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Lysis of Isolated BCG Cell Walls with Enzymes 2. Demonstration of 'Bound Wax D' as a Component of BCG Cell Walls*

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SUMMARY

The cell wall preparations isolated from 'delipidated' BCG cells which were exhaustively extracted with neutral, organic solvents at room temperature were submitted to successive treatment with egg white lysozyme and the L_{11} enzyme produced by a *Flavobacterium sp.* (L_{11} bacterium) which was primarily active against *Staphylococcus aureus*. By these enzyme treatments, about 40 per cent of the cell wall materials became soluble, leaving 60 per cent as an insoluble residue. By solubility tests in organic solvents, infrared spectrophotometry, determination of the mycolic acid content and other chemical analyses it was demonstrated that the insoluble residue consists mainly of materials essentially identical to the wax D fraction isolated from human type *Mycobacterium tuberculosis*. The residue was therefore designated as 'bound wax D', and its adjuvant activity tested. It was shown that the 'bound wax D' fraction exhibits a marked enhancing effect on both the production of circulating antibody and the development of a delayed type of hypersensitivity when injected into guinea pigs with egg white albumin or egg white lysozyme.

Immuno-diffusion experiments by the Ouchterlony technique showed that the materials liberated from 'delipidated' BCG cell walls under the action of lysozyme and the L_{11} enzyme contain at least one common antigen which is distinct from the cytoplasmic antigen.

INTRODUCTION

Previous work in this series (Kotani *et al.*, 1962) demonstrated that when a

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BCG cell wall suspension was incubated with egg white lysozyme there was about 35 per cent reduction in the optical density and that when the suspension of the cell walls previously treated with lysozyme was incubated with the L_{11} enzyme, which had been shown to have no lytic activity against untreated BCG cell walls, there was a further 25 per cent reduction in the optical density.

The pesent investigation was undertaken to determine the chemical nature of the cell wall component remaining as an insoluble residue after treatment of 'delipidated' BCG cell walls with lysozyme and the L_{11} enzyme, and to test the possible adjuvant activity of the insoluble residue in the production of circulating antibody and the development of a delayed type of hypersensitivity. To avoid contamination of the insoluble residue with free lipid fractions, cell wall preparations separated from 'delipidated' BCG cells which had been exhaustively extracted with ethanol-ether and chloroform at room temperature were used.

Preliminary experiments were also carried out to elucidate the chemical process inducing the optical density reduction of the suspension of BCG cell walls during incubation with lysozyme and the L_{11} enzyme, and to examine the serological reactivity of the materials liberated by the enzyme treatment.

MATERIALS AND METHODS

1. *'Delipidated' cell wall preparations*

BCG, strain Takeo, was used throughout this study. This strain was originally obtained from the Department of Tuberculosis, the Research Institute for Microbial Diseases, Osaka University, and serially subcultured on Ogawa's egg yolk medium in this laboratory. The organisms were grown as surface pellicles on Sauton synthetic liquid medium for 9 to 12 days at 37°C. The bacterial cells were harvested by filtration through a sintered glass filter and then washed with a large quantity of distilled water. Free lipids were removed as completely as possible from the washed cells by repeated extractions with ethanol-ether (1:1, v/v) and chloroform in the manner described by Anderson (1943). From the 'delipidated' bacterial cells thus obtained, the cell wall fractions were prepared according to the procedure described previously (Kotani *et al.*, 1959a) and used as 'delipidated' cell wall preparations.

2. *Lytic enzymes*

Partially purified preparations of the L_{11} enzyme were separated from culture supernatants of *Flavobacterium sp.* (L_{11} bacterium, Kotani *et al.*, 1959b) by ammonium sulfate precipitation and chromatographic fractionation on a hydroxylapatite column. Detailed descriptions of the procedures used for the separation have been given in the paper of Kato *et al.* (1962).

Crystalline egg white lysozyme specimens, purified by repeated recrystallization, were generously supplied by Dr. T. Amano, Department of Immunology, Research Institute for Microbial Diseases, Osaka University.

3. *Analytical methods*

Paper chromatography for detection of amino acids and sugars was carried out as described previously (Kato *et al.*, 1962) except that ethyl acetate-pyridine-water (8 : 2 : 1, v/v) was used as an additional developing solvent for sugar analysis (White and Secor, 1953).

Reducing substances, hexoses, pentoses, and hexosamines were determined by Nelson's modi-

fication of the Somogy technique (Nelson, 1944), the modified anthrone method (Ashwell, 1957), the orcin-HCl method of Bial as modified by Dische (Ashwell, 1957) and the method of Neuhaus and Retzring (1957), respectively. Ninhydrin-positive substances were estimated by the method of Moore and Stein (1948). The methods of Yokoi and Akashi (1955) and of Fiske-Subbarow (Fister, 1950) were used for determination of total nitrogen and phosphorus, respectively.

Infrared absorption spectra were measured in a Nippon Koken Infrared Spectrophotometer (Model DS-301, Japan Spectroscopic Manufacturing Co., Tokyo) according to the procedures described in the manfacturer's book. Specimens to be tested were incorporated into KBr disks.

4. *Physical methods*

Molecular weight was measured by the surface balance technique with a horizontal float type apparatus. Test specimens were dissolved at a concentration of 0.02 per cent (w/v) in petroleum ether (b.p. 40-60°C) containing 1 per cent (v/v) pyridine. Details of the procedures and the methods of calculation will be reported elswhere (Tanaka and Kitagawa, to be published).

