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Correlation of Adjuvant Activity and Chemical Structure of Wax D Fractions of Mycobacteria*

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Summary. By fractionation of wax D from mycobacteria by ultracentrifugation in ether, it is possible to prepare from wax D of human strains peptide-containing and non-peptide-containing compounds. The peptide-containing fractions, like the parent wax D, were able to act as adjuvants by increasing serum anti-ovalbumin levels, by increasing corneal hypersensitivity to ovalbumin, and by inducing encephalomyelitis after homologous guinea-pig brain injection. The peptide–carbohydrate moiety resulting from hydrolysis of the whole wax D was found inactive in all these biological effects.

When the same centrifugal technique was applied to several bovine types of *Mycobacterium tuberculosis*, *M. avium* and to atypical and saprophytic mycobacteria, analogous peptide and non-peptide-containing fractions were obtained. The amino acid patterns of these were of great variety, and in most cases differed from those present in human type wax D. In three instances a small proportion of a peptide-containing fraction was obtained (from *M. phlei*, *M. avium* and an atypical mycobacterium), which closely resembled a human type wax D. These fractions were found to have adjuvant activity. All other fractions of wax D of bovine, avian and saprophytic strains were inactive.

These facts support the role of a peptide of D- and L-alanine, D-glutamic acid and *meso*-α,α′-diaminopimelic acid in determining the adjuvant action of wax D fractions and whole mycobacteria. The structure of the peptidoglycolipid of an adjuvant-active mycobacterial wax corresponds closely in amino acid, amino sugar and hexose composition with the mucopeptide of the bacterial cell wall, and evidence is discussed for the concept of wax D fractions as partial replicas of a fundamental cell wall polymer.

INTRODUCTION

All mycobacteria contain a chloroform extractable, ether soluble, acetone insoluble glycolipid fraction, referred to in previous communications as wax D (Asselineau and Lederer, 1960). The most thoroughly analysed of wax D fractions were derived from human strains of *Mycobacterium tuberculosis* and shown to be peptido-glycolipids (Asselineau, Buc, Jollès and Lederer, 1958; Lederer, 1961).

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Previous investigations (White, Coons and Connolly, 1955; White, Bernstock, Johns and Lederer, 1958) have shown that wax D fractions of human strains of *M. tuberculosis* can reproduce the adjuvant effects of whole killed mycobacteria, including the production of increased serum antibody, when added to a water-in-oil emulsion of the antigen (ovalbumin) in mineral oil. The inactivity of other types of lipid fractions from human type *M. tuberculosis* attested to the specific activity of wax D or its components. Wax D fractions from several bovine strains of *M. tuberculosis*, from *M. avium*, as well as from saprophytic strains of mycobacteria and from *Nocardia* spp. were found inactive.

At that time, the principal chemical difference noted between wax D from human and from bovine, avian and saprophytic strains was the presence of a peptide moiety in the former and its absence in the latter strains. This peptide contains *meso*-α,α′-diaminopimelic acid, D-glutamic acid as well as D- and L-alanine and thus stands in obvious relationship to the basic 'mucopeptide' of the cell wall of Gram-positive bacteria (Salton, 1953; Cummins and Harris, 1956; Work, 1957).

The present investigation was planned in order to test further the hypothesis that the presence of the peptide moiety in wax D was necessary for adjuvant activity on serum antibody production and to allow more detailed correlation between chemical structure of wax D and biological activity. Fractionation of the previously used wax D was achieved by ultracentrifugation in ether (Jollès, Samour and Lederer, 1962) yielding one non-sedimentable, amino-acid-free fraction and several amino-acid-containing fractions from several human, bovine, avian and saprophytic strains.

MATERIALS AND METHODS

Preparation of Wax D

The details of the method of extraction from living 4-week cultures of *M. tuberculosis* of the chloroform soluble wax D, and its further resolution into fractions soluble (wax C) and insoluble in boiling acetone (wax D) have been given previously (Asselineau *et al.*, 1958; Jollès *et al.*, 1962).

Preparation of Fractions of Wax D by Ultracentrifugation

Purified wax D, prepared as above, was dissolved in 1–2 g. amounts in 50 ml. of ether. The solution was centrifuged at 50,000 *g* (Spinco rotor 40) for 15, 35, 70 and 150 minutes at 2°. Fraction D_s (s = supernatant) was obtained from the clear supernatant after 150 minutes as a colourless or yellow powder by precipitation with methanol. The sediments obtained after 15, 35, 70 and 150 minutes of centrifugation were fractions D_{p15}, D_{p35}, D_{p70} and D_{p150} (p = precipitate). Sometimes an intermediate viscous layer was obtained which could be decanted. Such preparations are designated as, for example D_{p35v} (v = viscous).

Preparation of the Hydrosoluble Moiety of Wax D Fractions

The preparation has been described previously (Jollès *et al.*, 1962). In order to avoid obtaining a fraction which was insoluble in methanol or in water it was necessary to increase the time of boiling wax D in benzene plus 5 per cent methanolic KOH to 2–4 minutes.

Separation of Amino Sugars by Column Chromatography

After hydrolysis of wax D (HCl 2 N, 110°, 4 hours) amino sugars were separated by

chromatography on a 55 × 0·9 cm. Amberlite CG-120 column; 0·33 N HCl was used as eluent, and 2 ml. fractions were collected. Glucosamine was eluted between 150 and 165 ml., muramic acid between 170 and 180 ml., and galactosamine between 180 and 200 ml. Measurement of optical density obtained with the Morgan and Elson reagent were read at 530 mμ after 30 minutes and at 505 mμ after 18 hours. The ratio of the optical density measurements after 30 minutes and 18 hours was found to be approximately 0·8 for glucosamine and galactosamine, and 3 for muramic acid.

