THE CHEMICAL STRUCTURE OF THE CORD FACTOR OF *MYCOBACTERIUM TUBERCULOSIS*

by

H. NOLL AND H. BLOCH

Division of Tuberculosis, The Public Health Research Institute of the City of New York, Inc., New York, N.Y. (U.S.A.)

and

J. ASSELINEAU AND E. LEDERER

Institut de Biologie Physico-Chimique, Paris (France)

The isolation and partial structural analysis of the cord factor of *Mycobacterium tuberculosis* has been described in previous publications[1-4]. Recently the final structure of cord factor has been established and announced in a short communication[5]. It was shown to be trehalose-6,6'-dimycolate (I). The present paper gives a full account of the experimental evidence leading to this result.

It has previously been found that cord factor, upon alkaline hydrolysis, was split into mycolic acid[2,4] and a non-reducing carbohydrate moiety which after subsequent acid hydrolysis yielded D-glucose[3]. The combustion analyses of the entire cord factor molecule repeatedly indicated the presence of a small amount of nitrogen (0.6 to 1.0%) corresponding to about one atom per molecular weight of about 1500 (ester of mycolic acid and glucose)[2-4]. It was assumed that the nitrogen resided in a small fragment attached to glucose through an acid-labile glucoside linkage. However, in continuing the degradation experiments no evidence of such a hypothetical nitrogenous fragment could be found. As more material became available the decisive experiment could be performed: a precise quantitative glucose determination on the non-reducing glycoside resulting from alkaline hydrolysis of cord factor. This gave the unexpected value of 106%, and thus it followed that glucose could only have been present in the form of its non-reducing disaccharide trehalose and that the small quantity of nitrogen was probably due to an impurity. This conclusion was confirmed by converting the non-reducing water-soluble fragment into a crystalline acetate which was identified as α,α-trehalose octaacetate by melting point (81–82°), mixed melting point, and by the identity of its infrared spectrum with that of an authentic specimen. Trehalose had already been isolated from various tubercle bacilli extracts by PANGBORN AND ANDERSON[6] and by ANDERSON AND NEWMAN[7] in 1933.

From the above findings it became clear that cord factor was an ester of myclioc acid and trehalose. Since our previous analytical data indicated a 1:1 molar ratio of mycolic acid and glucose, it followed that cord factor must be a trehalose dimycolate. It has been shown previously[3,4] that the mycolic acid isolated from cord factor

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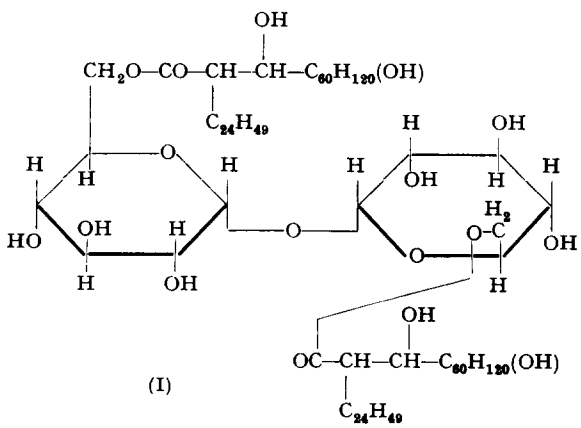
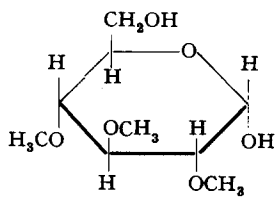
preparations is a 3,x-dihydroxy-mycolanoic acid* $C_{87}H_{174}O_4 \pm 5\ CH_2$. The molecular formula of cord factor is thus $C_{186}H_{366}O_{17} \pm 10\ CH_2$, which is in fair agreement with results of elementary analyses.

The remaining problem of determining the positions of mycolic acid on the trehalose molecule was solved by methylation. A completely methylated sample of cord factor was saponified and the resulting hexamethyltrehalose hydrolyzed by acid. Paper chromatography of the hydrolysate gave only one spot with an R_G value corresponding to a trimethylglucose. This proves that in cord factor the trehalose molecule is symmetrically esterified with two molecules of mycolic acid. By comparing the hydrolysate with known samples of the four isomers of tri-O-methyl-glucose** it was possible to identify the unknown methyl sugar as 2,3,4-tri-O-methyl-D-glucose (II). This result was arrived at by two independent methods: a combination of periodate oxidation and paper chromatography as described by LEMIEUX AND BAUER[8] and by infrared spectroscopy***. Identification of methylated sugars by the latter method proved to be of great value since the infrared spectra of the various isomers differ so greatly as to exclude any ambiguity. It should be stressed that both periodate oxidation and infrared spectroscopy further corroborated the homogeneity of the trimethylglucose isomer obtained from cord factor, thus confirming the conclusion that trehalose was symmetrically substituted.

The isolation of 2,3,4-tri-O-methyl-glucose after hydrolytic degradation of the methylated cord factor proved conclusively that mycolic acid is esterified with the two primary hydroxyl groups of trehalose. This conclusion is further supported by the results of periodate titrations published in a previous paper[4] showing that cord factor consumes more than one molecule of periodic acid per glucose residue. Hence, the structure of cord factor can be written as trehalose 6,6'-dimycolate (I).



(I)



(II)

* For a recent review on the chemistry of mycolic acids see J. ASSELINEAU AND E. LEDERER, *Experimental Tuberculosis*, A Ciba Foundation Symposium, J. & A. Churchill Ltd. London 1955, pp. 14–38.

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*** The authors are greatly indebted to Dr. R. C. GORE and Mr. N. B. COLTHUP, American Cyanamid Company, Stamford, Connecticut, for the courtesy of running a great number of infrared spectra and for their invaluable help in interpreting the results.

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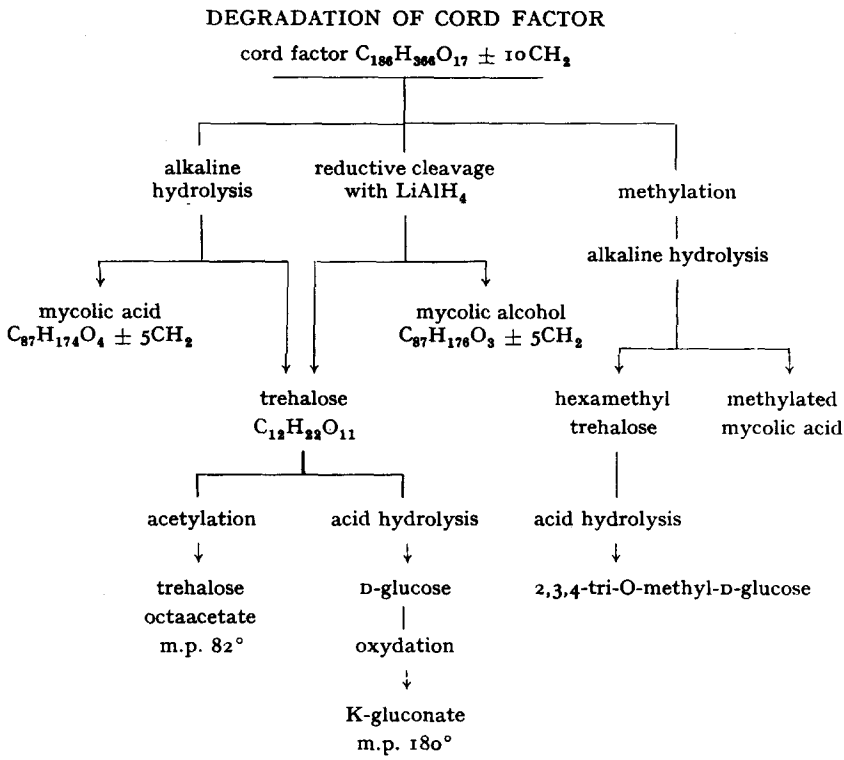
The course of chemical degradation of cord factor and the main analytical findings leading to this structure are summarized in the chart below.



The above results were obtained with cord factor preparations from the human strain "Brévannes". They were confirmed with analogous samples prepared from the BCG strain. The infrared spectrum as well as the toxicity for mice of cord factor from BCG was identical with the corresponding properties of cord factor previously isolated from six different virulent human and bovine strains[2-4].

Samples of cord factor from BCG when analyzed for nitrogen by the Dumas method gave negative results in contrast to the low positive values consistently obtained by the same method with the cord factor samples from other strains. This finding together with the negative results obtained by infrared spectroscopy and chemical degradation suggested that the previously found nitrogen values were either due to small impurities or to an experimental error (as liberation of small amounts of methane during combustion) inherent in the Dumas method[9] when working in a range close to its lower limit of sensitivity. Systematic nitrogen assays by three different methods with carefully repurified samples of cord factor Brévannes using mycolic acid for comparison showed indeed that the Dumas method gave erroneously positive values in the range of 0.3% for both cord factor and mycolic acid, while the other two methods[10,11] revealed only traces of nitrogen in the amount of 0.1% or less. Hence nitrogen was ruled out as part of the cord factor molecule.

With the analytical elucidation of the structure accomplished, the way was now

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open for the synthesis of cord factor. The results of the synthetical work* (which will be published shortly) are in excellent agreement with the analytical deductions presented in this paper**.

EXPERIMENTAL

I. THE STRUCTURE OF CORD FACTOR BRÉVANNES (by H. NOLL)***

A. Isolation of cord factor Brévannes

The purified wax was prepared from the chloroform extracts as reported previously[2]. Wax C and D were separated and cord factor obtained by chromatography of Wax C on magnesium silicate-celite as described[3]. For elementary analyses a sample was rechromatographed on silica gel Davison (preparation A). An aliquot of this sample was further purified by dissolving in ether and washing with a 10% solution of HCl. The moist ether phase was then passed through a column of Amberlite MB-3 anion-cation exchange resin, the effluent dried over anhydrous sodium sulfate, and the cord factor precipitated from the concentrated ether solution with methanol (preparation B).

Microanalysis of these highly purified samples gave the following values§:

	% C	% H
preparation A	77.02	12.66
preparation B	77.22	12.61
calculated for $C_{186}H_{366}O_{17}$	77.70	12.83

B. Nitrogen determination on highly purified samples of cord factor (Brévannes)

The above-described highly purified preparations A and B were assayed for nitrogen by three different micro methods, using nitrogen-free mycolic acid for comparison:

1. Method according to Dumas[9], modified for trace analysis by Schwarzkopf (unpublished results).
2. Method according to Kjeldahl[10].
3. Method according to LUBOCHINSKY AND ZALTA[11].

The results compiled in Table I show only traces of nitrogen. The variations between the data obtained with the different methods indicate that these values are due to experimental errors which are inevitable at the lower range of sensitivity of the methods used.

TABLE I

NITROGEN DETERMINATIONS ON HIGHLY PURIFIED SAMPLES OF CORD FACTOR (BRÉVANNES)

Sample		*Cord factor*		*Mycolic acid*
		A	*B*	
	% N found			
Method:	Dumas§	0.19	0.19	0.37
				0.33
	Kjeldahl§	0.30	0.09	0.10
	LUBOCHINSKY AND ZALTA[11]	<0.1	<0.1	

* Synthetic mycolates of glucose, galactose and glucosamine are described in a recent paper by ASSELINEAU AND LEDERER[19]; these substances are toxic[20].

** We are indebted to Ciba, Ltd., Basel, Switzerland, for microanalyses and to the "Fondation Waksman pour le Developpement des Recherches Microbiologiques en France" for several grants to the Laboratory of E. LEDERER.

*** Part of these experiments were performed at the Institut de Biologie Physico-Chimique, Paris, France.

§ Analysis by the Schwarzkopf Microanalytical Laboratory, Middle Village, New York.