Melting points were determined by the usual method.

5. *Serological methods*

Anti-BCG cell wall sera were prepared by two intramuscular injections of 0.3 ml each of a cell wall suspension (10 mg/ml) in incomplete Bacto-adjuvant (Freund) (Difco Laboratories, U. S. A.) into the thigh of rabbits with a 17 day interval between injections.

The qualitative precipitin test was performed by the ring method. Precipitation was examined at appropriate intervals and arbitrarily graded from − to ###. Immuno-diffusion experiments were performed by the Ouchterlony method. The antibody content of test serum specimens (not inactivated) was estimated in the presence of 0.01 M EDTA according to the description of Kabat and Mayer (1961). The washed precipitates were dissolved in 1 N sodium hydroxide solution and assayed for their protein content with Folin-Ciocalteu phenol reagent.

6. *Corneal test*

The method used was essentially identical to that described by White, Bernstock, Johns and Lederer (1958). The test was performed by injection of test antigen solutions (20 mg/ml egg white albumin, twice crystallized, Sigma Chemical Co., U. S. A. and 10 mg/ml egg white lysozyme) in amount sufficient to cause a disc of opacity, about 2 mm in diameter, when injected into the cornea instilled with a drop of ophthalmological Xylocaine solution (containing lidocaine hydrochloride at a concentration of 40 mg/ml, manufactured by Fujisawa Pharmaceutical Co., Osaka). The eyes were examined daily for a week for the extent and degree of corneal opacity and the presence or absence of chemosis. A drop of 1 per cent achromycin (crystalline tetracycline hydrochloride) ophthalmic oil suspension (Takeda Chemical Industries, Osaka) was instilled daily during the observation period to minimize the danger of bacterial infection of the cornea.

7. *Electron microscopy*

The procedures described previously (Kotani *et al.*, 1959a) were followed.

RESULTS

1. *Lysis of 'delipidated' BCG cell walls by successive treatment with lysozyme and L_{II} enzyme*

1) *Chemical process inducing the reduction in optical density of a cell wall suspension incubated with the enzymes*

A specimen of 60 mg of a 'delipidated' cell wall preparation was suspended in 12 ml of 0.025 M phosphate buffer, pH 6.8, containing 12 mg of egg white lysozyme and 0.1 per cent of sodium

Table I. Chemical Process Involved in the Lysis of 'Delipidated' BCG Cell Walls by Successive Treatment with Lysozyme and L_{11} Enzyme

Determination	Lysozyme-lyzate of 'delipidated' cell walls				L_{11} enzyme-lyzate of lysozyme-treated 'delipidated' cell walls			
mμ moles/mg cell walls	Time of incubation (hrs)				Time of incubation (hrs)			
	0	4	8	24	0	4	8	24
Nihydrin-positive substances (as leucine)	0	0	0	14.7	0	10.1	30.0	70.0
Reducing substances (as glucose)	0			18.0	0			21.4
Hexosamines (as glucosamine-HCl)	0	7.2	9.1	13.0	0	1.3	1.5	2.9

Determinations were made on unhydrolzyed specimens of materials released by the indicated times.

azide as a preservative. The suspension was incubated at 37°C for 24 hours. Samples (1 ml each) were taken after 0, 4, 8 and 24 hours, and were immediately centrifuged at 12,000 × *g* for 20 minutes in the cold. The supernatant fluids thus obtained were assayed without prior hydrolysis, for their content of reducing substances, hexosamines and ninhydrin-positive substances. The insoluble residue (lysozyme-treated cell walls) obtained by centrifuging the remaining 8 ml of the suspension after 24 hours incubation, was washed four times with 4 ml of distilled water. The washed sediment was resuspended in 8 ml of 0.025 M phosphate buffer, pH 6.8, containing 80 units of the L_{11} enzyme and 0.1 per cent sodium azide. Samples (1 ml each) were withdrawn from the suspension incubated at 37°C, as before, and mixed with 0.1 ml of 1 M sodium chloride solution to stop the enzyme action. The mixtures were centrifuged and the supernatants were analyzed chemically.

It is well established that the action of egg white lysozyme on microbial substrates generally involves the liberation of reducing groups and acetylamino sugars (Salton, 1960). It will be seen from Table 1 that treatment of 'delipidated' BCG cell walls with lysozyme causes uncovering of reducing groups and liberation of components reacting as hexosamines. Some amino groups were also uncovered by lysozyme treatment, but the extent of uncovering was far smaller than that obtained by digestion of the lysozyme-treated cell walls with the L_{11} enzyme.

The lysozyme-treated cell walls, on the other hand, released components with uncovered reducing and/or amino groups under the action of the L_{11} enzyme. There was little liberation of hexosamine-reacting materials. It was previously shown in this laboratory (Kato *et al.*, 1962) that the treatment of *Staphylococcus aureus*, strain Newman 1 (the original indicator strain for the assay of the lytic activity of the L_{11} enzyme), is almost exclusively accompanied by liberation of soluble products with free amino groups, but not of significant amounts of those with reducing and /or hexosamine-reacting groups. Studies were made by the fluoro-dinitrobenzene method on the N-terminal amino acids in small peptides isolated from the dialyzable fraction of the L_{11} enzyme-lyzate of *Staphylococcus aureus* cell walls. Evidence has been obtained that the L_{11} enzyme primarily attacks the linkage between the lactyl group of muramic acid and the amino group of alanine in cell wall mucopep-

tide (Kato *et al.*, unpublished data). The seemingly conflicting observation reported here that digestion of the lysozyme-treated BCG cell walls with the L_{II} enzyme was accompanied by uncovering of both reducing and free amino groups, may be explained by assuming that the cell wall components, the reducing groups of which were already uncovered by lysozyme treatment, passed into solution by further incubation with the L_{II} enzyme.

