CHEMISTRY OF PHOSPHATIDE AND WAX D OF *MYCOBACTERIUM TUBERCULOSIS* BCG IN ONE WEEK CULTURE

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“Purified wax” as isolated by Anderson (1929), is a complex lipid extracted with chloroform from cultures of acid fast bacilli. Extracting the purified wax with boiling acetone, Asselineau (1951) obtained a soluble portion termed “Wax C” and an insoluble residue, the “Wax D” fraction. Likewise, the alcohol-ether soluble part of acid fast bacilli was classified into several fractions. “Phosphatide” is a boiling acetone insoluble residue in the alcohol-ether soluble fraction, corresponding to Wax D of the alcohol-ether insoluble and chloroform soluble fraction. Therefore, the substances contained in Wax D can be migrated from “phosphatide” depending on the conditions of extraction and on the metabolic stage of bacterial cells.

Works in this laboratory elucidated the chemical components of Wax A, C (Tsumita, 1956 a, b) and Wax D (Nojima, 1959 a, b) of BCG in 4-6 weeks culture. In this experiment a special consideration was paid on the migration of substances in Wax D from “phosphatide” depending on the extracting condition and on the age of the culture. Therefore, the present author intended to compare the yield and some chemical natures of “phosphatide” with those of Wax D in one week as well as in 4 weeks culture of the bacterial cells. The data of the latter were already precisely reported though its chromatographic fractionation has not been described (Nojima *et al.*, 1958).

Substances containing phosphorus in “phosphatide” and Wax D will play an important role in the metabolic process of lipid in *Mycobacterium*. After chromatographic partitioning of these fractions phosphorus and inositol contents were analyzed and compared. On the other hand, special emphasis was placed on the yield of cord factor contained in Wax D depending on the age of the culture.

RESULTS

The production of the bacterial cultures and other materials and methods were described in the previous paper (Nojima, 1959 a). In this work the incubation lasted for one week. 4w and 1w mean the culture of 4 weeks and one week, respectively. 1w-1, 1w-2 and 1w-3 mean Lot 1, 2 and 3, respectively, of the one week culture. “Phosphatide” means the fraction, alcohol-ether soluble, boiling acetone insoluble, being discriminated from phosphatide of chemical designation.

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The Yields of Phosphatide and of Wax D in One Week Culture of BCG

The total yield of bacterial cells in one week culture after lyophilization was 0.9 g/l on average, while that of 4 weeks culture was 5 g/l (Nojima *et al.*, 1958).

Table 1 presents some examples of the yields of phosphatide and of Wax D in one week culture of BCG. For comparison, the yields of other fractions are also indicated.

Table 1. The yield of lipid in one week culture of BCG

Culture	Fat	Wax A	Phosphatide	Wax B	Wax C	Wax D	Total lipid
4w	12.0	5.0	0.5	3.0	3.0	1.5	25.0
1w-1	3.9	1.7	1.1	1.1	1.0	0.8	9.6
1w-2	4.4	1.0	1.1	2.5	0.3	0.5	9.8
1w-3	2.2	1.0	0.6	1.1	1.5	2.0	8.4

4w: 4 weeks culture (Nojima *et al.*, 1958).

1w-1, 2, 3: One week culture Lot 1, 2, 3, respectively.

The yield percentage is expressed as calculated from lyophilized cells.

The results to be noticed was the following: 1. The yield of neutral fat in 4 weeks culture was extremely large resulting in much larger yield of total lipid than in one week culture. 2. Phosphatides contained in "phosphatide" and Wax D fraction were richer in one week culture than in 4 weeks culture. Phosphorus content in "phosphatide" plus Wax D was greater in one week culture than in 4 weeks culture (Table 2).

Table 2. Phosphorus content in each lipid fraction of BCG in one week culture

Culture	Fat	Wax A	Phosphatide	Wax B	Wax C	Wax D	Sum of Wax D and phosphatide*
4w**	0.00	0.00	2.73 (1.37)	—	0.00	0.80 (1.2)	(2.57)
1w-1	0.13	0.08	3.77 (4.14)	1.06	0.00	1.03 (0.83)	(4.97)
1w-2	0.06	0.00	3.97 (4.37)	0.18	0.13	0.31 (0.16)	(4.53)
1w-3	0.11	0.12	3.49 (2.09)	1.30	0.06	2.18 (4.36)	(6.45)

Figures shown are percentage content of phosphorus in each fraction.

Figures in parentheses are percentage of phosphorus $\times 10^2$ per dry weight of bacterial cells.

* Sum of phosphorus content in Wax D and phosphatide expressed as percentage to dry weight of bacterial cells.

** cited from Nojima *et al.* (1958).

Some analytical data of these fractions are shown in Table 3.

Chromatographic Partitioning of Wax D in One Week Culture of BCG

Wax D of BCG in one week culture was chromatographed (Table 4) in the same way as in the case of 4 weeks culture described in the previous report (Nojima, 1959 a).

