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Chemical and Biological Basis of Adjuvants

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With 24 Figures

Chapman & Hall Limited · London
Springer-Verlag Berlin · Heidelberg · New York
1973

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ISBN 0-412-10180-7 Chapman & Hall Ltd. London

ISBN 3-540-06308-0 Springer-Verlag Berlin Heidelberg New York

ISBN 0-387-06308-0 Springer-Verlag New York Heidelberg Berlin

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Contents

Introduction and Historical Notes	1
List of Abbreviations	4
Chapter I Substances Exhibiting an Adjuvant Effect: Preparation of "Crude Material"	5
A. Cell Walls of Mycobacteria	5
1. Preparation of Cell Walls	5
2. Composition and Structure of the Walls	5
3. Digestion of Cell Walls by Enzymes	6
4. Biological Properties	8
5. "Bound Wax D"	8
B. Wax D Fractions of Mycobacteria	8
1. Paraffin Oil Extracts	9
2. Extraction of Wax Fractions from Mycobacteria: The Biologically Active Wax D Fraction	9
C. Water-soluble Active Fractions Obtained from Mycobacteria ..	11
1. A Macromolecular, Water-soluble, Immuno-adjuvant Fraction from the Cell Wall	11
2. A Water-soluble, Adjuvant-active "Polysaccharide-peptidoglycan" from the Cells	11
D. Other Substances Showing Adjuvant Activity	11
1. Substances with Known Adjuvant Activity	12
a) Microbial Products as Adjuvants	12
b) Nucleic Acids	12
c) Mineral Substances	13
d) Emulsions	13
e) Surface-active Agents as Adjuvants	14
f) Organic Compounds	16
g) Vitamins as Adjuvants	17
h) Phytohemagglutinin from <i>Phaseolus vulgaris</i>	17
2. Substances which Might Have Adjuvant Activity	17
a) Gram-positive Bacteria	17
b) Fungal and Protozoal Substances	17
c) Viruses	17

Chapter II Detailed Study of the Wax D Fractions and Water-soluble Fractions of Mycobacteria which Exhibit Adjuvant Activity	19
A. Isolation and Purification of Wax D	19
1. Purification of Wax D by Ultracentrifugation	19
2. Purification of Wax D by Acetylation	22
B. Analysis of Wax D	22
1. Elemental Analysis and Melting Points	22
2. Lipidic Part	23
3. Water-soluble Part	28
C. Molecular Weight of Wax D	32
D. Chemical Structure of Wax D	32
1. The Linkage between the Lipidic Moiety and the other Constituents. Isolation of Arabinose Mycolate	34
2. Chemical Structure of the Polysaccharide	34
3. Structural Studies of the Peptide Moiety	34
4. Tentative Structure of Wax D Showing Adjuvant Activity	38
E. Chemical Structure Data Concerning "Bound" Wax D	38
F. Physical and Chemical Data Concerning Water-soluble Fractions	38
1. From Cell Walls	38
2. From Delipidated Cells	39
Chapter III Correlation between Chemical Structure and Adjuvant Activity	40
A. Analytical and Structural Differences between Wax D Fractions with and without Adjuvant Activity	40
1. The Importance of Amino acids and Amino Sugars	40
2. The Lipid and Sugar Moieties of Active and Inactive Wax D Fractions	45
3. Residual Activity of Chemically Modified Active Waxes D Fractions	45
4. Electron Microscope Studies with Active and Inactive Fractions of Wax D	46
5. Comparison of Adjuvant Activity of Wax D, Whole Cells, and Active Mycobacterial Cell Walls	47
6. Structure of Substances Related to Wax D Showing no Adjuvant Activity	48
a) Substances of Mycobacterial Origin	48
b) Microbial Substances not of Mycobacterial Origin	49
c) Synthetic Compounds	50
B. Analytical and Structural Differences between Water-soluble Substances with and without Adjuvant Activity	50