2) *Total solids liberated from 'delipidated' cell walls by the action of lysozyme or the L_{II} enzyme*

A 422 mg specimen of a 'delipidated' cell wall preparation was incubated with stirring at 37°C with 101.3 mg of lysozyme (0.24 mg/mg cell walls) in a total volume of 84.4 ml of 0.01 M ammonium acetate solution, pH 6.3. A few drops of chloroform were added to the mixture as preservative. At the end of the 60 hour incubation period, the reaction mixture was centrifuged at $12,000 \times g$ for 20 minutes. The sediment was washed three times with 40 ml volumes of distilled water. The supernatant fluid, combined with the washings, was lyophilized (lysozyme-lyzate). The washed sediment was resuspended in a small amount of distilled water and exactly one-fifth of the suspension was lyophilized for weighing and further study (lysozyme-treated cell walls). The remaining four-fifth was reconstituted in the reaction mixture in a volume of 67.5 ml, containing 506 units of the L_{II} enzyme (1.5 units/mg original cell walls), 0.03 M phosphate buffer, pH 6.8, and a few drops of chloroform. The reaction mixture was incubated with stirring at 37°C for 72 hours and then centrifuged at $12,000 \times g$ for 20 minutes. The sediment was washed three times with 34 ml volumes of distilled water. The washed sediment was lyophilized (insoluble residue), weighed and submitted to chemical analysis. The supernatant fluid combined with the washings, was lyophilized (L_{II} enzyme-lyzate).

The weight of total solids liberated from the 'delipidated' BCG cell walls under the action of lysozyme and the L_{II} enzyme was calculated on the basis of the difference between the weight of the cell walls before and after treatment with each enzyme. It was found in the experiment described above that about 10 and 32 per cent of the cell wall constituents passed into solution on digestion with lysozyme and the L_{II} enzyme respectively. It should be pointed out, however in this connection, that the extent of liberation of cell wall materials by each of the enzymes

Table 2. Qualitative Precipitin Reaction between Anti-BCG Cell Wall Rabbit Serum and Materials Released from 'Delipidated' BCG Cell Walls by Successive Treatment with Lysozyme and L_{II} Enzyme

Anti-BCG cell wall serum	Antigen	Dilution of antigen (100×2^X)													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
No. 1	Lysozyme-lyzate	+	+	++	++	++	++	++	++	++	++	++	+	+	−
	L_{II} enzyme-lyzate	+	+	+	++	++	++	++	++	++	++	++	+	−	−
No. 2	Lysozyme-lyzate	+	+	++	++	++	++	++	++	++	++	++	+	+	−
	L_{II} enzyme-lyzate	+	+	+	++	++	++	++	++	++	++	++	+	+	−

Performed by the ring test. Dilution of the antigens was based on the calculated weight of the lyzates (*cf.* 1-2).

varies in different experiments, although the sum of total solids released by the two enzymes seems to be fairly constant. For instance, 21 and 23 per cent of the cell wall constituents were released by lysozyme and the L_{II} enzyme respectively in one experiment with a separate preparation of 'delipidated' BCG cell walls.

3) *Liberation of serological reactive materials by digestion with lysozyme or the L_{II} enzyme*

Table 2 presents a record of the precipitin reaction between anti-BCG cell wall rabbit serum and serial two-fold dilutions of the lysozyme- or L_{II} enzyme-lyzates obtained in the experiment described in the preceding section. It can be seen that both lysozyme and the L_{II} enzyme treatments liberate serological reactive materials from 'delipidated' BCG cell walls.

Since a positive reaction was obtained in the ring test on the lyzates, immunodiffusion experiments by the Ouchterlony method were carried out to clarify the antigenic relationship between the lysozyme- and L_{II} enzyme-lyzates. A specimen of the cytoplasmic fraction which was obtained by centrifuging a sonicated (for 10 minutes) BCG cell suspension at $80{,}000 \times g$ for 90 minutes served as control antigen. It may be added in this connection that some anti-cell wall serum specimens contain the antibody reactive with this soluble, cytoplasmic fraction, as previously reported (Kotani *et al.*, 1960). As illustrated in Fig. 1, a precititin line common to both of the cell wall lyzates developed and this common band of identity did not fuse with the line of the precipitate formed by the cytoplasmic fraction. An additinal precipitin line appeared between the lysozyme-lyzate and the antiserum. From the pattern shown in Fig. 1 it is unknown whether the antigen giving this line is identical to the antigen found in the cytoplasmic fraction.

