TABLE I

MATERIALS USED, PRODUCTS AND YIELDS

Bromide		Isopropyl	Ethyl	*n*-Propyl	*n*-Butyl
Moles used		8.8	11.0	8.8	17.25
Material		*n*-Heptaldehyde	Me-*n*-Hex	Me-*n*-Am ketones	EtOAc
Moles used		8.0	9.4	6.2	8.3
Intermediate products	( )-Methylnonanol-( )	2,3	3, 3	4, 4	5, 5
	Yield, %	66	77	76	79
	B. p. (10 mm.), °C.	90–95	87–88	86–87	83–86
	$n^{20}D$	1.435	1.436	1.434	1.434
Final products	( )-Methylnonane	2	3	4	5
	Yield, %	29	83	89	82

TABLE II

PHYSICAL PROPERTIES OF THE METHYLNONANES

	Total amounts	M. p., °C.[9]	B. p., °C. (760 mm.)	d^{20}_4	$n^{20}D$	C. T. S. aniline, °C.
2-Methylnonane	221	− 74.69 ± 0.05	166.8	0.72805	1.4099	80.3
3-Methylnonane	855	− 84.86 ± .03[a]	167.8	.73335	1.1425	78.25
4-Methylnonane	573	−101.62 ± .05[a]	165.7	.73234	1.4123	78.3
5-Methylnonane	698	− 86.80 ± .03	165.1	.73255	1.4122	77.9
n-Decane	...	− 30.1[10]	174.1[11]	.73014[11]	1.41203[11]	77.5[11]

[a] The nature of the freezing and melting curves indicates the presence of more than one allotrope, including one other form of the 4-methyl isomer melting at −99.0 ±0.1°.

Samples of these compounds will be loaned upon request to laboratories interested in determining some of their properties.

(9) The authors wish to express their appreciation to Dr. J. D. White, of the American Petroleum Institute Research Project 6 at the National Bureau of Standards, for the melting point determinations.

(10) I. Simon, *Bull. soc. chim. Belg.*, **38**, 47 (1929).

(11) A. L. Henne, A. F. Shepard and T. Midgley, THIS JOURNAL, **53**, 1951 (1931).

Summary

The four isomeric methyl nonanes have been prepared and purified, and some of their physical properties have been determined. The influence of structure upon their physical properties is discussed.

DETROIT, MICHIGAN RECEIVED MARCH 2, 1936

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

The Chemistry of the Lipids of Tubercle Bacilli. XLIV. Comparative Study of the Lipids of the Human Tubercle Bacillus[1]

BY J. A. CROWDER,[2] F. H. STODOLA,[3] M. C. PANGBORN[4] AND R. J. ANDERSON

Previous work in this Laboratory on the lipids of the human tubercle bacillus[5] was carried out on only one type of bacillus, namely, the old strain H-37, a strain which has been cultivated for many years on artificial media. Since it is possible that the organism might have undergone changes in metabolic activity and in virulence during its artificial cultivation, it seemed of importance to determine whether recently isolated bacilli when grown on the Long[6] synthetic medium would produce lipids possessing chemical and biological properties identical with those previously isolated from H-37. