STUDIES ON THE LIPID OF *BCG*

II. ON THE MYCOLIC ACIDS OF WAX A¹⁾ FRACTION OF *BCG*

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In the previous study on mycolic acid complex in *Mycobacterium tuberculosis* it was established that glyceryl mono-mycolate was a component in wax C fraction of *BCG* (Tsumita, 1956). However, considering the solubility of glyceryl mono-mycolate, it can be considered that a substance closely related with it might be present also in wax A fraction of *BCG*, which is extractable with alcohol-ether (1:1) from tubercle bacilli and soluble in hot acetone.

Asselineau (1951) reported that wax A fraction of a human virulent strain of tubercle bacilli, *Test*, was composed of free mycolic acid, phthiocerylesters of fatty acids and a lipidic substance with a molecular formula of $C_{60}H_{120}O$.

On saponification of alcohol-ether soluble wax, Wieghard and Anderson (1938) obtained mycolic, hexacosanoic, stearic and palmitic acids from the fatty acid fraction and phthiocerol from the neutral fraction and glycerol and sugars from the water soluble fraction.

The author fractionated wax A of *BCG* into three portions by aluminum oxide chromatography. It is the purpose of this paper to describe the fact that one of them was glyceryl mono-mycolate, which gave two kinds of mycolic acid on hydrolysis.

MATERIALS AND METHODS

The strain and culture of *BCG* as well as the method for the extraction of wax A were described in detail in the previous paper (Tsumita, 1956).

EXPERIMENTAL RESULTS

Fractionation of wax A of BCG by chromatography: Wax A of *BCG* was fractionated by chromatography using aluminum oxide (Brockmann) as an absorbent. For this chromatography, 7.0 g of wax A was dissolved in 60 cc of benzene and absorbed on aluminum oxide column. The successive elutions were made by

¹⁾ Wax A is a lipidic fraction of *Mycobacterium tuberculosis*, which can be extracted by alcohol-ether and separated from phosphatide and fat which are also found in alcohol-ether extracts along with it.

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benzene, benzene-ether (1:1), ether and finally ether-acetic acid (2%). Each eluate was concentrated *in vacuo* and precipitated by adding a large volume of methanol, except the ether-acetic acid eluates. No. 4 to 6 were at first neutralized with sodium bicarbonate solution and after drying treated in the same way as the others. 102% of the material was recovered (Table 1).

Fractions 4, 5 and 6 were pooled, I, and rechromatographed in the same way. The results are shown in Table 2. The low recovery rate (81%) seemed

Table 1. Chromatographic fractionation of 7.0 g of wax A (BCG)

Fraction	Solvent for elution	Volume (cc)	Material found (g)
1	Benzene	60	3.2
2	Benzene-ether(1:1)	120	2.1
3	Ether	150	0.1
4	Ether-AcOH (2%)	100	1.6
5	Ether-AcOH (2%)	100	0.1
6	Ether-AcOH (2%)	100	0.1

(I)

Recovery rate: 102%

Table. 2. Rechromatographic fractionation of 3.5 g of I

Fraction	Solvent for elution	Volume (cc)	Material found (g)
1	Benzene	100	Trace
2	Benzene	50	0
3	Benzene-ether (1:1)	100	0.02
4	Benzene-ether (1:1)	100	0.15
5	Ether	100	} 0.02
6	Ether-EtOH (2%)	100	
7	Ether-EtOH (2%)	100	0.05
8	Ether-AcOH (2%)	100	2.1
9	Ether-AcOH (2%)	100	0.3
10	Ether-AcOH (2%)	100	0.2
11	Ether-AcOH (4%)	100	0.015

(II)

Recovery rate: 81%

to be attributable to the high absorbing quality of aluminum oxide for the fraction I. Fractions 8, 9 and 10 were pooled, II, dissolved in ether and shaken with 1N KOH solution to be freed from an acidic material. The ether solution was washed, dried and concentrated. On adding methanol to the concentrated ether solution, the white powder was precipitated and dried *in vacuo*. M.p. 45–46°C. III.

Anal. Calcd. for glyceryl mono-mycolate, $C_{90}H_{180}O_6$, C 79.65, H 13.27, N 0, Glycerol 6.8.

Found C 79.65, 79.73, H 13.72, 13.06, N 0, Glycerol 5.9.

Nitrogen content was determined by Dumas' method. Glycerol content was measured by the method according to Blix (1937) with a modification that the heating time was 4.5 hours.

Hydrolysis of III by alcoholic KOH: 1.0 g of II was dissolved in a mixture of 15 cc of butanol, 5 cc of ethanol, 1 cc of water and 0.5 g of KOH, and refluxed on water bath for 2 hours. After cooling, it was added with a large volume of water and concentrated *in vacuo* to remove butanol and ethanol therefrom. The alkaline water suspension was extracted with ether. The ether solution was washed with diluted hydrochloric acid and water successively and dried on sodium sulfate. After concentrating and leaving it standing at room temperature, white granular precipitate was formed, which was filtered off. Another crop was obtained from further concentration of the ether solution. Both precipitates were pooled, dissolved in a small volume of warm ether and cooled at room temperature. The reprecipitation procedure was carried out once more and gave purified white powder, 0.16 g, M.p. 73–74°C, IV.

Anal. Calcd. for mycolic acid, $C_{87}H_{174}O_4$, C 81.44, H 13.57, OCH_3 0, Neutral equivalent 1282. Found C 80.90, H 13.52, OCH_3 0, Neutral equivalent 1200.