C. Relationship between Chemical Structure, Antigenicity and Adjuvant Activity	50
D. Substances Exerting a Protective Effect against the Adjuvant Activity of Wax D Fractions	51
1. Acetylated Mycobacterial Wax D Fractions	51
2. Mycobacterial Cell Walls and Bacterial Residues	51
3. Lipids	52
Chapter IV Biological Activity of Adjuvants	53
A. Adjuvants and Antibody Synthesis	53
1. Adjuvant Effect on Primary Stimulation Response	53
a) Quantitative Changes	53
b) Qualitative Changes	58
2. Adjuvant Effect on Secondary Response	60
a) <i>Ip-vivo</i> Systems	60
b) <i>In-vitro</i> Systems	62
3. The Relation of Adjuvant Activity to the Enhancement of Anaphylactic Reactions	64
4. Adjuvant Activity on Immune Acquired Tolerance and Spontaneous Immunological Reactivation	66
5. Immune Deviation	68
6. Auto-Antibodies	70
B. Adjuvants and Delayed Immune Reactions	70
C. Adjuvants, Graft Reactions and Tumor Growth	75
D. Adjuvants in Induction of Ascites and Plasmocytomas	77
1. Adjuvants Inducing Ascites	77
2. Adjuvants Inducing Plasmocytomas	77
E. Adjuvants and Interferon Synthesis	77
Chapter V Mechanism of Adjuvant Activity	81
A. General Background	81
1. Quantitative and Qualitative Changes among Serum Components in the Stimulated Host	81
2. Quantitative and Qualitative Changes among the Cell Population in the Stimulated Host	82
3. Neurohormonal Changes Induced by Adjuvants	84
B. Action of Adjuvants on Antigens	85
1. Modification of the Antigenic Properties of the Molecule ..	85
a) Conformational Changes and Adjuvanticity	85
b) Modification of the Net Electrical Charge of the Antigen Molecule	89
c) Special Studies on the Active Site	90

2. Modifications of Antigen-processing by Adjuvants	91
a) Antigen Maintenance <i>in situ</i>	91
b) Modifications of Antigen Localization	92
c) Modification of Antigen Catabolism	93
C. Action of Adjuvants on Immunocompetent Cells	94
1. Action of Adjuvants on Cell Transformation	94
a) Transformation of Phagocytes	94
b) Transformation of Lymphocytes	95
c) Modification of a Nonspecific Repressor-like System ...	97
2. Action of Adjuvants on Cell Multiplication	98
D. Conclusions Concerning the Mechanism of Adjuvant Activity	100
 Chapter VI The Practical Uses of Adjuvants	105
A. Adjuvants and Production of Therapeutic Antisera	105
B. Adjuvants and Active Immunization in Man and Animals	106
1. Alum Adjuvants in Human Immunization	106
2. Oily Adjuvants in Human Immunization	108
3. Use of Adjuvants in Veterinary Medicine	109
C. Tests of Safety and Potency in Adjuvant Standardization	110
1. Safety Tests	110
2. Potency Tests	112
a) Properties of the Antigen	112
b) Dosage of the Adjuvant for a Given Dose of Antigen ..	112
3. Adjuvants and Biological Standardization	114
a) Standardization of the Components of a Vaccine	114
b) Safety Test Standardization	115
c) Potency Test Standardization	115
 Conclusion	117
 Bibliography	119
 Subject Index	149

Introduction and Historical Notes

What are adjuvants? As WHITE [1967 (2)] said, "the list of them reads like a medieval alchemist's shopping list." We would like first to give a clear and simple definition which yet takes into account the complexities of a field where many authors have used the same words for completely different concepts. RAMON (1926), whose primary goal was the enhancement of antibody synthesis against diphtheria or tetanus toxoid, called "adjuvant and immunity stimulating substances" products which, used in combination with specific antigen vaccines, enhance immunity levels above those that the vaccines are capable of developing when injected alone. Even at that early date, he concluded: "Si intéressant que soit ce procédé du point de vue pratique, il ne l'est pas moins du point de vue théorique, à cause des recherches qu'il peut susciter pour essayer de pénétrer le mécanisme intime, soit de l'augmentation d'antitoxine ainsi provoquée, soit de l'élaboration des antitoxines au sein de l'organisme". In other words, RAMON thought that adjuvants could be used as a tool to gain new insights into the mechanism of antibody response. FREUND (1956), without giving any definition, emphasized the different manifestations of adjuvant effect:

- (a) enhancement of antibody formation and alteration of sensitization to proteins;
- (b) sensitization to simple chemical compounds;
- (c) allergy.