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C. Chemical degradation of cord factor

1. Isolation and analysis of the water-soluble moiety

619 mg of cord factor (Brévannes) were saponified in alkaline solution and the water-soluble moiety isolated by lyoph[illegible]e deionized aqueous solution as described previously[3]. This yielded 75 mg of a colorless syrup corresponding to 12.1 % of the starting material (theory for trehalose dimycolate: 11.8%). After drying this viscous syrup *in vacuo* and keeping over P_2O_5 for several weeks, it became a solid glass. Microanalysis of a sample of the original syrup which had been dried in vacuo at 65° C overnight gave the following values:

	% C	% H	% N
found	39.49	6.95	0
calculated for trehalose $C_{12}H_{22}O_{11} \cdot 2H_2O$	38.10	6.94	0

These data indicated that the water-soluble fragment had a C, H composition characteristic for carbohydrates and was free of nitrogen.

2. Quantitative glucose determination on the water-soluble carbohydrate fragment

10.10 mg of the above-described thoroughly dried material were dissolved in 2.00 ml of distilled water. Aliquots of 5, 10, and 25 mm^3 containing 25.3, 50.5 and 126.3 μg of the dissolved substance were then withdrawn with a micropipette and their glucose content determined using the sulfuric acid test according to MENDEL *et al.*[12]. The optical density of the characteristic absorption maxima at 255, 312, and 520 $m\mu$ was measured in a Beckman model DU spectrophotometer, and the values plotted against the weight of the samples. The glucose content of the unknown sample was then determined by graphically evaluating the ratio between the slope of the curve for the sample and the slope of the glucose standard curve obtained under identical conditions. Representative data taken from the graph for C = 100 μg substance per 4.0 ml are compiled in Table II.

As can be seen, these data agree well with the theoretical value of 105 % for the glucose equivalent of anhydrous trehalose.

TABLE II

GLUCOSE DETERMINATION ON WATER-SOLUBLE FRAGMENT BY SULFURIC ACID TEST[12]

Graphically evaluated optical densities for C = 100 $\mu g/4$ ml

	Optical density measured at		
mμ:	*255*	*312*	*520*
A. Water-soluble from cord factor	0.315	0.942	0.141
B. Glucose	0.300	0.888	0.131
Ratio A:B in % (glucose equivalent of water-soluble)	105 ± 3%	106 ± 3%	108 ± 3%

3. Identification of water-soluble moiety as crystalline trehalose octaacetate

A sample of 22.4 mg of the dried water-soluble carbohydrate from cord factor was acetylated according to ANDERSON AND NEWMAN[13]. Upon concentration of the chloroform extracts containing the reaction product, crystallization started immediately. After two recrystallizations from hot methanol, 23 mg of the acetate were recovered in the form of long prismatic needles melting at 81–82°. A mixed melting point with authentic trehalose octaacetate gave no depression. Its infrared spectrum was also identical with the spectrum of trehalose-octaacetate.

A few milligrams of a syrupy acetate of the water-soluble moiety of cord factor had already been prepared some time ago and its infrared spectrum published[2,3]. At that time it was not possible to identify it for lack of a reference spectrum, but this previously published spectrum was now found to be identical with the spectrum of trehalose octaacetate.

D. Determination of the position of the ester linkage between trehalose and mycolic acid

1. Methylation of cord factor

610 mg of cord factor, dissolved in 7 ml of CH_3I, were refluxed for three days in the presence of Ag_2O with magnetic stirring. Three portions of CH_3I and of freshly precipitated Ag_2O were added over the three-day period. The reaction product was then filtered off, washed with chloro-

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form, and taken to dryness. The waxy residue was dissolved in a small volume of ether, precipitated in the cold with an excess of methanol, filtered, and washed with methanol. The dried reaction product was a white powder of m.p. 33–35°.

The infrared spectrum of the methylated cord factor still showed some OH-absorption indicating that the methylation was not complete. In order to eliminate incompletely methylated material for subsequent hydrolysis and isolation of the methylated glucose, the crude methylation product was further purified by chromatography.

2. Chromatography of methylated cord factor

625 mg cord factor were dissolved in petrolether and chromatographed on silicic acid (Mallinckrodt).

Fraction	Eluted with	mg
1	500 ml petrolether	trace
2	500 ml petrolether-benzene 1:1	14
3	500 ml benzene-ether 1:1	220
4	500 ml ether	286
5	500 ml ether	15
6	500 ml ether containing 20% MeOH	19
	Recovered:	554 mg

Infrared spectroscopy showed that the strong bonded –OH band at 3350 cm^{-1} had disappeared in the spectra of fractions 2 and 3, while the spectra of fractions 4–6 still contained a hydroxyl band of greatly reduced but still considerable intensity. The fully-methylated fraction 3 was therefore used for the subsequent degradation studies.