The ether filtrate was concentrated to dryness, and the residue was redissolved in a small volume of ether and precipitated by adding methanol to yield white powder after drying. It was further purified by dissolving in hot butanol and precipitating from the butanol solution by cooling. 0.7 g of white powder was obtained and melted at 55–56°C.

Anal. Calcd. for mycolic acid, $C_{87}H_{174}O_4$, C 81.44, H 13.57, OCH_3 0, Neutral equivalent 1282. Found C 81.25, H 13.22, OCH_3 0.29, Neutral equivalent 1280.

In fact, two kinds of mycolic acid were isolated from glyceryl mono-mycolate in wax A of BCG.

The water solution was neutralized by passing through cation exchange resin IR-120, and concentrated *in vacuo* to dryness. The oily residue, 54 mg, was obtained after drying *in vacuo* on P_2O_5 . It was heated with 3 cc of 3N hydrochloric acid for 3 hours on boiling water bath. After the end of heating, the solution was diluted with water and neutralized by anion exchange resin Dowex 2. On evaporating it *in vacuo*, a colorless oil was obtained, which was negative in both Molish and Fehling tests but strong positive in the acrolein test. It was dissolved in a small volume of KOH solution and esterified by benzoyl-chloride in the usual way. The benzoyl derivative, colorless needles, melted at 73°C after recrystallization from ligroin. Mixed fusion with the authentic glyceryl tri-benzoate melted without depression.

Anal. Calcd. for glyceryl tri-benzoate, C 71.29, H 4.95. Found C 70.92, H 4.90.

DISCUSSION

Noll and Bloch (1955) reported that the cord factor, trehalose-6,6'-dimycolate, was extracted to some extent in the alcohol-ether mixture (1:1), although the cord factor was chiefly found in the chloroform soluble wax.

From this view points, it was quite natural that glyceryl mono-mycolate, III, was a component in wax A as well as wax C of the lipid of *BCG*. The infra-red spectrum of III in Fig. 1 showed the exact correspondence with that of glyceryl mono-mycolate in wax C, reported in the previous paper (Tsumita, 1956). But it should be of interest that two kinds of mycolic acid were obtained from the hydrolysed products of glyceryl mono-mycolate in wax A. They are presented in Table 3 in comparison with the mycolic acid, VI, obtained from glyceryl mono-mycolate in wax C.

It was evident that both mycolic acids, IV and V, were isomers, having the molecular formula of $C_{87}H_{174}O_4$, because the melting points, the contents of methoxyl radical, solubilities in ether and infra-red spectra of both acids (Fig. 1) were conclusively different. But the same infra-red spectra of V and VI, as shown in Fig. 1, indicated that both were the same acid even though they were obtained from different sources, wax A and wax C.

Asselineau (1951) isolated three kinds of mycolic acid from the lipid of *BCG* (Table 4) and the two of them, φ - and ω -mycolic acids were obtained from the bound lipid.

Ginsberg and Lederer (1952) isolated a kind of mycolic acid, M.p. 70–72°C,

Table 3. Mycolic acids obtained from glyceryl mono-mycolates in the waxes of *BCG*

Glyceryl mono-mycolate	Derived from M. P.	Wax A 45–46°, III		Wax C 49–51°
	M. P.	73–74°, IV	55–56°, V	56–58°, VI
	OCH ₃	0.0	0.29	0.44
Mycolic acid obtained by hydrolysis	Molecular formula	$C_{87}H_{174}O_4$	$C_{87}H_{174}O_4$	$C_{87}H_{174}O_4$
	Solubility in ether	insoluble unless warmed	soluble	soluble

Table 4. Mycolic acids of the lipid of *BCG* (Lederer 1952)

Mycolic acid- <i>BCG</i>	φ	α	ω
M.p.	68–70°	50–52°	53–56°
OCH ₃	0	0.5	0.6
Molecular formula	$C_{87}H_{174}O_4$	$C_{87}H_{174}O_4$	$C_{87}H_{174}O_4$
Functional group	2 OH	2 OH	2 OH
Sources obtained	bound lipid		bound lipid

Fig. 1. Infrared spectra of mycolic acids (IV, V and VI) and glyceryl monomycolate (III).

VII, from the bound lipid of *BCG* and proposed a molecular formula of $C_{87}H_{172}O_4$, which was called mycolonic acid. IV, which was purified using the different solubility in ether, seemed to be corresponding to *q*-mycolic acid or VII and to be the more purified mycolic acid than others, because it showed the highest melting point among them. It was also of interest that IV was derived from the free lipid in contrast with *q*-mycolic acid and VII from the bound lipid of *BCG*. Although it was not determined whether V (or VI) was corresponding α - or ω -mycolic acid, it might be valid that V was another isomer, different from mycolic acids ever obtained.

CONCLUSION

1. Glyceryl mono-mycolate, M.p. 45–46°C, was isolated from the alcohol-ether soluble wax (wax A) of *BCG* and gave the same infra-red spectrum with that of glyceryl mono-mycolate in the chloroform soluble wax (Wax C).
2. Two kinds of mycolic acid isomer, M.p. 73–74°C and M.p. 55–56°C, were obtained from the hydrolysis products of glyceryl mono-mycolate in wax A. The acid, M.p. 73–74°C, seemed to be corresponding to *q*-mycolic acid or mycolonic acid obtained from the bound lipid of *BCG* and to be the more purified acid than whatever substance ever obtained.

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