Munoz (1964) defined an "adjuvant" as a substance that enhances the antibody response to antigen injected either simultaneously with it or within a period of time close to the injection of the antigen. This meaning is extended to all substances that enhance hypersensitivity reactions that are directly related to antibodies, or suspected to be associated with the antibody response. WHITE [1967 (1)] applied the term "adjuvant" (L. *adjuvare*: to help) only to substances which, when injected together with antigen (i) convert an apparently nonantigenic substance to an effective antigen, (ii) increase levels of circulating antibody, (iii) lead to the production of delayed hypersensitivity or to its increase, and (iv) lead to the production of certain disease states such as thyroiditis, aspermatogenesis, allergic encephalomyelitis, adrenalitis or arthritis and iridocyclitis.

As we have seen from the different definitions, the manifestations of adjuvant effects are numerous, and to the antibody response, delayed hypersensitivity and allergic diseases we could add homograft reactions, some growth processes, induction of plasmacytoma, ascites, and interferon synthesis.

Although an adjuvant may literally help the immune response, we feel compelled to discuss opposite effects too, since such very well-known adjuvants as Freund's adjuvant have been observed to lower the immune response (JANKOVIC, 1963). More recently extensive work has been done on phytohemagglutinin adjuvant activity; some authors have concluded that these heterogeneous substances exert an enhancing effect on the immune response [GAMBLE, 1966 (2); SINGHAL *et al.*, 1967],

while others have observed an opposite effect (MARKLEY *et al.*, 1967; JASIN and ZIFF, 1968).

Therefore the definition of an adjuvant must have two aspects. From a practical point of view, as pointed out by WHITE [1967 (1)], it refers to substances enhancing the immune response, whatever this may be.

From a theoretical point of view it should refer to any substance which acts on (i) the nonspecific part of the antigen, called "adjuvanticity" by DRESSER (1961), and (ii) the nonspecific activity of the cells involved in the immune response (mainly macrophages and lymphocytes) by enhancing cell multiplication or by stimulating cell transformation.

It is obviously difficult to make a clear distinction between such products as (i) phytohemagglutinin, which is able to enhance DNA, RNA and protein synthesis besides possessing blastogenic capacity; (ii) Freund's adjuvant, which stimulates hormonal secretions in addition to its activity on macrophages and lymphocytes; (iii) such hormones as somatotropin or folliculin, which enhance protein synthesis (including synthesis of globulin); and (iv) mitotic drugs, which will not be included as adjuvants. Thus we could define as adjuvants or immunity-stimulating substances *any product which acts (i) on a hapten or an antigen by enhancing its antigenic properties, or (ii) on the cells involved in the immune response (this being understood as including antibody synthesis, anaphylaxis, delayed hypersensitivity, allergic diseases, and graft reactions immunized)*.

RAMON [1925 (1)] noticed a correlation in immunized horses between a local abscess at the site of antigen injection and a high level of antibodies; he demonstrated that it was possible to artificially increase diphtheria or tetanus antitoxin levels by adding substances such as bread crumbs, aleurone seeds, agar, tapioca, starch oil, lecithin.