Analysis of Amino Acids

The amino acids of whole wax D or its hydrosoluble moiety were characterized and estimated after total hydrolysis (HCl 6 N, 100° for 24 hours) by the method of Piez and Morris (1960) using the Technicon Auto-analyzer.

Animals

Guinea-pigs, which were reared in the animal house of The London Hospital, belonged to an albino outbred strain. Animals weighing between 350 and 500 g. were distributed according to sex and weight in an attempt to achieve groups comparable in these respects. Diet consisted of mixed bran and oats with supplementary cabbage *ad lib.*

Antigen and Injection Procedure

The antigen used throughout this study was thrice-crystallized egg albumin prepared by the method of Kekwick and Cannan (1936). The dose was always 2 mg. injected in 0·2 ml. of water-in-oil emulsion into the left hind footpad of the guinea-pig. To prepare a batch of antigen emulsion 10 mg. of crystalline ovalbumin was dissolved in 0·2 ml. of saline, 0·2 ml. mannide monooleate (Arlacel Z, batch 9325, Atlas Powder Company, Wilmington, Delaware, U.S.A.) was added and mixed by drawing up into a 1 ml. syringe and expelling repeatedly into a glass tube. A volume of 0·6 ml. of Bayol 55 (Esso Petroleum Company Ltd., Purfleet, Essex) was then added and the whole emulsified as before. When tubercle bacilli or their lipid fractions were added to the mixture they were either suspended or dissolved in the Bayol 55 before emulsification. Hydrosoluble fractions were dissolved in the saline. Dried bacilli were suspended in oil with a Griffith-type glass tube and pestle.

Collection and Treatment of Sera

After 3 weeks the animals were killed with chloroform and blood collected from the incised heart in sterilized petri dishes. Serum was separated after standing 2 hours at 37° and overnight at 0–4°. Sera were stored frozen at −30° and thawed immediately before estimation of precipitins 2–4 weeks later.

Estimation of Serum Anti-Ovalbumin Levels

Analyses for anti-ovalbumin were done in triplicate by the quantitative method modified from Kabat and Meyer (1948). For the sake of simplicity no account was taken of complement content of sera and the addition of this to the weight of precipitates was ignored, though care was taken to handle all sera after collection in a uniform manner.

Based on preliminary assessment of optimal proportions in precipitin tests, analyses were done with 0·2–2·0 ml. samples of serum, such as would yield 50–200 μg. of nitrogen in the final precipitate. The amount of antigen for use in the estimation was calculated such as to give maximal precipitation of antibody with a slight excess of antigen. The precipitation was allowed to proceed for 3 days in the refrigerator at 4°. Precipitates were

washed with the minimum volume of saline in order to prevent loss due to solubility of precipitates at 0–4°. Tubes containing precipitates were spun in an International-type PR2 centrifuge at 1560 *g* for 30 minutes at 0–4°. Supernatant fluid was removed and the packed precipitates were broken up, and washed thrice with 1·0 ml. volumes of ice-cold saline. The packed precipitate was digested for 6 hours with 0·1 ml. of 1 per cent SeO_2 and 0·1 ml. of H_2SO_4 (B.D.H., 'M.A.R.'), and the ammonia nitrogen estimated colorimetrically after nesslerization.

Corneal Tests

After instillation of a drop of 4 per cent cocaine hydrochloride intra-corneal injections were made using a needle size 30 (Imperial standard wire gauge) attached to a tuberculin-type syringe. A solution of crystalline ovalbumin, 20 mg./ml. was injected in an amount sufficient to cause a disc of opacity in the cornea 2 mm. in diameter. The eyes were examined at 24 and 48 hours and the extent and degree of corneal opacity, and the presence of chemosis were recorded. The chemosis increased in degree up to 4–6 hours and subsequently decreased. Corneal opacity was not apparent at 4 hours and appeared usually in maximal extent at 24 hours. With a strong reaction (recorded as 3) the whole of the cornea was thickened, opaque, grey-white (resembling sodden blotting paper). The controls looked entirely normal at 24 and 48 hours except for an occasional residual trauma of the needle penetration. Histological section confirmed these findings. The strong reaction showed a cellular infiltration from limbus to limbus within a cornea of up to three times usual thickness. Corneal tests were in all instances done on the nineteenth day after the injection of antigen mixture into the footpad and hence 2 days before the animals were killed.

Production of Allergic Disseminate Encephalomyelitis

Sixteen mg. (wet weight) of guinea-pig brain homogenized in 0·04 ml. of 0·25 per cent phenol saline was prepared as a water-in-oil emulsion with 0·04 ml. of Arlacel A and 0·12 ml. of Bayol 55 mineral oil, with or without 1 mg. of added mycobacteria or mycobacterial fraction. Guinea-pigs were given a single injection of 0·2 ml. of this brain emulsion mixture into the left hind footpad. Animals were observed daily from the 12th day after injection for the appearance of tremors, torticollis or paralysis, usually bilateral, of the hind limbs, wasting of thigh muscles, soiling of hindquarters and rectal impaction of faeces. Animals with severe neurological symptoms, which usually appeared rapidly over 24 hours and progressed to a total inability to stand or to move forwards or backwards, were killed immediately. The brain and spinal cord were removed and placed in fixative for subsequent histological examination. Animals that showed no evidence of paralysis or only minor neurological signs were killed on the 30th day after injection when their brains and spinal cords were removed for similar histological study.