3. Alkaline hydrolysis of methylated cord factor

100 mg of fraction 3 were dissolved in 5 ml *iso*propylalcohol containing 0.1 ml water and 100 mg KOH, and refluxed with magnetic stirring for 80 minutes. After cooling, a white precipitate resulted. The reaction mixture was neutralized with a very slight excess of HCl, 5 ml each of methanol and water added, and after shaking, stored in the ice box overnight. The white precipitate was then filtered off and washed with several portions of methanol-water 1:1 (v/v) and finally with methanol.

(a) *Isolation of methoxy-mycolic acid.* After drying *in vacuo* over P_2O_5, the washed precipitate yielded 87 mg of a white powder which was characterized by infrared spectroscopy as methoxy-α-mycolic acid (no hydroxyl absorption).

(b) *Isolation of hexamethyl trehalose.* After removal of the insoluble mycolic acid, the pooled solutions were concentrated *in vacuo* at 40–50° and taken to dryness in a desiccator. The resulting residue consisted of a mixture of solid KCl crystals and a yellowish oil. The oily residue was separated from the inorganic salts by extraction with 3 × 5 ml of acetone. The yellowish acetone extract was filtered through paper and taken to dryness *in vacuo*, yielding a yellow oil. Addition of 2 ml of water gave a cloudy solution which contained some undissolved lipid-like particles which were removed by extracting the aqueous phase with petrol ether.

10 mm^3 of the aqueous solution were withdrawn for a quantitative determination of the glucose content by the sulfuric acid test. Heating with concentrated H_2SO_4 produced a bluish-pink color with a maximum at 520 mμ characteristic for glucose. Tetramethylglucose, which was used as a reference, produced an identical color. The extinction value corresponded to a total amount of 12 mg glucose per 2 ml of aqueous solution, which represents a theoretical yield.

4. Acid hydrolysis of hexamethyl trehalose

To 1.0 ml of the methylated trehalose solution containing 6 mg glucose equivalent, 0.13 ml concentrated HCl was added, and the solution heated in a sealed tube at 110° for four hours. At the end of this period the hydrolysis was complete.

This was assured in a separate experiment in which the course of hydrolysis was followed by iodometric determination of the liberated reducing sugar by a micro-adaptation of the method of Willstätter and Schudel[14]. This rapid and simple microtitration procedure permitted an accurate determination of as little as 10 micrograms of glucose and its methylated derivatives. It was found that the usual colorimetric reduction methods cannot be used with highly methylated sugars, a fact which seems to be in agreement with theoretical considerations concerning the mechanism of this reaction.

The hydrolysate was deionized by passing through Amberlite IR-4B and the neutral eluates were concentrated *in vacuo* to a yellowish oil which was dissolved in 5 ml of acetone.

A 100 mm^3 aliquot of this acetone solution was withdrawn for a quantitative glucose determination by the sulfuric acid test. The value obtained corresponded to a total amount of 5 mg glucose equivalent per 5 ml acetone extract.

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From this acetone solution various aliquots were withdrawn for paper chromatography and periodate oxidation as described below.

5. *Identification of the trimethylglucose obtained from acid hydrolysis of the hexamethyl trehalose*

(a) *Identification by paper chromatography combined with periodate oxidation.* Paper chromatography of the methyl-trehalose hydrolysate together with mono-, di-, tri-, and tetra-O-methyl-glucoses showed that the unknown methylglucose moved with the rate of a tri-O-methyl-D-glucose.

For the finer distinction between the possible four isomers of tri-O-methylglucose, analytical micro methods of greater specificity than the usual paper chromatography were needed. LEMIEUX AND BAUER[8] have published a method for the identification of small amounts of monomethyl-glucoses. They suggested that this technique could also be applied to the identification of trimethyl-glucoses. The method is based on the fact that trimethylglucoses, previously reduced to the corresponding methylsorbitols, upon subsequent periodate oxidation and saponification yield characteristic reaction products which can be qualitatively distinguished by paper chromatography.