SORDELLI and SERPA (1925) reported the antigenic value of the precipitate that occurs when diphtheria toxin and specific antitoxin are mixed together. HARTLEY (1952) and GLENNY (1926) pointed out that such a precipitate was antigenic and had a higher immunizing value than the supernatant liquid. Lipovaccines were discovered by LE MOIGNIC and PINOY [1916 (1, 2)]. LEWIS and LOOMIS (1924) observed that antibody formation against various antigens was remarkably intense in guinea pigs which had received an injection of living virulent tubercle bacilli into the peritoneal cavity a few days before they were given antigens. These lipovaccines were also used by RAMON and ZOELLER (1927), WALSH and FRAZER (1934), COULAUD (1935), and SAENZ (1937).

With these observations as a foundation, FREUND (see FREUND *et al.*, 1937) started his classic experimentation on the production of delayed hypersensitivity and on antibody synthesis which showed that the two effects of the allergic irritability due to the tuberculous infection, namely the enhancement of antibody production and the alteration of sensitization, can be reproduced in the absence of tuberculosis (FREUND and McDERMOTT, 1942). FREUND reviewed these observations in 1947 and 1956.

The powerful antibody-stimulating effect of Freund's adjuvant has not been equalled by any other adjuvant. This mixture has made it possible to produce antibodies even in animals considered poor producers, such as rats (HAVAS and ANDRE, 1955) or mice (ANACKER and MUNOZ, 1961; MUNOZ, 1963), and to induce auto-allergic diseases (KABAT *et al.*, 1946; MORGAN, 1946). The next step of the investi-

gations on adjuvant activity was the isolation of active material from various bacteria: *B. pertussis* (ELDERING, 1942), *Salmonella* (RIBI *et al.*, 1959), and *Mycobacteria* (RAFFEL, 1948, 1950; WHITE *et al.*, 1955; LEDERER, 1959). Finally for 10 years scientists have tried to understand the activity of adjuvants on nonantigenic molecules (DRESSER, 1961; SELA *et al.*, 1962) or the activity of adjuvants on the immuno-competent cells (UNANUE *et al.*, 1969; NOWELL, 1960).

The plan of this book is as follows:

- I Study of the substances exhibiting adjuvant activity with special reference to the preparation of a crude material from the *Mycobacteria*.
- II Detailed study of the adjuvant-active waxes D of *Mycobacteria*.
- III Correlation between chemical structure and adjuvant activity.
- IV Biological activity of adjuvants.
- V Mechanism of adjuvant activity.
- VI Practical use of adjuvants.

List of Abbreviations

Ala	Alanine
Glu	Glutamic acid
Gly	Glycine
DAP	Meso- α , α' -diaminopimelic acid
Ara	Arabinose
Gal	Galactose
Glc	Glucose
Lip	Lipid, lipidic moiety
Myc	Mycolic acid
PA	"Nitrogen-containing" moiety (peptide + amino sugar)
Poly	Polysaccharide moiety
HSA	Human serum albumin
BSA	Bovine serum albumin
HGG	Human gamma-globulin
BGG	Bovine gamma-globulin
OA	Ovalbumin
PHA	Phytohemagglutinin
BCG	Calmette and Guérin bacillus (non pathogenic Mycobacteria)
PFC	Plaque-forming cells
EAE	Experimental allergic encephalomyelitis
Poly A:U	Polyadenylic-polyuridylic acid

Substances Exhibiting an Adjuvant Effect: Preparation of "Crude Material"

Many adjuvants, or components of adjuvants, occur among the pharmaceutical emulsifying agents, which are surface-active substances capable of stabilizing an oil-water interface. This seems to be a property common to many adjuvants. They are very often made up of *separate lipophilic and hydrophilic moieties* which is what makes them surface-active. Mycobacteria are among the best adjuvants used and have been widely studied. We deal first with some of their components.

A. Cell Walls of Mycobacteria

Mycobacterial cell walls are known to be adjuvants and to induce delayed hypersensitivity [KOTANI *et al.*, 1960; LARSON *et al.*, 1963 (1)]. They seem chemically and antigenically more complex than other bacterial walls so far studied. The characteristic feature of their chemical composition is their high lipid content, as shown by KOTANI *et al.*, (1959); significant amounts of free lipids can be removed by treatment with hot acetone before chemical disintegration of the cells.