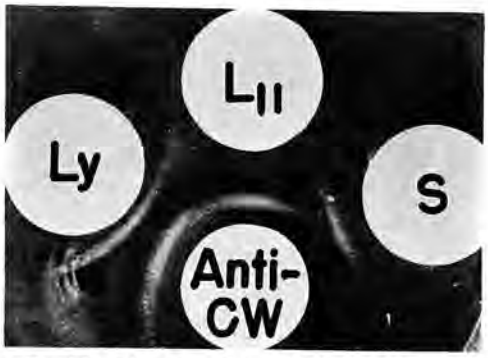

Fig. 1. Ouchterlony Double Diffusion Precipitation Pattern Showing the Antigenic Relation between the Lysozyme-Lyzate, L_{II} Enzyme-Lyzate, and Soluble, Cytoplasmic Fraction

Ly: Lysozyme-lyzate ($1:100 \times 2^8$); L_{II}: L_{II} enzyme-lyzate ($1:100 \times 2^8$); S: Soluble, cytoplasmic fraction isolated from sonicated BCG cells ($1:100 \times 2^6$); and Anti-CW: Anti-cell wall rabbit serum (1:1).

2. *Chemical nature of the insoluble residue isolated from 'delipidated' BCG cell walls digested with lysozyme and L_{11} enzyme*

As the first step in studies on the chemical nature of the insoluble residue, the solubility of a test preparation (50 mg) in various organic solvents was examined. It was found that more than 90 per cent of the preparation was readily soluble in chloroform-methanol-water (100 : 5 : 0.5, v/v), but not in ice-cold methanol or boiling acetone. This suggests that the insoluble residue may be mainly a material similar to the wax D fraction isolated from *Mycobacterial* cells by Asselineau (1951).

The infrared absorption spectrum of the insoluble redue was then examined. Fig. 2 illustrates the infrared spectra of two specimens of the insoluble residue and as references those of the wax D fractions isolated by the method of Asselineau (1951) from *Mycobacterium tuberculosis* (strain H37Ra) and from BCG (strain Takeo). The infrared spectra of the insoluble residue exhibit a series of bands all characteristic to the wax D of human type *Mycobacterium tuberculosis*, strain H37Ra. On the other hand, one distinct difference was noticed between the spectrum of the insoluble residue and that of the wax D fraction of BCG. The former, but not the latter, exhibits well defined absorption bands at about 1540 cm^{-1} and in the region of 1650 cm^{-1} that have been shown to be assignable to the presence of a peptide group.

The next step was to ascertain the presence of mycolic acid and to estimate the content of this acid in the insoluble residue.

A 150 mg specimen of the test preparation was heated under reflux for 46 hours with 12 ml of methanol-benzene (1 : 1, v/v) containing potassium hydroxide at a final concentration of 2.5 per cent. The reaction mixture was then centrifuged at $1,000 \times g$ for 5 minutes and the precipitate was exhaustively extracted by repeated washing with 20 ml volumes of benzene. The benzene was evaporated off from the combined extracts under reduced pressure. The dried material thus obtained was suspended in about 5 ml of distilled water and the solution acidified slightly by addition of an appropriate amount of hydrochloric acid. The suspension was then extracted three times with 20 ml of ether. The ether was evaporated and the ether-soluble split product was obtained as a residue.

The weight of the ether-soluble split product thus obtained was 79.9 mg (yield, 53.3 per cent). A similar experiment was carried out on a specimen (300 mg) of another preparation of the insoluble residue. The yield of the ether-soluble product in this experiment was found to be 45 per cent.

The presence or absence of mycolic acid and its content in the ether-soluble product separated from the insoluble residue were then examined by column chromatography in the following manner:

A 132 mg specimen of the ether-soluble product was dissolved in a small amount of benzene and applied to a column (5 mm in diameter) of 5 g of alumina (Standard Aluminium Oxide for chromatographic adsorption analysis, E. Merck, Germany) which had been treated with hydrochloric acid according to the description of Asselineau (1951). This material was then found to have activity II. The column was eluted successively with benzene, ether, acetic acid-ether (5 : 95, v/v) and acetic acid-ether (10 : 90, v/v) at a flow rate of one drop per 15 seconds. Fractions of 20 ml were collected and the content of materials eluted in each was measured by weight.

