DETECTION OF SPECIFIC LIPIDS IN MYCOBACTERIA BY INFRARED SPECTROSCOPY[1]

D. W. SMITH, H. M. RANDALL, A. P. MacLENNAN,[2]
R. K. PUTNEY,[3] AND S. V. RAO

Department of Medical Microbiology, University of Wisconsin, Madison, Wisconsin, and H. M. Randall Laboratory of Physics, University of Michigan, Ann Arbor, Michigan

Received for publication July 14, 1959

The chemical complexity of the lipids of mycobacteria has been well established by previous investigators. The pioneering studies of Anderson (1943) demonstrated the large number of compounds present in the tubercle bacillus and the unusual nature of some of them. More recent contributions are reported by Asselineau (1952) and Asselineau and Lederer (1953). Although most of the studies have concerned the lipids of a relatively small number of strains of *Mycobacterium tuberculosis*, comparisons have been made with *Mycobacterium bovis* and *Mycobacterium avium*. Anderson (1943) found phthiocerol to occur only in human and bovine strains of tubercle bacilli and Asselineau and Lederer (1953) state that certain lipopolysaccharides of human strains contain alanine, glutamic acid, and α,ϵ-diaminopimelic acid, whereas these amino acids do not occur in the lipopolysaccharides of bovine strains and saprophytes. Mycolic acids of human and bovine strains differ from those of saprophytic strains (Asselineau and Lederer, 1955).

These studies of limited numbers of strains of human, bovine, and avian species have been hampered because of their dependence upon classical methods of purification and analysis, but still suggest that more extended investigation might result in the discovery of additional characteristic lipids in these strains and others.

In recent years techniques have been introduced which provide a better means of separating the complex mixtures of lipids together with a rapid means of analysis. Adsorption chromatography has been used by Asselineau and Lederer (1949), Bloch *et al.* (1953), and Randall and Smith (1953) to separate the lipids of the tubercle bacillus into simpler mixtures and compounds. Randall *et al.* (1952) and Noll and Bloch (1953) have used infrared spectroscopy as a means of analysis of these lipid substances.

In a previous report (Smith *et al.*, 1957), results of a study of a small number of strains of acid-fast bacilli including human, bovine, and avian species, saprophytes, and representatives of the atypical group of mycobacteria[4] are summarized. This work based on a small number of strains indicated that the major categories of mycobacteria could possibly be distinguished from each other by the presence of one of a series of lipid substances characterized or identified by their infrared spectra. It was shown that *M. tuberculosis* could be identified by the presence of phthiocerol dimycocerosate (DIM), a compound reported previously by Noll (1957), and *M. bovis* could be identified by the presence of this compound and a component identified as Gb. Further, it was shown that *M. avium* could be tentatively characterized by the presence of a substance designated J which was also found in one of the groups of atypical mycobacteria, the nonphotochromogens. Another spectroscopically characteristic

[1] This work was supported in part by grants from National Institutes of Health, U. S. Public Health Service (E-1948), Research Corporation, Wisconsin Antituberculous Association, Washtenaw County, Michigan Tuberculosis Association, and Faculty Research Fund, University of Michigan.

[2] On leave (now returned) from Microbiological Research Establishment, Porton, Wiltshire, England.

[3] Present address: Glens Falls, New York.

[4] The atypical group of mycobacteria was subdivided by Timpe and Runyon (1954) on the basis of pigment formation *in vitro* into the photochromogens, scotochromogens, and nonphotochromogens. Atypical acid-fast bacilli of this general group are of interest because of their relatively frequent isolation from patients under conditions that indicate they are responsible for a disease that resembles clinical tuberculosis.

substance, designated Ga, was found to be present in the lipids of all four cultures of photochromogenic atypical mycobacteria studied.

One facet of this work which may have practical value concerns the identification of attenuated strains of mycobacteria by means of their specific lipid content. At the present time, the most widely accepted means of differentiating between the various groups of mycobacteria is on the basis of their virulence for various laboratory animals. If a strain becomes attenuated either purposefully as in the case of BCG (bacillus Calmette-Guèrin) or through chance loss of virulence on laboratory media, then it would appear that some other method of identification must be used. Strain identification by specific lipid content would be most valuable for an acid-fast culture isolated from an individual who had been vaccinated with BCG. A second illustration could be cited in the study by Feldman *et al.* (1943) who reported the isolation of an unusual mycobacterium from the sputum of a man suffering from pulmonary disease of long duration. This organism repeatedly failed to produce tuberculosis in guinea pigs and virulence studies in chickens were at first positive and then later negative.

The purpose of this paper is to report the study of lipids of a larger number of cultures (70 strains) and to report on progress of the characterization of some of the specific substances. In the present work infrared spectroscopy has been used in the following ways: (a) to detect the presence of unidentified substances, even when these are present in an impure state; (b) as an aid to the interpretation of chemical structure; and (c) to identify substances by comparing their spectra with those of materials of known composition.

MATERIALS AND METHODS

Bacteria. The strains of mycobacteria examined are listed in table 1 together with data on their nature and source.