1. Preparation of Cell Walls

Many techniques have been employed for isolating cell walls from *Mycobacterium tuberculosis*. Most of these are based on mechanical disintegration followed by differential centrifugation and treatment with proteolytic enzymes (CUMMINS and HARRIS, 1958; RIBI *et al.*, 1958; RIBI *et al.*, 1959; KOTANI *et al.*, 1959; KANAI and YOUNG, 1960; BELKNAP, CAMIEN and DUNN, 1961). The preparation of cell walls from the following strains has also been described: *M. phlei* (TAKEYA and HISATSUNE, 1963; DE WIJS and JOLLÈS, 1964), *M. butyricum* (LARSON *et al.*, 1963(1)), *M. tuberculosis*, BCG [LARSON *et al.*, 1963 (1); MISAKI *et al.*, 1966], *M. fortuitum* and *M. kansasii* (DE WIJS and JOLLÈS, 1964), *M. smegmatis* (PETIT *et al.*, 1969).

2. Composition and Structure of the Walls

(DE WIJS and JOLLÈS, 1964; MISAKI *et al.*, 1966; PETIT *et al.*, 1969)

The analyses of different cell wall preparations are presented in Table 1. Walls from undefatted cells had high lipid (48.7%) and low nitrogen (5.36%) contents compared with those from defatted cells.

1. *Amino acids*. The main components of purified cell walls are: Ala, Glu and DAP (8 to 12%); for most cell walls the molar ratios are approximately 3:2:2,

respectively (Table 2). They are very similar to those found in different waxes D of Mycobacteria.

2. *Amino sugars*. The major amino sugars found in the mycobacterial cell walls are N-acetylglucosamine and N-acetylmuramic acid; the molar ratios are nearly 1:1 (Table 2). Traces of galactosamine have been detected in the cell walls of *M. kansasii*.

3. *Reducing neutral sugars*. Chromatography of the hydrolyzed cell walls revealed that the main sugar constituents were arabinose and galactose (Table 1). In some cases, glucose and traces of mannose could be detected.

4. *Chemical structure studies*. (See for comparison Chap. II, part D). PETIT *et al.* (1969) studied the structure of the cell walls of *M. smegmatis*. A tripeptide Ala-Glu-

Table 1. Composition of cell walls of several Mycobacteria
(DE WIJS and JOLLÈS, 1964; MISAKI *et al.*, 1966)

	Bound lipids %	Neutral reducing sugars ^a %	Amino ^b sugars %	Amino acids	
				Ala, Glu, DAP %	Others %
<i>M. tuberculosis</i> var. <i>bovis</i>	31.0	31.2 ^c	2.58	^d	
BCG					
<i>M. phlei</i>	14.0	9.8	3.39	8.00	2.05
<i>M. smegmatis</i>	33.0	30.0 ^e	8.5	12.4	
<i>M. fortuitum</i>	24.0	11.9	1.67	3.65	1.32
<i>M. kansasii</i> No. 4	31.3	25.3	4.65	10.28	1.38

^a Calculated as galactose.

^b Calculated as glucosamine, HCl.

^c Molar ratios: Gal (1.00); Glc (0.71); Ara (2.12).

^d Nitrogen content of amino acids present in the walls: 3.71%.

^e Molar ratios: Gal (1); Ara (2).

DAP, a tetrapeptide Ala-Glu-DAP-Ala, and also a tetrasaccharide and a disaccharide which gave equimolecular amounts of glucosamine and muramic acid after hydrolysis, were obtained. The disaccharide can be distinguished from the typical β -1,4-N-acetylglucosaminyl-N-acetylmuramic acid by its R_f value in different solvents, as the muramic acid residue is N-glycolylated, instead of N-acetylated (ADAM *et al.*, 1969). The peptide subunits of mycobacterial cell walls were identified by mass spectrometry by WIETZERBIN-FALSZPAN *et al.* (1970).