Descending paper chromatography on Whatman No. 1 paper with butanol-acetic acid-water[15] was used. The spots were revealed with aniline phthalate. The material was applied to the paper in quantities of 50 μg dissolved in a volume of 3–5 mm^3 of water.

(i) *Reduction with $NaBH_4$.* 0.5 ml of the acetone solution containing 500 μg of the unknown trimethylglucose was taken to dryness and 2 mg $NaBH_4$ dissolved in 100 mm^3 of water added to the residue. A lively evolution of H_2 gas started immediately. The solution was kept at room temperature for an hour with occasional shaking. The excess $NaBH_4$ was destroyed by adding 5 mm^3 of 10% acetic acid three times, at which point the violent gas evolution stopped and the solution had a pH of 4–5. The solution was deionized by shaking with a few milligrams of Amberlite MB-3, then passed through a small paper filter containing a small amount of Amberlite MB-3 which was subsequently washed with 2 ml of water. The collected filtrates were taken to dryness *in vacuo*.

(ii) *Oxdiation with $NaIO_4$.* The syrupy residue was dissolved in 25 mm^3 of a saturated solution of $NaIO_4$. After one hour at 0°, 3–5 mm^3 glycerol were added to destroy the excess periodate and the solution was warmed to room temperature. After five minutes the solution was made alkaline to phenolphtalein by slowly adding 5% NaOH from a micropipette. After another five minutes 5 mm^3 of the solution (approximately 50 μg) was applied to the paper.

(iii) *Results of paper chromatography.* The results obtained by paper chromatography of the unknown trimethylglucose from cord factor together with the four known isomers are listed in Table III.

TABLE III

PAPER CHROMATOGRAPHY OF THE TRI-O-METHYL-GLUCOSE ISOMERS

	Tri-O-methyl-glucose				
	2,4,6-	*2,3,6-*	*3,4,6-*	*2,3,4-*	*from methylated cord factor*
R_G value	0.80	0.82	0.84	0.88	0.88
Reported in literature[17]	0.76	0.83	—	0.85	
Color of spot with aniline phthalate	red-brown	red-brown	walnut	red-brown	red-brown

In Table IV the R_G values of 2,3,4-tri-O-methylglucose are compared with those of the tri-O-methylglucose from cord factor before and after treatment according to LEMIEUX AND BAUER[8],

It can be seen from Table III that the spot produced on the paper by the cord factor-trimethylglucose is identical in position and color to the one obtained with 2,3,4-tri-O-methylglucose, having the highest R_G value (0.88) of all four isomers. In order to further test this correspondence, the trimethylglucose from cord factor and the 2,3,4-isomers were reduced and oxidized with $NaIO_4$. Subsequent chromatography of the saponified oxidation products again gave rise to identical spots (Table IV).

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TABLE IV

PAPER CHROMATOGRAPHY OF 2,3,4-TRI-O-METHYLGLUCOSE AND OF THE TRIMETHYLGLUCOSE OBTAINED FROM METHYLATED CORD FACTOR BEFORE AND AFTER TREATMENT ACCORDING TO LEMIEUX AND BAUER[8]

Tri-O-methyl-glucose:	*2,3,4-*	*from methylated cord factor*	*After oxidation with $NaIO_4$ and saponification:*		*Saponification products after reduction with $NaBH_4$ and oxidation with $NaIO_4$:*	
			2,3,4-	*from methylated cord factor*	*2,3,4-*	*from methylated cord factor*
R_G value	0.88	0.88	0.88	0.88	0.97	0.97
Color of spot with aniline phthalate	red-brown	red-brown	red-brown	red-brown	grey	grey

The 2,4,6- and 2,3,6-isomers could be excluded with reasonable certainty because their R_G values of 0.80 and 0.82 respectively were considerably lower than the value of 0.88 observed for cord factor-trimethylglucose. In addition, 2,4,6-tri-O-methylglucose is resistant towards periodate oxidation even after reduction with $NaBH_4$, while trimethylglucose from cord factor was oxidized (after reduction) as evidenced by the appearance of a new spot of higher R_G value (0.97) and different color (Table IV). This spot, which according to the theory is due to 2,3,4-tri-O-methyl-L-xylose, seemed also sufficiently different in position and color from the data reported on 2,3-di-O-methyl-L-threose which would be the corresponding reaction product from the 2,3,6-isomer[8].