Cultivation of bacteria. Mass cultures of each strain to be examined were prepared on the chemically defined medium of Wong and Weinzirl (1936). Usually 20 flasks (1-gallon milk bottles, later 1-gallon pyrex bottles) were inoculated and were incubated from 3 to 8 weeks depending upon the growth rate of the organism.

Extraction of lipids. When the cultures were ready for harvesting, the medium was withdrawn by means of a water aspirator pump and was subsequently autoclaved and discarded. The cells were transferred in ethanol to a single flask, an equal volume of diethyl ether was added, and the extraction was continued for 48 hr on a magnetic stirrer. The cells were allowed to settle, the supernatant solvent was withdrawn and replaced by 2 L of fresh ethanol-ether, and the second extraction was continued for 24 hr. Three additional 4-hr extractions with fresh solvent were made.

The combined ether-ethanol extracts were evaporated under water aspirator evacuation on a water bath at 40 C. The residue was partitioned between water and ether and the water layer was washed repeatedly with ether to remove as much lipid as possible. The combined ether extracts were dried over anhydrous magnesium sulfate and filtered through a Seitz clarifying pad under positive pressure. The filtrate was then evaporated to dryness and the deposited lipid (20 to 30 per cent of the dry bacillary weight) was stored at 5 C for chromatography.

Preparation of Materials for chromatography. (1) Adsorbents:—Magnesol (magnesium trisilicate, Westvaco Chemical Company, South Charleston, West Virginia) was usually mixed with Celite (Analytical Filter Aid, Johns Manville Company) in the ratio of 2 parts by weight of Magnesol to 1 part of Celite. The mixture was washed thoroughly with boiling distilled water until the wash water was about pH 7. Then later it was washed with methanol followed by diethyl ether. The washed, air-dried adsorbent was activated at 150 C for 30 min, immediately before use.

Silicic acid (analytical reagent grade 100 mesh, Mallinckrodt Chemical Company) washed successively with methanol and chloroform and dried at 110 C.

Hyflo-Super Cel (Johns Manville Company) washed with methanol and chloroform and dried at 100 C; mixture of 5:2 silicic acid-Hyflo Super Cel was used.

Alumina. Aluminum oxide, ignited powder (Baker and Adamson Chemical Company) was washed with methanol and then with chloroform, dried at 100 C and columns were packed with dry adsorbent.

(2) Solvents:—Hexane (technical grade normal hexane, Phillips Petroleum Company,

TABLE 1

Strains of mycobacteria and their sources

Strains	Nature	Source
Mycobacterium tuberculosis		
17 cultures	Virulent	Hospitals and laboratories in Michigan, Wisconsin, Ohio, France, England, and Scotland
H37Rv, 4 cultures	Virulent	Runyon, Salt Lake City, Utah
		Steenken, Trudeau Lab.
		Vandiviere, Chapel Hill, N. C.
		Stimpert, Park-Davis and Co.
H37Ra, 3 cultures	Avirulent	Vandiviere
		Steenken
		Runyon
R1Rv, 2 cultures	Attenuated	Vandiviere
		Runyon
Brevannes	Virulent	Noll, Pittsburgh, Pa.
Pearson	Virulent	Middlebrook, Denver, Col.
Mycobacterium bovis		
3 cultures	Virulent	U. S. Dept. of Agriculture
		Schaefer, Denver, Col.
BCG, 3 cultures	Attenuated	Lurie, Phipps Institute
		Birkhaug, New York Health Dept.
		Rosenthal, Tice Laboratories
Ravenel strain	Virulent	Runyon
Mycobacterium avium		
Kirschberg strain	Virulent	Runyon
4 cultures	Virulent	Feldman (Mayo)
1 culture	Virulent	Ellis, Animal Disease Lab., Madison, Wisc.
Atypical Acid-fast Bacilli		
Photochromogens		
3 cultures		Hall, Minneapolis, Minn.
2 cultures		Pollak, Kansas City, Kan.
9 cultures		Runyon
1 culture		Frenkel, Kansas City, Kan.
1 culture		Hobby, Pfizer, Brooklyn, N. Y.
1 culture		State Lab. Hygiene, Madison, Wisc.
Scotochromogens		
3 cultures		Runyon
Nonphotochromogens		
9 cultures		Runyon
1 culture		Hall
1 culture		Feldman
Saprophytes		
Mycobacterium phlei, 2 cultures		Gordon, Rutgers
Mycobacterium phlei, 1 culture		Runyon
Mycobacterium smegmatis		Gordon
Miscellaneous		
Mycobacterium marianum		Sister Marie Suzanne, Lyon, France
Mycobacterium sabotier		Sister Marie Suzanne, Lyon, France

Bartlesville, Oklahoma) was washed with concentrated sulfuric acid, 10 per cent KOH, and with water and then redistilled, and stored over metallic sodium before use.

Benzene (analytical reagent grade, Mallinckrodt Chemical Company) was redistilled and stored over metallic sodium before use.

Ether (reagent grade, Baker and Adamson Chemical Company) was stored over metallic sodium before use.

Methanol (reagent grade, Merck) was used without preliminary treatment.