RESULTS

PURIFICATION AND AMINO ACID COMPOSITION OF WAX D FRACTIONS FROM HUMAN STRAINS OF *M. tuberculosis*

High-speed centrifugation of solutions in ether of wax D from five human strains of *M. tuberculosis* (Canetti, $H_{37}R_a$, $H_{37}R_vS_r$, Test and Brévannes) was found to yield two distinct groups of resultant fractions. (1) D_s fractions remained ether soluble after centrifugation

for 150 minutes at 50,000 *g*. The yield of the D_s fractions from human strains did not exceed 10 per cent. They were found to be free from amino acids. Their low nitrogen content was accounted for entirely as amino sugars. (2) D_p fractions comprised a series of fractions which sedimented after different times of centrifugation. D_p fractions contained alanine, glutamic acid, *meso*-α,α′-diaminopimelic acid and glycine in molecular proportions close to 3·0:2·0:1·5:0·2 respectively. The yields of the different fractions and some amino acid analyses are indicated in Tables 1 and 2.

TABLE 1

YIELD (PER CENT OF WAX D) OF THE FRACTIONS OBTAINED AFTER ULTRACENTRIFUGATION OF PURIFIED WAX D FROM DIFFERENT STRAINS OF MYCOBACTERIA

Bacterial strain		D_{p15}	D_{p35}	D_{p35v}	D_{p70}	D_{p70v}	D_{p150}	D_{p150v}	D_s	*Total*
M. tuberculosis										
Human	Brévannes	49·8	13·6	12·6	14·5	0	4·6	0	3·6	98·7
	Canetti	17·6	12·2	31·0	6·8	0	7·7	3·8	6·9	86·0
	$H_{37}R_vS_r$	6·6	7·1	23·6	36·1	0	9·6	0	3	86·0
	Test	13·6	23·6	13·7	29·7	7·5	4·2	0	4·8	97·2
	$H_{37}R_a$	←			90			→	5·0	95·0
Bovine	Dupré	5·5	2·9	0	0	0	12·5	0	78·9	99·8
	BCG	2·3	0	0	0	0	36·7	0	54·9	93·9
	Marmorek	0	2·4	0	3·2	0	34·3	0	44·8	84·7
M. phlei		3·5	4·5	0	2·9	0	5·1	0	69·7	85·8
M. smegmatis		0	0	0	8·3	0	12·9	0	78·4	99·6
M. avium	No. 802	2·1	0	0	34·2	0	0	0	48·5	84·8
Mycobacterium: atypical photochromogenic	No. 4	6·7	13·8	24	15·3	29	4	0	0·8	93·6

TABLE 2

AMINO ACID COMPOSITION (MOLAR RATIOS) OF THE FRACTIONS OBTAINED AFTER ULTRACENTRIFUGATION OF PURIFIED WAX D FROM DIFFERENT STRAINS OF MYCOBACTERIA

Strain	*Ref. No.*	*Fraction*	*Alanine*	*Glutamic acid*	*Diamino-pimelic acid*	*Glycine**	*Aspartic acid*
M. tuberculosis var. *hominis*							
Canetti		D_{p15}	3·0	1·7	1·5	0·3	0
		D_{p35}	3·0	1·0	1·5	0·15	0
	WL 78	D_{p35v}	3·0	1·8	1·7	0·16	0
		D_{p70}	3·0	1·9	1·4	0·07	0
		D_{p150}	3·0	1·2	1·3	0·7	0
		D_{p150}	3·0	2·0	1·7	0·85	0
		D_s	0	0	0	0	0
Brevannes		Total D_p	3·0	1·9	1·7	Traces	0
$H_{37}R_vS_r$	WL 54	Total D_p	3·0	1·9	1·6	0·4	0
Test	WL 80	D_{p70}					
Atypical No. 4 (photochromogenic)		D_{p70}	3·0	2·4	1·8	0·6	0·45
M. phlei		D_{p15}	3·0	1·8	1·4	0·9	0·75
	WL 56	D_{p35}	+	+	+	+	+

* May contain some traces of glucosamine.

+ Indicates that the presence of the amino acid was established but a quantitative result is not available.

PURIFICATION AND AMINO ACID COMPOSITION OF WAX D FRACTIONS FROM BOVINE TYPES OF *M. tuberculosis*, OF *M. avium* AND OF *M. phlei* AND *M. smegmatis*

High-speed centrifugation has been applied to wax D fractions from three bovine strains of *M. tuberculosis* (BCG, Marmorek, Dupré, from *M. avium*, and from two saprophytic strains of mycobacteria (*M. phlei* and *M. smegmatis*). (For further details see Jolles, Samour and Lederer, 1963.) With all strains two distinct fractions were obtained. (1) D_S fractions remained soluble in ether after centrifugation for 150 minutes at 50,000 ***g***. The yield of these D_S fractions was 44–79 per cent. (2) D_S fractions comprised a group of fractions which sedimented after different periods of centrifugation. Whereas in the case of human strains these represented 90–95 per cent of the whole, and chemically different fractions appeared after each successive centrifugation period, the D_P fractions from saprophytic and bovine strains represented only 15–30 per cent of the total wax D and were not obtained after each centrifugation period. Table 1 indicates the yield of such fractions. It is important to note that some of these D_P fractions (e.g. from *M. phlei* and *M. avium*) are closely related chemically to human D_P fractions and possess the same amino acid composition : alanine, glutamic acid, and *meso*-α,α′-diaminopimelic acid in molar ratio near to 3 : 2 : 2 with variable amounts of glycine and also aspartic acid. It is the first time that such amino acid-containing fractions have been characterized in wax D from non-human bacillary strains. The corresponding D_S fractions of these particular strains do not contain amino acids.