This left the 2,3,4- and the 3,4,6-tri-O-methyl isomers as remaining possibilities for identification with the trimethylglucose from cord factor. However, the experimental finding that the paper chromatographic behavior of the trimethylglucose from cord factor was unaltered after periodate treatment and saponification (when not reduced prior to this treatment) indicated that it was resistant toward $NaIO_4$ oxidation. This again was in agreement with the behavior of 2,3,4-tri-O-methylglucose. Hence the 3,4,6-isomer could be ruled out, since it is the only isomer directly oxidized by periodate without previous reductive cleavage of the acetal ring[16]. (After saponification of the $NaIO_4$ oxidation product it would yield 2,3,5-tri-O-methyl-D-arabinose of higher R_G value.)

Thus it could safely be concluded that the trimethylglucose obtained from methylated cord factor is identical with 2,3,4-tri-O-methyl-D-glucose.

(b) *Identification by infrared spectroscopy.* Samples were prepared for spectroscopy by squeezing nujol mulls in a very thin layer between rock salt plates using about one milligram of the sugar derivative and a trace of nujol. Because of the strong absorption bands best results were obtained with thin samples. The spectra were taken with a Perkin-Elmer Model 21 recording infrared spectrophotometer.

Comparing the spectrum of the trimethylglucose from cord factor with the spectra of pure samples of the four tri-O-methyl-glucose isomers, the former was found to be identical with 2,3,4-tri-O-methyl-D-glucose in agreement with the results obtained from paper chromatography. The infrared spectrum of 2,3,4-tri-O-methyl-D-glucose is characterized by a series of distinct bands which differ considerably in position and intensity from those of the other isomers*. The maxima of the absorption

* The infrared spectra of the tri-O-methyl-D-glucose isomers will be published in a forthcoming paper.

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bands are defined by the following wave numbers: 1190, 1150, 1090, 1035, 990, 955, 935, 915, 890, 845, 760 cm^{-1}.

II. CORD FACTOR OF THE BCG STRAIN (by J. ASSELINEAU)

A. Isolation of cord factor BCG

1. *First experiment*

8.8 g of Wax C were chromatographed on 100 g magnesium silicate/Celite (2:1); elution with 200 ml each of petroleum ether, petroleum ether-benzene, ether, and ether-methanol (9:1) gave only inactive material. Elution with ether–methanol (4:1) gave 820 mg of a biologically active fraction which was rechromatographed on 250 g silicic acid (Mallinckrodt). After using the same series of solvents, 720 mg of eluate were dissolved in ether and precipitated by methanol. The cord factor thus obtained was a nearly colorless solid, m.p. 43–45°.

$C_{186}H_{366}O_{17}$ calculated C 77.70 %, H 12.83 %

found:	% C	% H	% N	% (O)CH_3
	77.76	12.40	0*	
	77.40	12.28	0	0**

2. *Second experiment*

17.2 g Wax C were chromatographed on 160 g magnesium silicate/Celite (2:1); 905 mg of active substance were eluted with ether–methanol (4:1). These were rechromatographed on 270 g of silicic acid (Mallinckrodt) from which ether-methanol (4:1) eluted 720 mg. After precipitation from ether solution with methanol an almost colorless solid was obtained, melting at 44–46°; $[\alpha]_J = +40° - 5°$ ($CHCl_3$; c = 1.375; l = 1).

found:	% C	% H	% N
	77.69	12.62	0
	77.43	12.52	

B. Chemical degradation

1. *Characterization of lipid moiety*

330 mg of cord factor, dissolved in 5 ml benzene, were saponified by adding 5 ml methanol containing 200 mg KOH and refluxing for 3 hours. The lipid-soluble portion was isolated after acidification and ether extraction in the usual manner. It was then resaponified and chromatographed on 2 g of alumina. By elution with ether containing 0.5 % acetic acid, and purification of the eluate by dissolution in ether and precipitation with methanol, a white solid was obtained, melting at 57–59°.