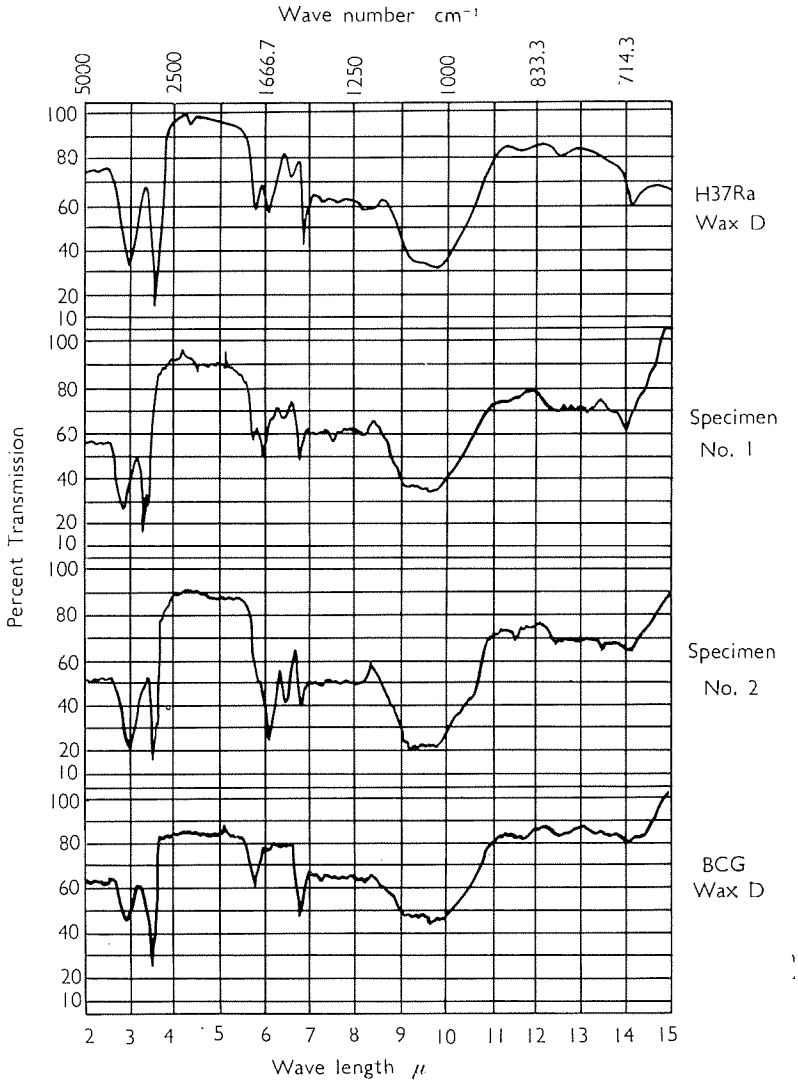

Fig. 2. Infrared Absorption Spectrum of the Insoluble Residue Isolated from 'Delipidated' BCG Cell Walls Digested with Lysozyme and L11 Enzyme

The results of the assay are presented in Table 3. Of the applied material 83 and 7 per cent respectively were recovered in fractions eluted with acetic acid-ether mixtures of ratios of 5 : 95 and 10 : 90, which are known to elute mycolic acid from an alumina column (Asselineau, 1951).

Fig. 3 illustrates the infrared absorption spectrum of the major fraction eluted from the alumina column, showing that the spectrum is essentially identical with the spectrum of the authentic sample of mycolic acid. Elementary alanlysis car-

Table 3. Chromatographic Analysis of the Ether-Soluble Fraction Isolated from the Alkali-Hydrolyzed Insoluble Residue of 'Delipidated' BCG Cell Walls Digested with Lysozyme and L11 Enzyme

Tube number	Solvent for elution	Volume of fractions ml	Weight of eluted material mg	
1	Benzene	20	3	
2	〃	〃	2	(6 per cent)
3	Ether	〃	3	
4	〃	〃	0	
5	〃	〃	0	
6	Acetic acid-ether (5:95)	〃	89	
7	〃	〃	9	
8	〃	〃	3	(83 per cent; m.p.: 52-55°C)
9	〃	〃	5	
10	〃	〃	3	
11	〃	〃	0	
12	〃	〃	0	
13	〃	〃	0	
14	〃	〃	0	
15	Acetic acid-ether (10:90)	〃	3	
16	〃	〃	4	(7 per cent; m.p.: 53-54°C)
17	〃	〃	2	
			Total 125	(95 per cent)

A specimen of 132 mg was applied on a column of 5.0 g of alumina (activity 11).

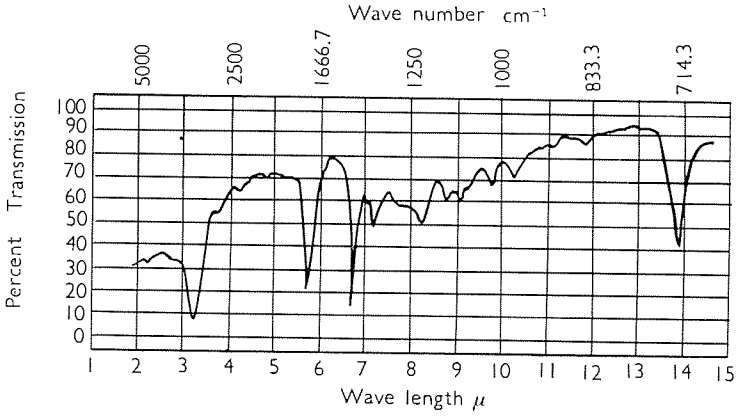

Fig. 3. Infrared Absorption Spectrum of the Major Fraction of the Ether-Souble Split Product of the Alkaline-Hydrolyzed Insolubel Residue Isolated from 'Delipidated' BCG Cell Walls Digested with Lysozyme and L11 Enzyme

ried out on the fraction, on the other hand, gave the values of C 80.98 per cent and H 13.36 per cent. The melting point was 52-55°C. These values agree fairly well with those described by Ginsburg (Asselineau, 1962) for his preparations of 1- or 2-mycolic acid isolated from BCG. On the minor fraction only the melting point was determined and a value of 53-54°C was obtained.

On the basis of the findings described above, it seems reasonable to conclude that the mycolic acid content of the insoluble residue isolated from 'delipidated' BCG cell walls by digestion with lysozyme and the L_{11} enzyme is within the range of $45 - 53 \times 0.9 = 41 - 48$ per cent.