Chloroform (reagent grade, Merck) was used without preliminary treatment.

Chromatography. Columns were developed with a series of solvents of increasing polarity and for this reason were packed as a slurry of adsorbent with the least polar solvent of the series to be used. The lipid charge was dissolved in the smallest possible volume of the same solvent and the column was developed with further solvent until the eluate no longer contained a significant quantity of lipid. At this point the second solvent in the series was applied to the column and the development continued.

Magnesol-Celite columns were invariably used for the preliminary fractionation of lipid extracts and the usual conditions were as follows. Column diameter 50 mm, length 180 mm, 85 g of adsorbent, 2 g of ethanol-ether extracted lipid in mixture of equal parts hexane and benzene, 250-ml eluate fractions and normally 1800 ml of each solvent or solvent pair was used. The sequence of solvents was as follows: equal parts hexane and benzene, benzene, benzene containing 5 per cent ether, benzene-ether, equal parts, ether containing 1 per cent methanol, ether-5 per cent methanol, ether-20 per cent methanol, and ether-1 per cent glacial acetic acid.

The eluate fractions were dried down by warming in a stream of filtered air, and the infrared spectrum of each eluate was recorded.

Infrared spectroscopy. The spectrograph used was the double-beam Perkin-Elmer model 21. This instrument records the spectrum from 2 to 15 μ on sheets of paper 8½ by 11 in with a linear scale of 2 cm per μ. This permits the measurement of the positions of sharp absorption bands with an accuracy of ± 0.02 μ, however, the measurements of these spectra are seldom made. Ordinarily the sample was spread evenly on one salt plate and pressed by a second plate to a thickness to give optimum resolution. If the sample was fluid, shims were used between the salt plates to give a film of the right thickness. At present the mass of sample for a satisfactory spectrum may be as small as 0.5 mg.

Purification. When indicated, samples of material were rechromatographed on silicic acid or alumina columns to remove impurities. Further purification of the material was done whenever possible by crystallization to constant melting point

An additional test of purity applied more recently was the use of Fiberglas paper impregnated with silicic acid or alumina. The paper was impregnated with silicic acid according to the method described by Dieckert and Rieser (1954) with some modifications, or with alumina by the method described by Bush (1952) again with modifications. Narrow strips of impregnated paper were used in 1 by 8 in test tubes and the material to be tested was spotted about 1 in from the end of the paper and developed by ascending technique in various solvents chosen according to the behavior of the compound on the column. The spots were detected by spraying with dilute sulfuric acid (50 per cent, v/v) and charring on a hot plate. A substance was considered to be at a reproducible state of purity if it satisfied the criterion of traveling as a single spot in several solvent systems. If it was desirable to record the infrared spectrum of a substance separated by Fiberglas paper chromatography, the following procedure was used: a 10 mg charge was applied on a thin line 1 in from the bottom of the paper (4 by 12 in) and developed by ascending technique in a suitable solvent. The bands were located by cutting a thin strip out of the center of the sheet and charring it as described earlier. The bands were cut out and eluted with polar solvents. The samples were dried and their infrared spectra recorded. The band showing the presence of the largest amount of material was usually found to represent the pure compound.

This technique of using large strips (4 by 12 in) and eluting the bands was found to provide an effective means of purifying small amounts (10 to 20 mg) of material.

RESULTS

Nonspecific lipids. Analysis of over 70 strains of mycobacteria has shown that many of the lipids found are common to all strains, that is, they are of widespread occurrence among the different groups of mycobacteria. Among these substances are fatty acids, representatives of the mycolic acid family of compounds, "cord factor," and the so-called "A," "B," "C," and "D" substances reported previously (Kubica *et al.*, 1956;

TABLE 2

*Distribution of lipid substances in various strains of mycobacteria**

Substance	Nature	Distribution	Frequency of Detection
F	Esters of fatty acids	Most strains	
DIM	Phthiocerol dimycocerosate	Human strains	28/30
		Virulent bovine strains	4/4
A	Triglyceride	Most strains	
B	Triglyceride	Limited but sporadic	
C	Diglyceride	Most strains	
Gb	Glycolipid	Virulent bovine strains	4/4
		BCG strains	3/3
Ga	Glycolipid	Photochromogens	17/17
D	Monoglyceride	Most strains	
Fatty acids	—	Most strains	
Jabs	Lipopeptide	Avian	
		Nonphotochromogen	
		Scotochromogen	
Jav	Glycolipid peptide	Avian	4/6
		Nonphotochromogens	3/6
Jat	Glycolipid peptide	Scotochromogens	3/3
Cord factor†	Trehalose-6,6-dimycolate	Many strains	
Mycolic acid		Many strains	

* Arranged in the order of their elution from a Magnesol column.

† Spectroscopically similar substances, presumed to be analogues, are included.

Smith *et al.*, 1957). Table 2 records the strain distribution of these various substances. For simple fatty acids, mycolic acids and cord factor, identification was based on comparison of their infrared spectra with spectra of authentic materials.