	% C	% H	mol. weight
calculated for mycolic acid ($C_{87}H_{174}O_4$):	81.36	13.66	1284
found:	81.30	13.38	1310 (titration)

(The substance contained no methoxyl.)

2. *Characterization of water-soluble moiety*

The hydrosoluble portion obtained from the saponification of the cord factor was freed from salts by passing its solution through a column of Amberlite MB-3; the filtrate was evaporated to dryness; the residue had $[\alpha]_J = +147° \pm 5°$ (water; c = 0.333; l = 1) (trehalose dihydrate: $[\alpha]_D = +178.3°$).

The substance was analyzed after drying for 72 hours in a high vacuum over P_2O_5 at room temperature and then 8 hours at 56° C.

	% C	% H
calculated for trehalose $C_{12}H_{22}O_{11} \cdot 2H_2O$:	38.09	6.92
found:	37.01	6.98

* Analysis by Ciba, Ltd., Basel, Switzerland.
** Analysis by Firmenich et Cie, Geneva, Switzerland.

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The substance was hydrolyzed with 5% HCl in a sealed tube for 3 hours at 110°. Paper chromatography of the hydrolysate gave only one spot with an R_F value identical to that of glucose.

Acetylation of the substance, as described by ANDERSON AND NEWMAN[13] for the acetylation of trehalose, gave an acetate which was extracted from the reaction mixture with chloroform and purified by recrystallization in methanol, yielding fine needles of m.p. 79–81°. The mixed melting point with authentic trehalose octaacetate (m.p. 80–81°) was not depressed and the infrared spectra* of both samples were identical.

	% C	% H
calculated for $C_{28}H_{38}O_{19}$:	49.56	5.64
found:	49.87	5.43

C. Methylation

230 mg of cord factor were methylated for 72 hours with methyl iodide in the presence of silver oxide as described above. The methylated cord factor, obtained after precipitation from ether with methanol, was a slightly yellow solid, melting at 36–37°. There was still a slight -OH band in the infrared spectrum similar to that of the methylated cord factor of the strain Brévannes.

	% C	% H	% $(O)CH_3$
calculated for $C_{192}H_{378}O_{17}$: (assuming 6 $(O)CH_3$ groups)	77.92	12.87	3.04
found:	77.50	12.26	3.25

186 mg of the methylated product were dissolved in 5 ml benzene and, after adding 100 mg KOH in 5 ml methanol, saponified by refluxing for 2 hours. The acidified lipid portion was extracted with petrol ether (165 mg) and chromatographed on 2 g alumina; ether containing 0.5% acetic acid eluted a white solid melting at 50–52°.

	% C	% H	% $(O)CH_3$
calculated for $C_{87}H_{174}O_4$:	81.36	13.65	
found:	81.55	13.60	0.4

The analysis shows that only about 30% of the mycolic acid had been methylated at one hydroxyl group (calculated for one $(O)CH_3$: 1, 16%).

The hydrosoluble portion was deionized by passage through an Amberlite MB-3 column and evaporated to dryness. The infrared spectrum was identical to the spectrum of the hexamethyltrehalose obtained from the methylated cord factor of the Brévannes strain. Paper chromatography of the acid hydrolysate using the system *n*-butanol-ethanol-water-NH_4OH (40:10:49:1)[18] showed one principal spot of R_G 0.90 corresponding to the R_G value of a trimethylglucose.

SUMMARY

The chemical structure of cord factor of *Mycobacterium tuberculosis* has been established as trehalose-6,6′-dimycolate (I), $C_{186}H_{366}O_{17} \pm 10CH_2$.

Alkaline hydrolysis of cord factor yields two molecules of mycolic acid and one molecule of the disaccharide trehalose. Methylation studies show that the two mycolic acid molecules are attached through an ester linkage to the primary hydroxyl groups of trehalose.

Identical results were obtained with cord factor from the human strain Brévannes, and the BCG strain.

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