- and A/B *trans*-, C/D *cis*-ring junction on the other, did not influence the shapes of *p*H-activity curves and the positions of *p*H optima which were largely dependent on the steric configuration of the hydroxyl group at carbon 3. The *p*H optimum for the dehydrogenation of 3α-hydroxysteroids was at 9.6 with TPN or at 8.6 with DPN and that of 3β-hydroxysteroids was at 10.2 with DPN. Irrespective of the ring isomerization, the 3α-hydroxysteroids were dehydrogenated with TPN as cofactor much faster than when DPN was used. The 3β-hydroxysteroids were not significantly oxidized by the liver supernatant fraction in the presence of TPN. The *cis*- or *trans*- junction of the rings A and B or C and D influenced the reaction rate only. Extent and direction of this influence was not predictable from the conformation of the substrate. The present evidence favors the conclusion that in the supernatant fraction two types of 3α-hydroxysteroid dehydrogenases are involved, differing in their specificity to TPN and DPN, and one type of DPN-dependent 3β-hydroxysteroid dehydrogenase. No distinction, either in shape of *p*H curve, *p*H maximum, or nucleotide specificity could be observed between steroids of the hormone and cardenolide types having the same steric orientation of the hydroxyl group at C-3.

Metabolic experiments carried out with steroids of the hormone series possessing a C/D *trans* configuration have shown the presence of various 3-hydroxysteroid dehydrogenases in rat liver. Tomkins (1956) partially purified a soluble 3α-dehydrogenase. This enzyme used C_{19} and C_{21} compounds as substrates but did not attack two C_{27} steroids. A 3β-hydroxysteroid dehydrogenase has not yet been isolated; the existence of such an enzyme n rat liver may, however, be deduced from the results obtained in metabolic experiments using some C/D *trans*-steroids (Kochakian and Aposhian, 1952; Schneider, 1952; Harold *et al.*, 1956). These results indicated that rat liver contains 3β-hydroxysteroid dehydrogenases which may use C_{19}, C_{21}, and C_{27} steroids of the C/D *trans* series as substrates. The identity and the distribution of these 3β-enzymes in the soluble and particulate fractions of the liver is not yet clear. A 3α-hydroxysteroid dehydrogenase preparation obtained from the soluble fraction of a liver homogenate (105,000 × *g*) also used epiandrosterone as a substrate in addition to 3α-hydroxysteroids but did not metabolize testosterone (Hurlock and Talalay, 1958). The metabolism of the 3β-hydroxysteroid was probably due to the impurity of the enzyme preparation. Thus it is likely that the rat liver contains at least one soluble 3β-hydroxysteroid dehydrogenase which is distinctly different from the 17β-enzymes of the rat liver.

Oxidation-reduction reactions on C-3 are not limited to the C/D *trans*-steroid series but also have been observed with certain steroids of the cardenolide series distinguished by a C/D *cis* configuration. These C_{23} steroids show in addition to the difference in the C/D configuration a tertiary hydroxyl group at C-14 and a butenolide group on C-17 (Fig. 1). Upon incubation of 3β-hydroxycardenolide genins (especially digitoxigenin, gitoxigenin, digoxigenin, and diginatigenin) with liver slices, occasionally in addition to traces of 3-keto derivatives a substantial production of the 3α-epimers of these substrates could be detected (Lauterbach and Repke, 1960). Because of the peculiar constitution of the genins it has been supposed that

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