A limited chemical study of nonspecific lipids has been made. "A" and "B" have been tentatively shown to be triglycerides but the identification of the constituent fatty acids has not been made. "C" has been shown by Noll and Jackim (1958) to be a diglyceride and is tentatively identified as 1-stearo-3-palmitin. "D" has been shown by Noll and Jackim (1958) to be a 1-monoglyceride of stearic or palmitic acid. Further work in our laboratory has shown "D" to be a monoglyceride of palmitic acid. The infrared spectrum of the fatty acid and its mobility on reverse phase paper chromatography were identical with authentic palmitic acid. The alcohol was identified as glycerol on the basis of infrared spectrum. The melting point (mp) of "D" (70 to 71.5 C) suggests this compound to be 2-monopalmitin.

Specific lipids. Attention has been concentrated on those substances which show a characteristic strain distribution, so far six in number. The spectra of these substances (figure 1) indicate chemical compositions quite different from those of the nonspecific lipids described above. The distribution of these substances can be seen in table 2.

Phthiocerol dimycocerosate. This compound has been detected in the lipids of 28 out of 30 human and virulent bovine strains of mycobacteria. The exceptions are the Brevannes strain and the Pearson strain. The lipids of the Pearson strain contain another substance having a similar chromatographic behavior to phthiocerol dimycocerosate.

The structure of the phthiocerol part of the molecule has been determined with some degree of certainty by Demarteau-Ginsburg and Lederer (1955) and Hall and Polgar (1954).

In the present study, identification of phthiocerol dimycocerosate has been based on the identity of spectra with the spectrum of an authentic sample of the compound. Saponification of a sample of this compound obtained from one of the human strains yielded an acid whose spectrum was identical to mycocerosic acid, and an alcohol whose spectrum was that of phthiocerol:

Phthiocerol, found: C, 76.81 per cent; H, 13.68 per cent; mp, 72 to 73 C (reported, 72 to