3. Digestion of Cell Walls by Enzymes

Lysozymes (EC 3.2.1.17) of different origins (bird egg-whites, human milk) are able to digest mycobacterial cell walls [KOTANI *et al.*, 1963 (1); DE WIJS and JOLLÈS, 1964; ADAM *et al.*, 1972]. Other enzymes, such as the L_{11} enzyme separated from culture supernatants of *Flavobacterium* spp. and pronase, also attack these cells. (See also preparation of adjuvant active "bound wax D", Section 5.)

Table 2. Content of major amino acids and amino sugars of mycobacterial cell walls (DE WIJJS and JOLLÈS, 1964)

Amino acid or amino sugar	<i>M. phlei</i>		<i>M. fortuitum</i>		<i>M. kansasii</i>		<i>M. smegmatis</i>	
	$\mu\text{mole/mg}$	Molar ^a ratio	$\mu\text{mole/mg}$	Molar ^a ratio	$\mu\text{mole/mg}$	Molar ^a ratio	$\mu\text{mole/mg}$	Molar ^a ratio
Ala	0.263	3.0	0.10	3.0	0.327	3.0	1.6	1.6
Glu	0.19	2.2	0.10	3.0	0.251	2.3	1.0	1.0
DAP	0.142	1.65	0.055	1.65	0.179	1.65	1.0	1.0
Gly	0.027	0.31	0.032	0.9	0.038	0.35	tr.	tr.
Glucosamine	0.076	0.87	0.035	0.98	0.079	0.71	1.0	1.0
Muramic acid	0.06	0.70	0.032	0.90	0.095	0.87	1.0	1.0
Galactosamine	0	0	0	0	0.01	0.09	—	—

^a Based on alanine = 3.0.

4. Biological Properties

WHITE *et al.* (1958) showed that delipidated bacterial cells of bovine, avian and saprophytic strains of *Mycobacteria* have high adjuvant activity. Similar observations were made by different authors and especially by MISAKI *et al.* (1966); the BCG-cell wall preparations were able to increase the resistance of mice to staphylococcal infection to a value as high as that of the intact BCG cells. Both the periodate-degraded walls and the mucopeptide fraction elicit in mice a resistance to staphylococcal infection similar to that produced by the original cell walls. This observation of MISAKI *et al.*, (1966) clearly indicates that the mucopeptide is responsible for the protective effect of BCG cells. In another experiment, the mucopeptide fraction was shown to produce tuberculin-type hypersensitivity when injected intradermally into guinea pigs sensitized with killed BCG. However, skin tests in guinea pigs showed that the mucopeptide fraction failed to induce the delayed hypersensitivity. This result, together with analytical data concerning the amino acid composition, suggested to MISAKI *et al.* (1966) that the delayed hypersensitivity elicited by the cell walls is due mainly to a protein moiety associated with the mucopeptide. This suggestion is not in accordance with the demonstration that "bound wax D" is a cell wall constituent which can produce a delayed type of hypersensitivity [KOTANI *et al.*, 1963 (1)]. BONHOMME *et al.* (1969) were able to induce experimental arthritis (termed adjuvant arthritis) in rats with cell walls of *M. tuberculosis var. hominis*, strain Test, of *M. tuberculosis var. bovis*, strain BCG and of *M. avium*.

5. "Bound Wax D"

[KOTANI *et al.*, 1963 (2)]

BCG cells were extracted with neutral organic solvents at room temperature to obtain "delipidated" BCG cells; these latter were submitted to successive treatments with hen egg white lysozyme and the L_{11} enzyme produced by *Flavobacterium* spp. These enzyme treatments rendered about 40% of the cell wall material soluble. Solubility tests in organic solvents and various physical and chemical determinations (see Chap. II, Part E) demonstrated that the insoluble residue consisted mainly of materials essentially identical to the wax D fractions isolated from human type *M. tuberculosis*. The residue was therefore designated "bound wax D", and its adjuvant activity was 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 with egg white albumin or hen egg white lysozyme into guinea pigs.