Table 4 summarizes the results of qualitative and quantitative chemical analyses carried out on two preparations of the insoluble residue, with those of some physical analyses. Paper chromatographic analysis showed that the principal

Table 4. Chemical and Physical Properties of the Insoluble Residue Isolated from 'Delipidated' BCG Cell Walls Digested with Lysozyme and L_{11} Enzyme

Analysis	Specimen 1	Specimen 2
Chemical properties		
Qualitative		
Pricipal component amino acids	Alanine, Glutamic acid, α, ε-diaminopimelic acid	
Principal component sugars	Arabinose, Galactose, Glucosamine	
Quantitative		
Nihydrin positive substances (as leucine) *1	17 per cent	21 per cent
Hexoses (as galactose) *2		20 〃 〃
Pentoses (as arabinose) *3	14 〃 〃	16 〃 〃
Hexosamines (as glucosamine-HCl) *4	2.0 〃 〃	2.6 〃 〃
Total nitrogen		2.3 〃 〃
Total phosphorus	0.6 〃 〃	0.5 〃 〃
Mycolic acid	48 〃 〃	41 〃 〃
Physical properties		
Melting point	227 - 228°C	
Molecular weight		
Apparent M. W.	32,000	
(True M. W.)	(16,000)	

*1 Hydrolyzed in 6 N HCl at 100°C for 16 hours.
*2 Hydrolyzed in 2 N HCl at 100°C for 3 to 10 hours. The maximum value is presented.
*3 Hydrolyzed in 2 N HCl at 100°C for 2 to 8 hours. The maximum value is presented.
*4 Hydrolyzed in 4 N HCl at 100°C for 10 hours.

component amino acids are alanine, glutamic acid, α, ε-diaminopimelic acid, and the principal component sugars are arabinose, galactose and glucosamine. The content of ninhydrin-positive substances, hexoses, pentoses, hexosamines, total nitrogen and phosphorus is as shown in Table 4. It should be pointed out that the results of quantitative analysis may not be very significant since the purity and homogeneity of the test preparations have not yet been fully established.

The molecular weight determination was carried out by the surface balance technique. The $\pi A - \pi$ curves, where π is the surface pressure (dyne/cm) and A is the area occupied by the specimen (m^2/mg), of a specimen of the insoluble residue and by a wax D preparation of H37Ra are shown in Fig. 4. The curve exhibited by the insoluble residue is very similar to that of the wax D preparation of H37Ra. The apparent molecular weight of both test and reference specimens is about 32,000 on the basis of measurements made at the range of 0.05 to 0.15 dyne/cm. The curves at the lower surface pressure region (less than 0.05 dyne/cm), on the other hand, show a gradual upward turn and tended roughly towards a value $(A)_{\pi=0}$ of 0.15. The results suggest that at a very low surface pressure, both test and reference preparations exist as monomer units with molecular weights of about 16,000 (for further discussion, see Tanaka and Kitagawa, to be published).

The above findings seem to justify the conclusion that the insoluble residue isolated from 'delipidated' BCG cell walls digested with lysozyme and the L_{11} enzyme consists mainly, if not exclusively, of materials essentially identical with

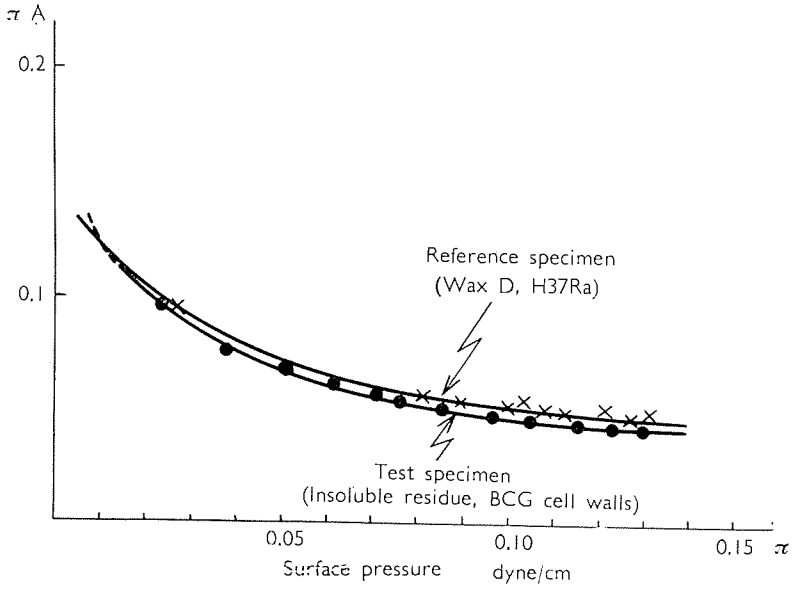

Fig. 4. Determination of the Molecular Weight of the 'Bound Wax D' Fraction by the Surface Balance Technique (πA—π Curves)

the wax D fraction of human type *Mycobacterium tuberculosis* (a peptide-type). The insoluble residue, therefore, will be referred to below as 'bound wax D'.

3. *Electron-microscopy of the 'bound wax D' fraction*

Fig. 5 illustrates an electron micrograph of a highly diluted specimen of a suspension of the 'bound wax D' fraction in distilled water. The fraction is composed of morphological units with apparently a membraneous appearance. The observation that the fraction chemically identical to the wax D of a peptide type has a membraneous structure, seems to be contradictory to the general view that the mucopeptide portion of bacterial cell walls is responsible for their morphological integrity, and this point requires further investigation.

4. *Adjuvant activity of 'bound wax D'*

The striking resemblance of the insoluble residue isolated from the 'delipidated' BCG cell walls digested with lysozyme and the L_{11} enzyme to the wax D of human type *Mycobacterium tuberculosis* stimulated an investigation of the adjuvant effect of the fraction in the production of circulating antibody and the development of a delayed type of hypersensitivity towards crystalline egg white albumin and crystalline egg white lysozyme.