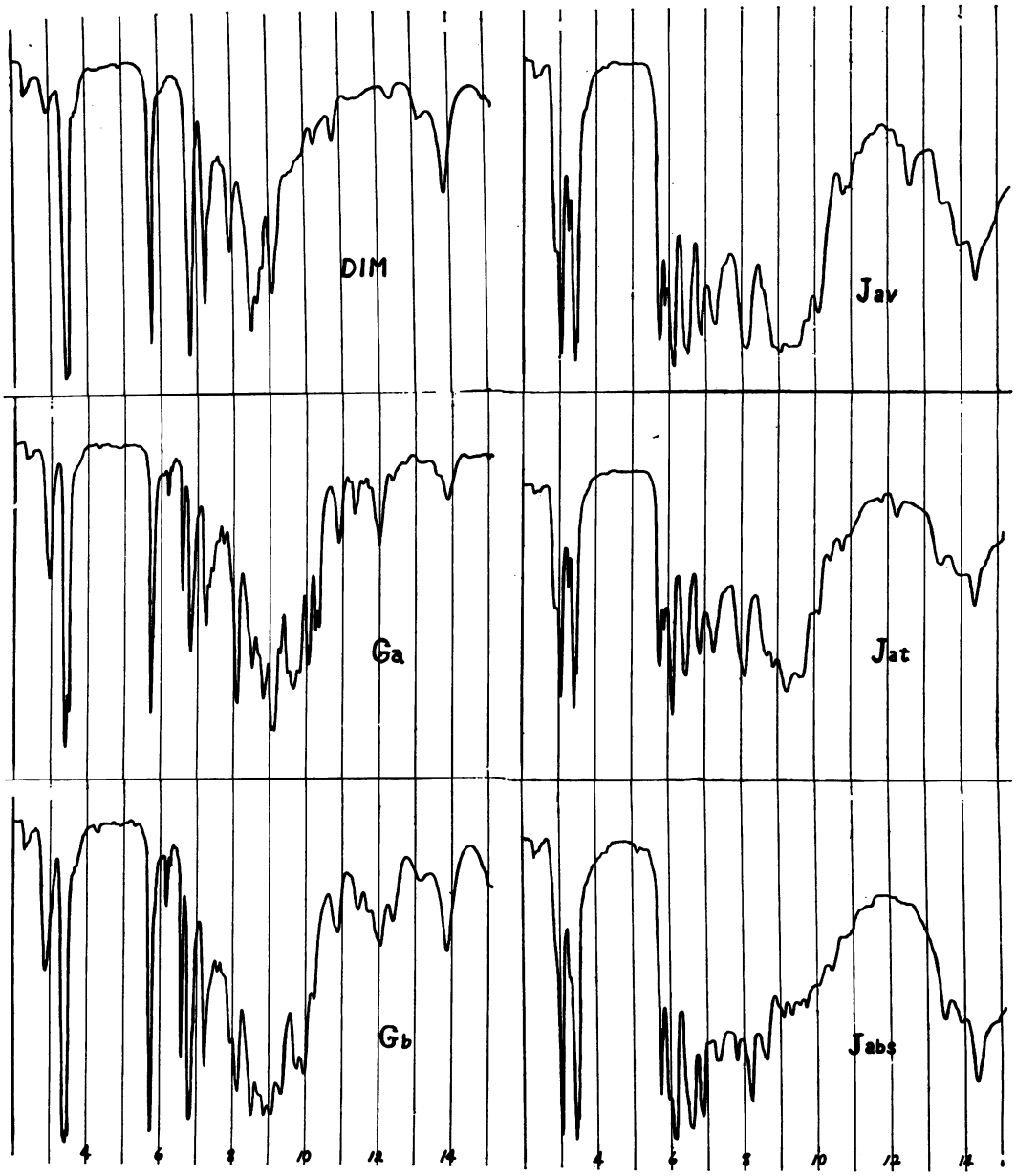

Figure 1. Infrared spectra of group specific lipids and glycolipids identified in extracts of various types of mycobacteria. DIM, phthiocerol dimycocerosate—present in lipids of human strains and bovine strains (except BCG); *Ga*, glycolipid present only in the lipids of photochromogenic atypical acid-fast bacilli; *Gb*, glycolipid present only in the lipids of bovine tubercle bacilli, both virulent and BCG strains; *Jav*, glycolipid peptide present in the lipids of some avian strains and atypical acid-fast bacilli of the nonphotochromogenic variety; *Jat*, glycolipid peptide present in the lipids of some scotochromogenic atypical acid-fast bacilli; and, *Jabs*, lipopeptide found in some of the strains shown to contain either Jat or Jav.

TABLE 3

Analytical data for glycolipids Ga and Gb

	C	H	N	P	Methoxyl	Melting Point	α_D^{20}	Ultraviolet Absorption
	%	%	%	%	%	C		mμ
Ga	72.6	10.8	<0.1	None	8.6	109 ± 1	37.2	275; 282
Gb	77.1	12.3	<0.1	None	3.96	23 ± 1	25.7	275; 282

75 C). Calculated ($C_{35}H_{72}O_3$): C, 77.71 per cent; H, 13.42 per cent.

Mycocerosic acid, found: C, 79.57 per cent; H, 13.25 per cent; mp, 32 to 36 C (reported, 28 to 36 C); mol wt, 459. Calculated ($C_{30}H_{60}O_2$): C, 79.57 per cent; H, 13.35 per cent; mol wt, 452.8.

Saponification of phthiocerol dimycocerosate yielded 2 parts of the acid for each part of the alcohol. Phthiocerol dimycocerosate was ordinarily found in late hexane-benzene eluates or early in the benzene eluates and represented up to 10 per cent of the charge.

G Substances. Ga is found in the atypical group of mycobacteria classified as photochromogens. Thus far Ga has been identified in the lipids of 17 strains, where it usually appeared in the early ether-1 per cent methanol eluates and represented about 5 per cent by weight of the charge.

Gb is found only in strains of the bovine species of tubercle bacillus. In virulent strains it is accompanied by the presence of phthiocerol demycocerosate but in BCG strains, Gb is the only specific lipid substance recognized. Gb is usually eluted in benzene-ether (1:1) and may represent from 1 to 5 per cent of the charge.

The structures of Ga and Gb are not known but the sharp bands at 6.2 and 6.6 μ of their infrared spectra, and their ultraviolet absorptions at 275 and 282 mμ, suggest that an aromatic ether is a constituent common to both.[5]

Table 3 presents analytical data for the G substances prepared by purification of the crude materials on alumina and silicic acid columns.

Saponification of Gb with benzene-methanolic KOH or Ga with KOH in moist isopropanol (Noll and Bloch, 1955) yields an acid with infrared spectrum identical to mycocerosic acid (prepared by hydrolysis of phthiocerol dimycocerosate). Analysis supported this conclusion (Ga acid, found: C, 79.78 per cent; H, 13.3 per cent; mol wt, 465. Gb acid, found: C, 79.73 per cent; H, 13.18 per cent; mol wt, 467.5. Expected: C, 79.57 per cent; H, 13.35 per cent; mol wt, 452.8).

In addition to mycocerosic acid and an aromatic ether, the G compounds contain carbohydrate. Acid hydrolysis of 20 to 50-mg samples in sealed ampoules (1 N HCl, 16 hr, 100 C) followed by removal of lipid components by $CHCl_3$ extraction left a water soluble residue in which sugar components could be detected by conventional paper chromatography using a variety of spray reagents. In three different solvent systems the hydrolyzed Ga material gave 3 spots when sprayed with *p*-anisidine hydrochloride, whereas Gb formed only 1 of these spots. The R_f values of the G sugars were all greater than that of rhamnose but the strongly positive reaction given in the H_2SO_4-cysteine test (Dische and Shettles, 1948) by samples of the sugars eluted from chromatograms suggest that they are indeed derivatives (probably O-methylated derivatives) of one or more of the 6-deoxyhexoses. A detailed consideration of these sugars will be presented elsewhere. The physical properties of the G substances, their elementary analysis, and the evidence provided by paper chromatography of acid hydrolyzates, suggest very strongly that these substances differ in their carbohydrate composition although having lipid components in common.