D_P fractions which do not contain diaminopimelic acid (e.g. from *M. smegmatis*, and from the bovine strain BCG) contain traces of a great number of amino acids (aspartic acid, threonine, serine, glycine, alanine, valine, leucine, lysine). The bovine strain Dupré contains, besides such traces, a greater amount of lysine. The corresponding D_S fractions of these strains have nearly the same composition. It has not been established if the amino acids really constitute a peptide moiety.

TESTS FOR BIOLOGICAL ACTIVITY OF CENTRIFUGAL FRACTIONS OF WAX D: ADJUVANT EFFECT ON SERUM ANTI-OVALBUMIN LEVELS AND ON CORNEAL REACTIONS TO OVALBUMIN, GRANULOMA PRODUCTION AND INDUCTION OF ALLERGIC ENCEPHALOMYELITIS

The levels of serum antibody in test and control groups of guinea-pigs on the 21st day after injection are shown in Tables 3 and 4, together with the results of corneal tests done on the 19th day after injection, and read 24 and 48 hours later. Since the results of the 24 hour reading were in almost all cases identical with those at the 48 hour interval they were omitted from the table.

Table 3 includes the results of tests done with human type whole killed bacilli of *M. tuberculosis* (included as a positive control), and with centrifugal fractions of wax D of various human strains of *M. tuberculosis* $H_{37}R_vS_r$ (D_S, D_{p150} and the total D_P), Canetti (D_{p35v}) and Test (D_{p70}), together with negative controls.

The results show that the inclusion of the human type D_P fractions of $H_{37}R_vS_r$ (D_{p150}, total D_P), of Canetti (D_{p35v}) and Test (D_{p70}) (containing alanine, glutamic acid, *meso*-α,α′-diaminopimelic acid and glycine) all produced an increase in antibody levels (366–510 μg. N/ml.) at 3 weeks after a single injection of ovalbumin in water-in-oil emulsion, as compared with the levels in the animals injected with ovalbumin emulsions similar except in their lacking this fraction. Serum anti-ovalbumin values in control animals ranged from 0 to 232 μg. Ab. N per ml., with one exceptional high value of 303. The mean value for all

control animals was 65 μg. Ab. N per ml. By contrast, the D_S fraction (devoid of amino acids) was inactive, the antibody levels in the serum being below those of the control group of animals. It was further shown that the inclusion of the same D_p fractions of $H_{37}R_vS_r$ in the injection mixture resulted in high levels of corneal reactivity to ovalbumin, and in the case of the total D_p fraction of $H_{37}R_vS_r$ a high incidence of allergic disseminated encephalomyelitis in guinea-pigs receiving injections of homologous brain tissue in water-in-oil emulsion (four out of five guinea-pigs). The presence of encephalomyelitis was accepted if animals developed characteristic signs of paralysis (see 'Materials and Methods')

TABLE 3

EFFECT OF WHOLE HEAT-KILLED BACILLI AND VARIOUS WAX D FRACTIONS OF HUMAN TYPE *N. tuberculosis* ON SERUM ANTI-OVALBUMIN PRECIPITIN LEVELS, DELAYED-TYPE HYPERESENSITIVITY (CORNEAL TEST) AND INDUCTION OF ENCEPHALOMYELITIS BY HOMOLOGOUS BRAIN

No. in group	*Fraction and dose*	*Ref. No.*	*Anti-ovalbumin (μg. N/ml. serum)*			*Corneal reaction at 48 hours*	*Encephalomyelitis*
			Mean	*Range*	*S.D.*		
4	5 mg. whole bacilli *M. tuberculosis* human type	WL 1	359	267–445	80	3C*, 3C, 3C, 3C	3/5
5	Contemporaneous controls	—	153	24–303	96	0·5, 0, 0, 0, 0	
5	200 μg. total D_p wax D $H_{37}R_vS_r$	WL 54	510	341–621	102	3C, 2·5C, 2·5C, 3C	4/5
5	Contemporaneous controls	—	153	24–303	96	0·5, 0, 0, 0, 0	
5	200 μg. D_S $H_{37}R_vS_r$	WL 55	81	60–175	65	0, 0, 0, 0, 0,	0/5
5	Contemporaneous controls	—	40	0–112	48	0, 0, 0, 0, 0	
4	200 μg. hydrosoluble moiety from D_{p35} wax fraction	WL 57	95	57–152	35	0, 0, 2, 0	0/5
4	Contemporaneous controls	—	79	52–124	32	0·5, 0, 0, 0,	
4	200 μg. D_{p35v} human type, Canetti	WL 78	366	330–421	34	3C, 3C, 2C, 2C	
4	Contemporaneous controls	—	122	34–232	75	0, 0, 0, 0	
4	200 μg. D_{p150} $H_{37}R_vS_r$	WL 79	440	350–600	95	3C, 3C, 2C, 2C	
4	Contemporaneous controls	—	122	34–232	75	0, 0, 0, 0	
4	200 μg. D_{p70} human type, Text	WL 80	389	304–590	94	3C, 3C, 3C, 3C	
4	Contemporaneous controls	—	122	34–232	75	0, 0, 0, 0	