Twenty-four guinea pigs, weighing about 400 g, were divided into four groups of 6 animals. The first and third groups were sensitized with antigen alone (5 mg per animal), and the second and fourth groups received the antigen (5 mg) with the 'bound wax D' fraction (0.4 mg). The 'bound wax D' fraction was dissolved in chloroform-methanol-water (100 : 5 : 0.5, v/v), and the solution was filtered through Toyo Roshi filter paper of Seitz EK type. The fraction recovered from the filtrate (82.8 per cent recovery) was tested for its adjuvant activity. All animals were sensitized by a single injection into the left hind foot pad of 0.2 ml each of the water in oil emulsion, containing the indicated dose of the 'bound wax D' fraction and/or the antigens. The emulsions were prepared by mixing 0.4 ml of saline containing the antigens, 0.4 ml of Arlacel A (Atlas Powder Co., U. S. A.) and 1.2 ml of Bayol F (Esso Standard Oil Co., U. S. A.). The 'bound wax D' fraction was dissolved in Bayol F when this fraction was to be incorporated in the sensitizing antigens.

Three weeks after the sensitizing injection, the animals were examined by corneal and intracutaneous tests for the development of a delayed type of hypersensitivity towards the respective antigens. One week later, blood specimens were collected from all animals by heart puncture to measure the content of circulating antibodies against the sensitizing antigens.

A summary of the results of the experiments are presented in Table 5. Inclusion of the 'bound wax D' fraction in the injection mixture resulted in much higher levels of corneal and skin reactivity of a delayed type to both of the test antigens. The adjuvant effect of the reaction was the most striking in the corneal reaction: the animals sensitized with the antigen alone exhibited no visible reaction, in sharp contrast to the intense reaction with chemosis in the animals sensitized with the antigens combined with the 'bound wax D' fraction. The 'bound wax D' fraction exhibited a powerful adjuvant effect also on the production of circulating antibody towards egg white albumin. The average antibody level in serum specimens taken from animals receiving albumin mixed with the 'bound wax D'

Table 5. Adjuvant Activity of 'Bound Wax D' Isolated from 'Delipidated' BCG Cell Walls Digested with Lysozyme and L_{11} Enzyme

Sensitizing antigen (mg/animal)	Guinea pig No.	Antibody content (μg protein/ml)	Corneal reaction *1	Skin reaction *2	
				Antigen (0.5 mg/0.1 ml)	Sauton old tuberculin (1 : 10)
Crystalline egg white albumin (5 mg)	E. 1	0	0	0	
	E. 2	153	0	$\frac{\pm}{(25\times16)}$	
	E. 3	282	0	$\frac{-}{(26\times16)}$	
	E. 4	53	0	0	
	E. 5	19	0	$\frac{-}{(10\times7)}$	
	E. 6	— (Mean : 101)	0	0	
Crystalline egg white albumin (5 mg) + 'Bound wax D' (0.4 mg)	EB. 1	1240	3 C*3	$\frac{+N}{14\times13\ (38\times36)}$	
	EB. 2	900	2	$\frac{-}{14\times10\ (44\times28)}$	
	EB. 3	1460	3 C	$\frac{\pm\pm}{14\times13\ (36\times27)}$	
	EB. 4	1120	2 C	$\frac{\pm}{13\times9}$	0
	EB. 5	1040	1	$\frac{\pm}{16\times14}$	$\frac{\pm}{(17\times11)}$
	EB. 6	950 (Mean : 1102)	3 C	$\frac{\pm\pm}{17\times16}$	0
Crystalline egg white lysozyme (5 mg)	L. 1		0	0	
	L. 2		0	0	
	L. 3		0	0	
	L. 4		0	0	
	L. 5		0	$\frac{-}{8\times6}$	
	L. 6		0	0	
Crystalline egg white lysozyme (5 mg) + 'Bound wax D' (0.4 mg)	LB. 1		3 C	$\frac{-}{14\times11}$	
	LB. 2		3 C	$\frac{-}{13\times11}$	
	LB. 3		1	$\frac{+}{11\times10}$	0
	LB. 4		3 C	$\frac{+}{11\times9\ (19\times14)}$	$\frac{-}{(12\times11)}$
	LB. 5		3 C	$\frac{\pm N}{12\times9}$	0
	LB. 6		3 C	$\frac{+}{12\times9}$	0

*1 The concentration of the antigen solutions used for corneal test; egg white albumin: 20 mg/ml; egg white lysozyme: 10 mg/ml.

*2 $\frac{\text{Intensity of induration}}{\text{Size of redness (size of weak redness) mm} \times \text{mm}}$ N: necrosis

*3 C: chemosis.

was almost 11-fold that of the animals sensitized with albumin alone. A similar tendency for increased antibody production on inclusion of the 'bound wax D' fraction in the sensitizing antigen was noticed in animals sensitized with egg white lysozyme. However, the exact antibody content was not measured in the serum specimens.

The last column of the Table shows the results of an intracutaneous test performed with a 1:10 dilution of Sauton old tuberculin on animals receiving injections of the 'bound wax D' fraction irrespective of the sort of antigen used. Among seven animals tested, five showed no reaction at all and the remaining two gave only a very slight reaction. The finding that the animals injected with the 'bound wax D' fraction did not react significantly to tuberculin suggests that the adjuvant effect observed in the present experiment is certainly due to the fraction itself, and not to the possible presence of contaminating cell walls which escaped enzyme action, since BCG cell walls, even without their free lipids, have been shown to induce tuberculin hypersensitivity (Kotani *et al.*, 1960).