J Substances. Grounds for the group specificity of the J substances are less certain than in the case of the G compounds and phthiocerol dimycocerosate. Jat is found only in the scotochromogenic group of anonymous acid-fast organisms where it is eluted in ether-methanol-20 per cent and may represent up to 10 per cent of the charge.

Jav is found in avian strains, in some nonphotochromogenic strains, *Mycobacterium marianum*, and in the strain isolated by Feldman *et al.* (1943). Jav is eluted in ether-methanol-20 per cent in amounts equal to about 3 per cent of the charge.

Elementary analysis of Jav gave (C, 66.68 per cent; H, 9.66 per cent; N, 3.71 per cent) and for Jat (C, 65.43 per cent; H, 9.81 per cent; N, 3.95 per cent; mol wt, 1250 ± 125 (Rast)) and acid hydrolysis (1 N or 6 N HCl) liberated amino acids which were detected by paper chroma-

[5] The chemical structure of these compounds are being studied in collaboration with E. Lederer.

tography. Lederer (*personal communication*) has found alanine, threonine, and phenylalanine in material of similar origin and identical infrared spectrum to Jav. Our results agree with Lederer's findings but note that other ninhydrin reacting materials are present in the J substances. Although these other ninhydrin reacting materials are present in smaller amounts, the strikingly quantitative similarity of Jav and Jat hydrolyzates in the intensity of their reactions with ninhydrin suggests that they are not the result of contamination of J preparations with protein. This possibility is rendered even less likely by the demonstration that the amino acid composition of the J substances was unchanged when they were dissolved in $CHCl_3$-butanol (4:1 by volume) and shaken vigorously with water, a procedure known to remove protein under other circumstances (Sevag, 1934).

Hydrolysis with 1 N HCl also liberates sugars from the J substances which in their reactions with *p*-anisidine hydrochloride on chromatograms and with reaction in the H_2SO_4-cysteine reagent, and in their R_f values resemble the sugars of the G compounds. Whereas Jav appears to have at least 3 such sugars, not all of these have been noted in Jat. Therefore it appears that Jav and Jat may bear the same relationship to each other as Ga and Gb, that is, they differ primarily in sugar components. A naturally occurring substance referred to as Jabs which has been found in eluates of columns charged with lipids of avian, nonphotochromogen, and scotochromogen strains, appears to contain the same amino acids as Jav and Jat, but is lacking the sugar components. The spectrum of Jabs resembles the spectra of substances Jav and Jat from which the sugars have been removed.

Factors studied for their effect on the development of specific lipids. (1) Age of culture:—Since it is known that strains of acid-fast bacilli differ in growth rate, ranging from rapid growing saprophytes and *Mycobacterium fortuitum* strains to intermediate photochromogenic bacilli and finally to the slow growing recent isolate human strains, and it is also known that the lipid content does vary with age (Asselineau, 1951), it appeared necessary to investigate the possibility that the specific lipids are only manifestations of the physiological age of the culture and represent different stages in a single biosynthetic process (i. e., phthiocerol dimycocerosate → Gb → Ga).

Mass cultures started at one time were harvested at intervals from 2 to 10 weeks of age and the extracted lipids were subjected to the usual chromatographic procedures. Phthiocerol dimycocerosate was found in the lipids of a virulent human strain (H37Rv) of tubercle bacillus after 3, 6, and 10 weeks of growth, whereas none of the other specific lipids were detected at any time. A culture of the photochromogenic strain P4 (Runyon, see table 1) was harvested at 15, 30, and 45 days and again no difference was seen in the occurrence of specific lipids, that is, Ga and no other specific lipid occurred in all 3 samples.

(2) Medium composition:—In a study following the extraction schema of Aebi *et al.* (1953), the Brevannes strain was cultured on Sauton medium for 6 weeks. Lipids were extracted by ethanol-ether and chloroform and the extracts were chromatographed in the usual manner. Phthiocerol dimycocerosate, a compound heretofore found in all strains of the human variety, could not be detected in any of the column eluates. Cultures of the Brevannes strain and H37Rv, a strain known to produce phthiocerol dimycocerosate on Wong-Weinzirl medium, were then prepared on both Sauton medium and on Wong-Weinzirl medium to determine whether the lipid content varied significantly between two similar chemically defined media. It was found that H37Rv produced phthiocerol dimycocerosate on both Wong-Weinzirl and Sauton medium and that the Brevannes strain did not produce this compound on either medium. Within the narrow limits examined, medium composition did not influence the production of specific compound phthiocerol dimycocerosate.

(3) Light:—Pigment production by photochromogenic cultures is photoactivated and it is largely by this character that the group is defined. The finding that Ga is produced by all photochromogens provides another character by which these organisms may be recognized and its value is greater if it is entirely independent of pigment formation and of photoactivation.

To investigate the possible relationship between pigment production in a photochromogenic strain and the occurrence of the specific lipid Ga, a mass culture inoculated at one time was divided at the beginning of incubation so that culture flasks on alternate shelves in the

incubator were shielded from the light by heavy paper while the intervening shelves were exposed to the usual amount of light coming from a fluorescent fixture in the ceiling. At the time of harvest, the heavy paper was removed from the shelves and a marked difference in pigment was noted. The cultures exposed to light had a definite yellow to orange color, whereas those shielded from the light were nonpigmented and appeared in fact to have less pigment than a human strain cultured in the usual manner.