* C indicates the presence of chemosis.

within the period of 14–20 days after injection of homologous brain antigen and if histological section revealed characteristic lesions of this disease (White and Marshall, 1958). Moreover, the inclusion of all of the above D_p fractions resulted in the development of characteristic firm granulomata at the local site of injection in the hind footpad and within the regional lymph nodes (popliteal, inguinal and iliac nodes). Histological examination showed these to consists of epithelioid and giant cells (Suter and White, 1954; White *et al.*, 1958). Control animals showed slight local swelling in the injected foot. Histological examination revealed some increase in macrophages ('foam cells') at this local injection site and within the peripheral sinuses of the regional lymph nodes. These differences between test and control animals in respect of type and extent of granulomatous tissue formation were readily apparent either by inspection with the naked eye or by

examination of sections under the microscope. The D_S fraction of $H_{37}R_vS_r$ (devoid of amino acids) was found to be inactive in inducing encephalomyelitis, in so far as no evidence of muscle wasting or paralysis was seen in these animals during 30 days after injection. The animals, which appeared healthy at the end of this period, were killed. Histological examination of brain and cord for the lesions of encephalomyelitis was negative.

TABLE 4

EFFECT OF CENTRIFUGAL FRACTIONS OF WAX D FROM BOVINE TYPE *M. tuberculosis*, *M. avium*, ATYPICAL, AND SAPROPHYTIC MYCOBACTERIA ON SERUM PRECIPITIN LEVELS, DELAYED-TYPE HYPERSENSITIVITY (CORNEAL TEST) AND INDUCTION OF ENCEPHALOMYELITIS BY HOMOLOGOUS BRAIN

No. in group	*Fraction and dose*	*Ref. No.*	*Anti-ovalbumin (μg. N/ml. serum)*			*Corneal reaction at 48 hours*	*Encephalomyelitis*
			Mean	*Range*	*S.D.*		
4	200 μg. D_S *M. tuberculosis* bovine 'Marmorek'	WL 60	102	18–155	54	0, 0·5, 0, 0·5	
4	Contemporaneous controls	—	79	52–124	29	0·5, 0, 0, 0	
4	200 μg. D_{p150} *M. tuberculosis* bovine 'Marmorek'	WL 58	96	16–242	88	0·5, 0, 0, 0	
4	Contemporaneous controls	—	79	52–124	29	0·5, 0, 0, 0	
4	200 μg. D_{p35} *M. tubercolosis* bovine, Dupré	WL 68	55	0–130	60	1, 0, 0, 0	
4	Contemporaneous controls	—	122	34–232	75	0, 0, 0, 0	
5	200 μg. D_{p70} *M. avium*	WL 63	166	13–284	65	2·5C*, 0, 0, 1C, 3C	
4	Contemporaneous controls	—	122	34–232	75	0·5, 0, 0, 0	
4	200 μg. D_{p150} *M. smegmatis* saprophytic	WL 59	102	45–142	41	0·5, 0, 0, 0·5	
4	Contemporaneous controls	—	79	52–124	29	0·5, 0, 0, 0	
4	200 μg. B_{35} *M. smegmatis* saprophytic	WL 64	92	39–152	50	0, 0·5, 0, 0	
4	Contemporaneous controls		122	34–232	75	0·5, 0, 0, 0	
5	200 μg. D_{p35} *M. phlei* saprophytic	WL 56	359	278–414	51	2C, 3C, 3C, 2C, 2C	5/5
5	Contemporaneous controls	—	39	0–112	45	0, 0, 0, 0, 0	
4	200 μg. D_{p35} Atypical No. 4 (photochromogenic)	WL 73	321	227–383	63	3C, 2·5C, 2C, 2C	
4	Contemporaneous controls	—	122	34–232	75	0·5, 0, 0, 0	
5	200 μg. total D_p $H_{37}R_vS_r$ *M. tuberculosis* human type	WL 54	510	341–621	102	3C, 2·5C, 2·5C, 3C	4/5
5	Contemporaneous controls	—	153	24–303	96	0·5, 0, 0, 0, 0	

* C indicates the presence of chemosis.

Table 3 also includes the results of similar tests of a product of hydrolysis of the D_{p35} fraction of $H_{37}R_vS_r$—the hydrosoluble moiety, consisting of the peptido-polysaccharide containing the amino acids: alanine, glutamic acid, *meso*-α,α′-diaminopimelic acid and glycine. This was without significant effect in raising serum antibody levels, or in inducing positive corneal reactions to ovalbumin. Moreover, none of the five guinea-pigs injected with this fraction and homologous brain developed allergic encephalomyelitis.