Table 3. Several analytical data of lipid fractions in one week culture of BCG (1w-1)

	Fat	Wax A	Phosphatide	Wax B	Wax C	Wax D
P %	0.13	0.08	3.77	1.06	0.00	1.03
N %	0.28	0.25	0.96	0.26	0.05	0.31
Inositol %	0.05	0.02	4.17	0.47	0.00	2.57

Table 4. Chromatography of 0.3 g of Wax D of 1w-BCG dissolved in chloroform, absorbed on 6.6 g of Silicic acid and 3.3 g of Hyflosupercel

Fraction	Tube No.	Eluted with 50 cc of	Weight (mg)	mp. °C	P %	N %	Inositol %
		Original	300	48—73	1.03	0.31	2.57
“A”	1—3	Chloroform-methanol 98:2	10	27—31	—	—	—
	4—10	Chloroform-methanol 93:7	226	43—44.5	0.31	0.01	0.00
“B”	11—14	Chloroform-methanol 78:22	42	>225	1.70	0.00	2.28
	15—18	Chloroform-methanol 48:52	36	220 (decomp.)	1.80	0.09	5.02

In the case of 4 weeks culture the fraction “A” as demonstrated in Table 4 contained cord factor which was identified by rechromatography (Florasil-Hyflosupercel 2:1, ether-methanol 9:1). In the present case, fraction “A” contained cord factor as identified by infrared spectrum (Fig. 1, 1w-1, 1w-2 and 1w-3) using tube No. 4 for 1w-1, No. 3 for 1w-3 and whole substance for 1w-2. These tubes showed the maximum yield of the first peak of the chromatography. Although some contaminants containing phosphorus and a negligible amount of nitrogen can be expected, the fraction “A” can represent “cord factor fraction”, as evidenced by the absorption of infrared spectrum identical to that of the standard (Fig. 1). Fraction “B” contained oligomannoinositide in the case of 4 weeks culture (Nojima, 1959 a).

Since a negligible amount of hexose was detected in Wax C (Table 5), the total yield of cord factor is considered to have been restricted in Wax D in the present experiment. The amounts of “cord factor fraction” in fraction “A” of Table 4 were compared with each other among 4w, 1w-1, 1w-2 and 1w-3 (Table 5). As seen in the table, the amount of “cord factor” remained constant (0.5–0.6% to dry weight of bacterial cells) regardless of culture age.

Chromatographic Partitioning of “Phosphatide” Fraction in One Week and 4 Weeks Culture of BCG

“Phosphatide” fraction, alcohol-ether soluble and boiling acetone insoluble, of BCG in one week as well as in 4 weeks culture was chromatographed. The yield patterns are presented in Figs. 2 and 3. Three main peaks were obtained. The tubes of the maximum yield in each peak were subjected to several chemical analyses (Table 6).

Table 5. Yield of cord factor in Wax D of 1w BCG as compared with that in 4 weeks culture

Culture	Wax D yield % to dry weight of bacterial cells	Cord factor		Hexose content in Wax C %
		Yield % to Wax D	Yield % to dry weight of bacterial cells	
4w*	1.5	41 (average)	0.61	0
1w-1	0.8	79	0.63	0.58
1w-2	0.5	ca. 100	0.5	1.09
1w-3	2.0	31	0.62	0.50

* cited from Nojima (1959 a)

Fig. 2. Chromatography of 0.45 g of phosphatide of 1w-BCG dissolved in chloroform, absorbed on 20 g of Silicic acid and 10 g of Hyflosupercel.

Each fraction of one week culture was shown to contain a considerable amount of phosphorus. The smaller amount of phosphorus in 4w-No. 2 (Table 6) may be due to the presence of a large amount of glyceryl monomycolate therein (Kondo, 1960).

DISCUSSION

The total amount of lipid from one week culture of BCG was far less (9% in Table 1) than that of 4 weeks culture (25% in Table 1). It is mainly due to the increase of neutral fat in 4 weeks culture (12% in Table 1) in parallel with the age of the culture (1w-1 : 3.9% ; 1w-2 : 4.4% ; 1w-3 : 2.2% in Table 1).

Fig. 2. Chromatography of 2.9 g of phosphatide of 4w-BCG dissolved in chloroform, absorbed on 60 g of Silicic acid and 30 g of Hyflosupercel.

Table 6. Several analytical data of those tubes of maximum yield in three main peaks of phosphatide (1w and 4w) chromatographed on Silicic acid and Hyflosupercel (2:1)

1w					4w				
Tube No.	mp. °C	P %	Glycerol %	Inositol %	Tube No.	mp. °C	P %	Glycerol %	Inositol %
2	118	2.95	13.78	0.00	2	40—46	0.43	6.91	0.00
10	<27	3.00	12.17	0.92	15	127—170	1.84	10.13	0.00
14	160 (decomp.)	4.90	15.22	3.73	24	>240	5.98	6.67	0.61

Details of chromatography are illustrated in Figs. 2 and 3.

On the other hand, the lipid-phosphorus content of one week culture of BCG is greater than that of 4 weeks culture, especially in the sum of "phosphatide"-P and Wax D-P (Table 2).

Table 5 showed that the cord factor content remains constant regardless of the culture age. It seems that this contradicts the common knowledge (*e.g.* Noll & Bloch, 1953, 1955) that the cord factor (Wax C) content is greater in young than in old culture. But their data using a virulent human type of bacilli were obtained by the comparison of 2 to 3 weeks old (probably in logarithmic phase) with 12 weeks old (probably in stationary to declining phase) bacilli, while ours using BCG were one week (logarithmic phase) *versus* 4 weeks (stationary to declining phase) culture.

SUMMARY

1. Lipid fractions, especially "phosphatide" and Wax D, fractionated by Anderson-Lederer method from one week culture of BCG, were isolated and analyzed for some chemical behaviours which were compared with those of 4 weeks culture.

2. Neutral fat content of 4 weeks culture was much higher than that of one week culture, resulting in that the amount of total lipid was far greater in 4 weeks culture than in one week culture.

3. The sum of phosphorus contents in Wax D and "phosphatide" was much larger in one week culture than in 4 weeks culture.

4. The cord factor content remained constant regardless of the culture age.

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