Ga was found in the lipids of both the "light" culture and the "dark" culture.

(4) Drug resistance:—Comparisons were made of the lipid content of three pairs of human tubercle bacillus cultures, each pair consisting of a parent isoniazid sensitive culture and a post-treatment culture isolated from the same patient and shown to be resistant to isoniazid. Phthiocerol dimycocerosate and no other specific lipid was isolated from each of the six cultures.

(5) Micromethods for detection of specific lipids:—The usual chromatographic procedure used for the study of cultures reported here involves the separation of lipids in a 2-g sample in a column procedure requiring 4 to 6 weeks for completion. Once the specific lipids were recognized it seemed worthwhile to devise methods that would permit an identification using less material and in a shorter period of time.

It has been found that microcolumns (1 by 6 mm) with 2 g of silicic acid (100 to 200 mesh) can be used to detect Ga, i. e. give a good spectrum, in 15 to 30 mg of total lipids within 2 hr.

One of the tests by which the presence of 6-deoxyhexose derivatives in the G and J substances was determined was the reaction of these substances in the cysteine-sulfuric acid test of Dische and Shettles (1948). Since it has been determined that 5 μg of rhamnose (6-deoxy-L-mannose) can be detected in the presence of 250 μg of rhamnose-free lipid (phthiocerol dimycocerosate), it is possible that an adaptation of the Dische and Shettles test may permit the rapid separation of acid-fast organisms into Dische and Shettles positive and Dische and Shettles negative strains. Evaluation of this procedure is presently being made.

DISCUSSION

The first paper in this series appeared in 1951 and described the conditions necessary to obtain reproducible spectra from complex extracts of mycobacteria. (Randall *et al.*, 1951). Since that time numerous reports have appeared describing techniques for the study of various types of bacteria by means of infrared adsorption spectra of the whole cells (Stevenson and Bolduan, 1952; Levine *et al.*, 1953; Bailey *et al.*, 1953; Greenstreet and Norris, 1957; and Haynes *et al.*, 1958).

Others have felt that elimination of the common protein background spectrum would improve the conditions for studying bacteria by infrared spectroscopy (Schneider and McLaughlin, 1955; Levine *et al.*, 1953; O'Connor *et al.*, 1957). In considering the mycobacteria, the advantage of nonprotein extracts as against whole bacterial cells for showing strain differences is illustrated in the paper of Smith *et al.* (1954).

It is recognized that the infrared spectrum of an organic compound is one of its most definitive properties. This project is based upon the use of infrared spectra to discover substances in the lipid extracts of the mycobacteria studied. When the spectra of the eluates of the many columns are studied it is found that some of the eluates will have quite similar spectra. Such eluates are found at common stages of development of their respective columns. Experience has shown that the material of these recurring spectra, when purified, are compounds. Compounds thus isolated which agree in spectra and other physical and chemical properties with known compounds are given the names of those compounds. The other isolates are called A, B, etc., in the order of their discovery. This ability to recognize the eluates containing a compound simply through the recurrence of an infrared spectrum has made it possible to study more than 70 strains of mycobacteria and to isolate numerous compounds from them. Infrared spectra are used also to disclose the functional groups of the isolated compounds and thus to gain some knowledge of their structure. In addition, the spectra are relied upon to follow the various operations to which the substances are subjected during purification and analyses.

With regard to the application of infrared spectroscopy to the identification and characterization of large molecules, one question that has to be kept in mind is what is the magnitude and type of chemical difference that can be detected within the range of spectrum examined and with the resolution commonly employed? One example

of the type of chemical difference that can be detected by infrared spectroscopy is demonstrated in the work of Sinclair *et al.* (1952) and Meikeljohn *et al.* (1957), in which a relationship is shown between the number of methylene groups in long chain fatty acids and esters and a progression of bands in the region 7.5 to 8.5 μ. The latter authors have extended the study to include free fatty acids and soaps (odd and even numbers of carbon atoms) in the series from C_2 to C_{36} and have shown that the number of bands in the progression series is directly related to the number of carbon atoms in the chain. A second example of the type of chemical difference that can be detected by infrared spectroscopy is the recognition of the different polymorphic forms of fatty acids. Sinclair *et al.* (1952) and Chapman (1958) reported difference in infrared spectra of polymorphic varieties of the same acid. An example of a limitation of the use of infrared spectroscopy to characterize large molecules is concerned with the glycerides present in extracts of mycobacteria. These substances (referred to earlier as "A," "B," "C," and "D") may each be a family of closely related compounds, for example "A" may be dipalmitostearin or distearopalmitin, and the spectrum may not be sufficiently distinct to differentiate between them. Glyceride identification did not present a problem in this work because it was not necessary to rely on these compounds to differentiate between any of the groups of mycobacteria.