Table 4 includes the results of biological tests of centrifugal fractions of wax D from

bovine strains of *M. tuberculosis*, from *M. avium*, from an atypical (photochromogenic) strain and from two saprophytic mycobacteria (*M. smegmatis* and *M. phlei*) and of the total D_p fraction of wax D of the human strain $H_{37}R_vS_r$ which was included as a positive control. The results show that the D_s and D_{p150} fractions of the bovine strains 'Marmorek' and the D_{p35} fraction of the bovine strain 'Dupré' were all inactive in respect of adjuvant effect on serum anti-ovalbumin levels, on corneal reactivity to ovalbumin, and on the induction of allergic encephalomyelitis in guinea-pigs injected with homologous brain. None of these fractions contain all three amino acids: alanine, glutamic acid and *meso*-α,α′-diaminopimelic acid. The D_s fraction of the bovine strain 'Marmorek' was found to be devoid of amino acids of any kind. The D_{p150} fraction of Marmorek contained traces

TABLE 5

EFFECT OF VARIOUS AMINO ACID-CONTAINING LIPIDS (OTHER THAN WAX D DERIVATIVES) ON SERUM PRECIPITIN LEVELS AND DELAYED-TYPE HYPERSENSITIVITY (CORNEAL TEST)

No. in group	*Fraction and dose*	*Ref. No.*	*Anti-ovalbumin (μg. N/ml. serum)*			*Corneal reaction at 48 hours*
			Mean	*Range*	*S.D.*	
4	Mycoside of *M. avium* M. 928 (C_2)	WL 70	29	0–101	42	0, 0·5, 0, 0
4	Contemporaneous controls	—	95	0–233	75	0·5, 0, 0, 0·5
4	Fortuitine	WL 65	25	0–49	29	0, 0, 0, 0·5
4	Contemporaneous controls	—	95	0–233	75	0·5, 0, 0, 0·5
5	Acetone insoluble fractions of *C. ovis**	WL 69	83	26–169	19	0, 0·5, 1, 1·5, 0
4	Contemporaneous controls	—	95	0–233	75	0·5, 0, 0, 0·5
5	Černý ID L380	WL 74	80	23–102	29	0, 0·5, 1, 0, 0
4	Contemporaneous controls	—	122	34–232	75	0·5, 0, 0, 0
5	Černý IIID L382	WL 76	53	0–95	33	0, 0, 1, 0, 0·5
4	Contemporaneous controls	—	122	34–232	75	0·5, 0, 0, 0

* Amino acid-containing phospholipid isolated by Professor J. Asselineau (Toulouse).

of numerous amino acids but was devoid of *meso*-α,α′-diaminopimelic acid. The D_{p35} fraction of *M. phlei* was found to be highly active in all tests for biological activity. This fraction caused raised anti-ovalbumin levels in serum, high levels of corneal reactivity to ovalbumin, as well as inducing encephalomyelitis in all of five guinea-pigs injected with homologous brain emulsion. Moreover, the injected footpad and draining regional lymph nodes showed the characteristic macroscopical and microscopical changes of epithelioid granuloma formation. As seen from Table 2 this fraction yielded on hydrolysis a qualitative and quantitative pattern of amino acids very similar to those of the human-type wax D hydrolysates previously discussed—Ala(3), Glu(2·0), DAP(1·5) and Gly(1·0).

The D_{p35} and D_{p150} fractions from wax D of *M. smegmatis* were found, on the contrary, to be devoid of adjuvant activity in respect of the ability to raise serum anti-ovalbumin levels or to induce corneal hypersensitivity to ovalbumin. Although these fractions contained amino acids, the pattern of these differed markedly from that of human wax D centrifugal fractions, being devoid of *meso*-α,α′-diaminopimelic acid.

The D_{p70} fraction of wax D of *M. avium* was found (Table 4) to possess some adjuvant activity in raising serum levels of anti-ovalbumin (two aminals out of five were above level

of controls) and in inducing a high level of corneal reactivity to ovalbumin (in two of the five animals). In this case, the presence of alanine, glutamic acid and *meso*-α,α′-diaminopimelic acid in hydrolysates was established by paper chromatography but insufficient material was available for a quantitative determination of amino acids.

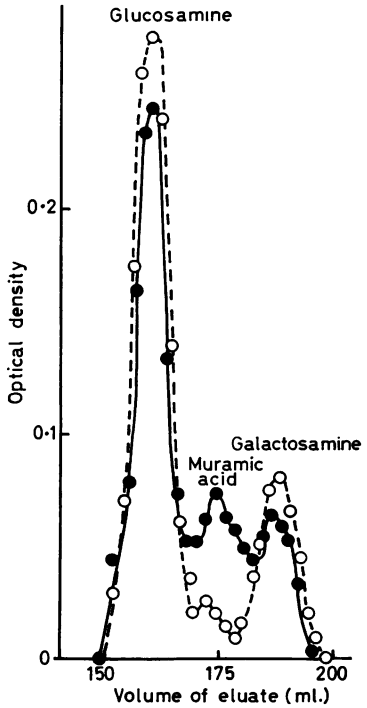

FIG. 1. Separation of amino sugars: glucosamine, galactosamine and muramic acid by chromatography on an Amberlite column CG120 (55 × 0·9 cm.) following elution with N/3 hydrochloric acid. The colour reaction obtained by the Elson and Morgan method was estimated at 530 mμ after 30 minutes (○ - - - - ○) and at 505 mμ after 18 hours. (●——●). Ratio O.D. 505/O.D. 530 is approximately 3 for muramic and 0·8 for glucosamine and galactosamine. The column was loaded with the hydrolysate from 0·189 g. of wax D strain $H_{37}R_vS_r$ fraction D_{p35v}.

The D_{p35} fraction of wax D of the atypical strain of *M. tuberculosis* (No. 4, photochromogen; Runyon classification) was found to have adjuvant activity (Table 4) in raising serum levels of anti-ovalbumin and in inducing a high level of corneal reactivity. Table 2 shows this fraction to contain alanine, glutamic acid and *meso*-α,α′-diaminopimelic acid in the molar proportions near to 3:2:2 (together with smaller proportions of glycine and aspartic acid). Moreover, as seen from Table 1 the wax D of this atypical strain has practically the same ultracentrifugal pattern as wax D of human strains (D_s is nearly absent). The mycolic acid of this strain proves to be unlike that which is typical of human strains and to be comparable with that derived from saprophytic strains (Jollès *et al.*, 1963).