B. Wax D Fractions of *Mycobacteria*

There are two possible interpretations of the chemical nature of the adjuvant component: (1) the adjuvant activity is related to a chemically well-defined substance which is present in different amounts in different bacterial cells; (2) the difference in the adjuvant activity of the assayed bacteria is due to differences in the chemical structure of the active principle. Only *Mycobacteria* have been intensively studied as regards point (2). We shall now try to establish whether the adjuvant activities of different strains of *Mycobacteria* can be related to a well-defined compound.

1. Paraffin Oil Extracts

COULAUD (1934) and SAENZ (1939) observed that paraffin oil extracts of dead cells of Mycobacteria possess some adjuvant activity. It was thus suggested that some lipids may be involved in this activity. The main work in this area was done by CHOUKROUN (1939, 1946, 1947, 1948), who described the preparation of a biologically active fraction called "Pmko"; according to ASSELINEAU, CHOUKROUN and LEDERER (1950), this fraction was a mixture containing mycolic acids (45%) and nitrogen- and phosphorus-rich lipopolysaccharide (55%).

Paraffin oil extraction of BCG cell walls also gave [LARSON *et al.*, 1963 (2)] biologically active extracts.

RAFFEL (1948) found that bacilli which had the lipids removed by chloroform extraction lost the ability to induce tuberculin hypersensitivity, although the protein antigenicity of the treated bacillary bodies remained intact. By adding extracted constituents to these defatted bacilli, it was found that a fatty substance obtained by ANDERSON (1941) as "purified wax fraction" was essential for the sensitizing process.

2. Extraction of Wax Fractions from Mycobacteria: the Biologically Active Wax D Fraction

Recent studies intended to define the active principle of Mycobacteria have used materials prepared by prolonged extraction with neutral organic solvents of cultures usually 4 to 6 weeks old. Extraction with an ethanol-ether mixture followed by chloroform, in accordance with the procedure developed by ANDERSON (1927, 1929, 1941), yields a series of waxy materials. ASSELINEAU and LEDERER (1953) described the preparation of four waxy substances referred to as waxes A, B, C, and D (Table 3). Wax A, which contains mainly phitioceryl dimycocerosate, is soluble in a mixture of alcohol-ether (1:1, v/v) and can be separated from waxes B, C and D, which are soluble in chloroform. Wax B can be separated as indicated in Table 3; it is also soluble in cold acetone. It is mainly composed of phitioceryl, glycerol and esters of fatty and mycolic acids. As wax D remains insoluble in boiling acetone, it can finally be separated from wax C, which is soluble in this solvent; wax C contains a high proportion of "cord factor", which is trehalose dimycolate (NOLL *et al.*, 1956) (Fig. 1).

RAFFEL (1950) demonstrated that wax D from the human strain Test of *M. tuberculosis*, when mixed with tuberculo-protein, induces tuberculin hypersensitivity. When wax D was isolated, it was hoped that the adjuvant active principle of Mycobacteria had been obtained. However, WHITE *et al.* (1958) established that only wax D fractions from human strains of *M. tuberculosis* possessed this activity, *i.e.* the ability to induce delayed hypersensitivity to a protein antigen (ovalbumin) and to produce allergic encephalitis. Many mycobacterial extracts, other than wax D, were tested and found inactive.

These observations made it necessary to further purify wax D from Mycobacteria, called at this stage "crude wax D" (Chap. II, part A). Later, wax D from other strains of *M. tuberculosis* (var. *bovis*) [MIGLIORE and JOLLÈS, unpublished data, 1969 (2); MIGLIORE *et al.*, 1971] and other Mycobacteria (*M. kansasii*) (WHITE *et al.*, 1964) were also found to be active (see Chap. III).

Table 3. Extraction of waxes A, B, C and D based on the work of ASSELINÉAU and LEDERER (1953)