Another type of problem is due to the variation in the composition of glycerides at different stages in the physiological maturity of the culture and, as is suggested by Lovern (1955), this variation may depend upon the opportunity for fatty acid radicals to migrate from one position to another on the glycerol molecule or from one triglyceride to another. This lability of glyceride structure was another reason for not making a study of this group of compounds.

Recognizing the above limitations, the approach in this research has been to screen a large number of strains for the occurrence of specific substances as revealed by infrared spectra. When found, a simple chromatographic test of homogeneity is applied together with crystallizations when possible and the material submitted to chemical analysis.

Two examples of how a determination of specific lipid content may be useful in relating cultures to a recognized group are as follows: First Suzanne and Penso (1953) isolated from lepromas two cultures, one was called *M. marianum* and the other *Mycobacterium sabotier*. Lipid studies would tentatively indicate that *M. marianum* may be in the nonphotochromogenic or avian group because of the presence of Jav. *M. sabotier* has none of the strain characterizing lipids and for this reason might be considered to belong to the *Mycobacterium phlei* group of cultures. Secondly, Feldman *et al.* (1943) reported the isolation of an unusual mycobacterium from the sputum of a man suffering from pulmonary disease of long duration. This organism repeatedly failed to produce tuberculosis in guinea pigs and virulence studies in chickens were at first positive and then later negative. Jav was isolated from this strain indicating it to be a nonphotochromogen strain, or an attenuated avian strain.

In general the preparatory columns used in this study gave separations of the major classes of lipids and fatty acids. The combination of chromatography on Magnesol-Celite plus examination of column eluates by infrared spectroscopy permitted the recognition of several hitherto unsuspected lipids called Ga, Gb, Jav, and Jat, each of which has been shown to be present in the lipids of a single group of acid-fast bacilli.

Chemical studies completed to date indicate that Ga, the substance found only in photochromogenic atypical bacilli, and Gb, found only in bovine tubercle bacilli, are glycolipids differing from each other only in the composition of the carbohydrate part of the molecule. The sugars of Ga and Gb appear to be *O*-methylated derivatives of 6-deoxyhexoses.

Likewise, Jav, the substance found in nonphotochromogen and avian strains, and Jat, found only in scotochromogen strains, appear to differ from each other only in their content of *O*-methylated derivatives of 6-deoxyhexoses. In addition to these sugars, Jav and Jat contain an unknown lipid moiety and the amino acids, alanine, threonine, and phenylalanine, together with other ninhydrin reacting materials, present in smaller amounts.

The presence of Ga in the photochromogen strains, and including the culture grown in the absence of light, suggests that this substance will be helpful in defining this group of mycobacteria. The recognition of Ga in the lipids of a given strain will become more significant if further

proof can be obtained that photoactivation of pigment production and the presence of Ga are independent properties.

The presence of phthiocerol dimycocerosate in a given culture of mycobacteria would suggest that the strain is either the human or bovine type of tubercle bacillus. The distinction between these two is based on the presence of Gb in bovine strains. Other strains in which Gb is detected but not phthiocerol dimycocerosate have thus far been BCG strains. The two human strains (Pearson and Brevannes) studied thus far that lack phthiocerol dimycocerosate will have to be examined further before significance can be attached to this finding.

Additional work will be required to establish the full significance of the J compounds. Too few strains have been examined and in the case of Jav, certain strains classed as avian or nonphotochromogens seem not to have detectable amounts of this material in the ethanol-ether extractable lipids. It should be emphasized that in general only the ethanol-ether extracted lipids were examined in this work. Additional specific lipid substances may be present in the $CHCl_3$ extracts.

The occurrence of sugars in the G and J substances suggests the possibility of serological specificity for these materials. Parlett *et al.* (1958) by means of an agar double diffusion precipitation technique have already shown the possibility of strain differentiation using concentrated culture filtrates of mycobacteria.

There is hope that a fairly simple laboratory method can be developed for classifying strains of mycobacteria by their lipid content. New attachments to the spectrophotometer may reduce the quantity of lipid extract and hence of bacterial growth to a very small value. The coupling of the Dische and Shettles test with Fiberglas chromatography has promise.

Of great interest is the possibility of examining lipids produced by bacilli grown *in vivo*. Thus far attempts to demonstrate, in infected tissues, the presence of any of the compounds known to be characteristic for mycobacteria have been negative (Anderson *et al.*, 1943).

ACKNOWLEDGMENTS

We wish to thank Professor E. Lederer, Institute de Biologie Physico-Chimie, Paris, France, for his generous contributions of spectra and samples of pure compounds. We are also grateful to Professor W. J. Nungester, Department of Bacteriology, University of Michigan, for making available to us the infrared spectrophotometer facilities. We are indebted to the Spang Analytical Laboratory, Ann Arbor, Michigan, for carrying out the chemical microanalyses.

SUMMARY

Using the combination of column chromatography and infrared spectroscopy, it has been possible to demonstrate the presence of group specific lipids or glycolipids in mycobacteria including *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium*, and representatives of the atypical acid-fast bacilli.

A total of 72 strains were examined including virulent and attenuated cultures of the major types of acid-fast bacilli.

The significance of these findings is discussed.

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