Table 5 includes the results with some other natural and synthetic amino acid or peptide containing lipids which were tested for adjuvant activity in respect of their ability to increase levels of serum anti-ovalbumin and corneal reactivity to ovalbumin in the guinea-pig. The results include the mycoside C_2 of *M. avium* (WL 70) (Smith, Randall, MacLennan

and Lederer, 1960; Chaput, Michel and Lederer, 1963); fortuitine (WL 65) (Vilkas, Miguel and Lederer, 1963); the acetone insoluble fraction of *Corynebacterium ovis* (WL 69), an amino acid-containing phospholipid isolated by Professor J. Asselineau; methyl-2-O-α-D(-)glutamoyl-6-O-stearoyl-β-D-galactopyranoside, designated Černý ID (WL 74) (Černý, Moron and Lederer, 1963); and methyl-2-O-α-D(-)glutamoyl-(N-benzoyloxycarbonyl-γ-benzyl)-3,4-O-isopropylidene-6-O-stearoyl-β-D-galactopyranoside, designated Černý III D (WL 76) (Černý *et al.*, 1963). None of these were found to possess any evidence of adjuvant activity and none produced characteristic local or disseminated epithelioid granulomata.

HYDROLYSIS OF WAX D, SEPARATION AND IDENTIFICATION OF AMINO SUGARS BY COLUMN CHROMATOGRAPHY

As shown in Fig. 1, elution with 0·33 N hydrochloric acid from an Amberlite column CG-120 of the hydrolysis products of wax D from the human type *M. tuberculosis* $H_{37}R_vS_r$ fraction D_{p35v} yielded in succession glucosamine (150–165 ml.), muramic acid (170–180 ml.) and galactosamine (180–200 ml.). The ratio of optical densities as obtained with the Elson and Morgan reagent, read at 530 mμ after 30 minutes and 505 mμ after 18 hours, were 0.8 for glucosamine and galactosamine, and 3 for glutamic acid.

DISCUSSION

In a previous communication (White *et al.*, 1958) it was reported that the wax D fractions of a variety of human strains of *M. tuberculosis* could replace whole killed mycobacteria in Freund-type adjuvant mixture, as judged by their ability in guinea-pigs to increase the levels of serum anti-ovalbumin, to induce a high degree of corneal reactivity to ovalbumin, 3 weeks after a single immunizing injection into one hind footpad. Lipid fractions of mycobacteria other than wax D and the wax D fractions from bovine type *M. tuberculosis* or *M. avium* or saprophytic mycobacteria were inactive in these experiments.

At that time, it was considered that the principal chemical difference between wax D of human and of bovine, avian or saprophytic strains was determined by the presence of a peptide moiety in human strains only. On the basis of this correlation, it was considered that the presence of the peptide moiety was necessary for biological activity.

Before attempting the interpretation of the results of the present investigation, it might be useful to give a brief summary of the chemistry of wax D of mycobacteria. Wax D is isolated from the chloroform extract of bacteria which have previously been extracted repeatedly with ethanol–ether mixture. Wax D is obtained from the chloroform extracts, as the fraction which remains insoluble following repeated boiling in acetone. Several years ago it was found that wax D fractions of human strains of *M. tuberculosis* were peptidoglycolipids, whereas the corresponding wax D fractions of other strains were simply glycolipids (Asselineau and Lederer, 1953).

In a recent paper (Jollès *et al.*, 1962) it was reported that fractional ultracentrifugation of ether solutions of wax D at 50,000 *g* gives a series of different fractions. Of these, the fraction remaining unsedimented after 150 minutes of centrifugation (D_s) contains no amino acids, whereas the sedimentable fractions all contain varying proportions of an oligo (probably hepta) peptide containing *meso*-α,α′-diaminopimelic acid, glutamic acid, alanine and sometimes glycine.

The same fractionation method has now been applied to several wax D fractions of

bovine strains of *M. tuberculosis*, of *M. avium* and of saprophytic mycobacteria and found to yield amino acid-free and various amino acid-containing wax D fractions.

The evolution of our concepts of the chemistry of wax D fractions of various mycobacterial strains can be schematically indicated as follows. (Myc) will represent the mycolic acid moiety which is esterified to polysaccharide (poly); (pep) will represent the peptide moiety which if present is covalently linked to the polysaccharide.

Until quite recently it was thought that (myc)–(poly)–(pep) represented wax D of all human strains and (myc)–(poly) represented wax D of all bovine, avian and saprophytic strains. However, from the data given in this communication and that of Jollès *et al.* (1962) it is concluded that in reality wax D of human strains is composed of about 90 per cent of a mixture of (D_p) fractions (myc)–(poly)–$(pep)_n$, where *n* varies from 1 to 3, and 3–8 per cent of (D_s): (myc)–(poly). In other mycobacterial strains (bovine types of *M. tuberculosis*, *M. avium* and saprophytic strains *M. phlei* and *M. smegmatis*, more than 60 per cent of the wax D has the structure (myc)–(poly), whereas 10–30 per cent has attached peptide components and a schematic structure (myc)–(poly)–(pep). The peptide composition of these latter fractions is variable and usually different from that which is characteristic for human type wax D, and in particular *meso*-α,α′-diaminopimelic acid is missing. In one case (wax D of *M. phlei*) 3·9 per cent of the wax D (fraction D_{p35}) was found to contain a peptide with alanine, glutamic acid, *meso*-α,α′-diaminopimelic acid and glycine in molecular ratios closely resembling those of a typical wax D of human type. In two other cases (*M. avium*, D_{p70}) and the atypical photochromogen No. 4 (*Mycobacterium kansasii*, D_{p35}) wax fractions were obtained in small quantity and shown to contain alanine, glutamic acid and *meso*-α,α′-diaminopimelic acid. Of these, wax D of *M. avium* (D_{p70}) was able to induce corneal reactivity to ovalbumin in two of five animals and characteristic epithelioid granulomata locally and in draining lymph nodes. Serum precipitin levels of anti-ovalbumin were considerably above control levels in two animals. Wax D of the atypical mycobacterium No. 4 (D_{p35}) was found to possess definite adjuvant activity (Table 4).