DISCUSSION

The data presented in this paper clearly show that material essentially identical to the wax D fraction of human type *Mycobacterium tuberculosis* is the major component of 'delipidate' BCG cell walls. The 'bound wax D', as a component of BCG cell walls, is definitely distinct from the usual wax D that is located, as it were, as a capsule over the cell wall proper of bovine type *Mycobacterium tuberculosis* including BCG, since the former, unlike the latter, contains a peptide consisting of cell wall amino acids and exhibits a marked adjuvant activity in both production of circulating antibody and development of a delayed type of hypersensitivity. White and cowerkers (1958) demsontrated that wax D fractions of bovine, avian and saprophytic strains of *Mycobacterium* failed to increase either anti-ovalbumin antibody or the corneal reaction to ovalbumin under conditions in which wax D fractions from various strains of human type *Mycobacterium tuberculosis* exhibited a marked adjuvant effect. The reasons for the observed striking difference between the strong adjuvant activity of the wax D of human strains and the inactivity of the wax D of bovine, avian and saprophytic strains have been discussed in their paper and it has been suggested that the presence of a peptide consisting of alanine, glutamic acid and α, ε-diaminopimelic acid, linked to glycopeptide, is essential for the adjuvant activity. The findings reported here that the 'bound wax D' isolated from 'delipidated' BCG cell walls under the action of the cell wall lytic enzymes, unlike the wax D separated from chloroform extracts of bacterial cells, contains a peptide consisting of cell wall amino acids and exhibits a marked adjuvant effect offers further evidence in support of the above mentioned view of White *et al.*

White and cowerkers (1958) also showed that 'delipidated' bacterial cells of bovine, avian and saprophytic strains of *Mycobacterium* still contain firmly bound lipids and have high adjuvant activity. In the light of the present study, the fraction that has so far been known as firmly bound lipid may be regarded as a cleavage product derived by hydrolysis from the 'bound wax D' present as cell wall component.

Studies on the chemical nature of the cell wall components liberated by the digestion of 'delipidated' BCG cell walls with lysozyme and the L_{11} enzyme are now in progress. The linkage between these cell wall components and 'bound wax D', and the relation of 'bound wax D' with 'capsular' wax D must be the subject of future study.

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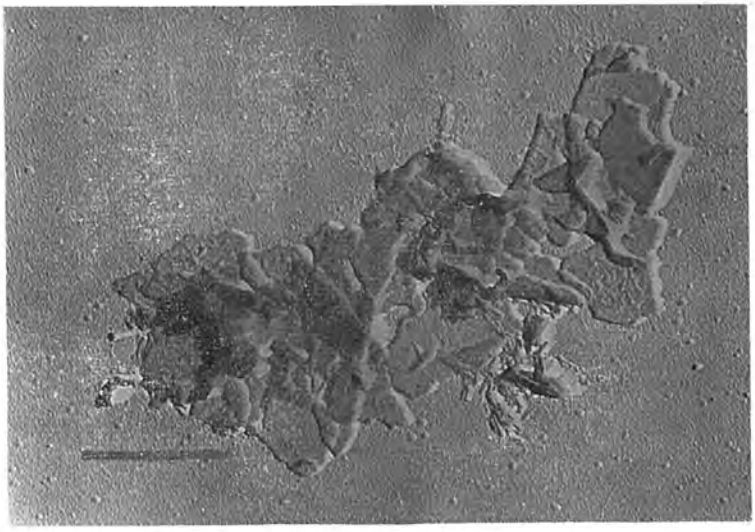
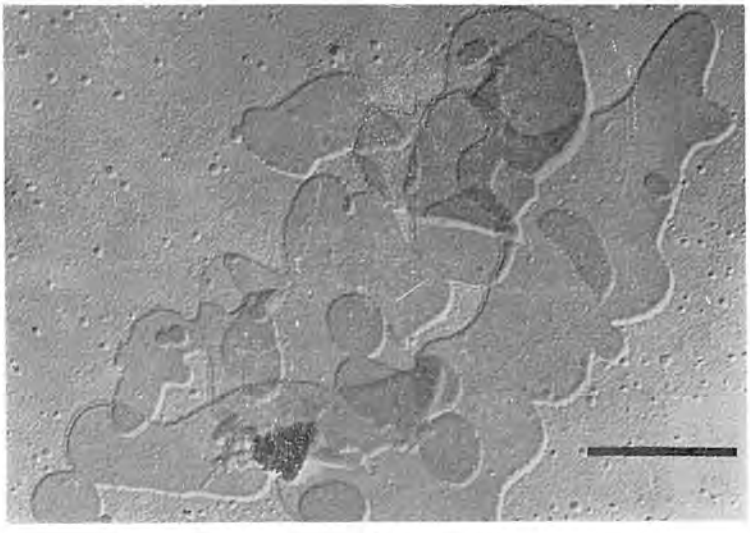


(A)



(B)

Fig. 5. Electron Microscopic Appearance of 'Delipidated' BCG Cell Walls Before and After Digestion with Lysozyme and L_{11} Enzyme

(A) : Non-treated 'delipidated' cell walls; (B) : Insoluble residue ('bound wax D') isolated from 'delipidated' cell walls digested with both lysozyme and L_{11} enzyme. The specimens were mounted on collodion films and shadowed with chromium. Scale: 1 μ.