The results of the present investigation therefore provide further examples of the correlation of adjuvant activity with wax D fractions containing a characteristic peptide moiety of alanine, glutamic acid, *meso*-α,α′-diaminopimelic acid and glycine, and all fractions with such adjuvant activity have consistently yielded a similar qualitative and quantitative pattern of amino acids (although some variation is apparent in the molar proportions of glycine).

The results of a previous investigation (White *et al.*, 1958) showed that tests of chemical fractions of mycobacteria other than wax D (wax C, phosphatide, phthiocerol diacetate, cord factor or trehalose dimycolate) failed to yield evidence of adjuvant activity. The results included in Table 5 of various amino acid- or peptide-containing lipids, which all failed to show any evidence of adjuvant activity, reinforce the view that this is a specific attribute of peptidoglycolipids of the type found in wax D of human strains.

The chemical analogy between the hydrosoluble moiety of wax D and the cell wall of mycobacteria has been stressed in previous papers (White *et al.*, 1958; Asselineau and Lederer, 1960). All fractions which were found to possess adjuvant activity in this investigation were also found to contain alanine, glutamic acid and *meso*-α,α′-diaminopimelic acid in molar proportions close to 3:2:1·5–2, and thus have an obvious resemblance to the basic mucopeptides of the cell wall of Gram-positive bacteria (Salton, 1953; Cummins and Harris, 1956; Work, 1957). Wax D fractions that were found to possess adjuvant

activity also contain hexosamines (glucosamine and galactosamine). Both hexosamines and also *meso*-α,α′-diaminopimelic acid are confined to the cell wall in bacterial species such as *Corynebacterium diphtheriae* (Holdsworth, 1952).

Adjuvant-active wax D peptidoglycolipids also resemble bacterial cells walls in the stereochemical configuration of their constituent amino acids, particularly in the presence of D-alanine and D-glutamic acid. The latter are characteristically found in wall preparations from a wide variety of bacterial species, both Gram-positive and Gram-negative (Salton, 1957). Amino acids in the D-configuration are notably inconspicuous among the cytoplasmic components of bacteria. Moreover, D-alanine has been found to act as an essential growth factor for some lactobacilli in the absence of vitamin B_6 (Snell, 1945), and in such experiments almost all labelled D-alanine incorporated by the bacteria appears in the cell walls (Snell, Radin and Ikawa, 1955; Ikawa and Snell, 1956). Similarly, D-glutamic acid is recorded as a typical cell wall component by Ikawa and Snell (1956), Salton (1957) and Ikawa and Snell (1960).

Recently, further evidence of chemical similarity between wax D peptidoglycolipids and the bacterial cell wall has been derived from the chromatographic isolation from the former of the 3-O-carboxyethyl derivative of glucosamine (muramic acid). This amino sugar was first described along with alanine, glutamic acid, α,α′-diaminopimelic acid and glucosamine in the hydrolysis mixture from germinating spores of the *Bacillus* genus (*B. subtilis*, *B. megaterium* and *B. cereus*) (Straing and Powell, 1954; Strange and Dark, 1956a, b).

The chromatographic behaviour of this new amino sugar was identical with that of an unknown amino sugar which Cummins and Harris (1956) had found to be present in the hydrolysates of the wall preparations they had examined. It is of interest that chromatograms prepared by Professor H. Harris at this time (1956) from acid hydrolysates of wax D of the human strain Brévannes by the technique of Cummins and Harris (1956) clearly showed the presence of the 'unknown amino sugar', now identifiable as muramic acid, as well as glucosamine and galactosamine. Recent investigations in both the laboratories involved in this communication have demonstrated by paper chromatography and by quantitative analysis of the hydrolysis products separated on a column of amberlite CG-120, that muramic is generally present in wax D peptidoglycolipids from a variety of human type mycobacteria but is generally absent from wax D fractions of bovine and saprophytic mycobacteria (Jollès *et al.*, 1963; Stewart-Tull and White, 1963). Fig. 1 shows that muramic acid is present in equal amounts with galactosamine, but in lower amounts than glucosamine.

The basic structure of adjuvant-active waxes therefore bears a considerable resemblance to the mucopeptides of the bacterial cell wall. The results of this and previous work (White *et al.*, 1958) show that the hydrosoluble moiety which is obtained by acid hydrolysis of wax D is inactive as an adjuvant (Table 3, WL 57). It has also been found (White *et al.*, 1958) that mycolic acid, methyl mycolate or trehalose di-mycolate are also inactive, which indicates therefore that activity is to be ascribed to the intact mycolic ester of the peptide-linked polysaccharide (or mucopeptide).

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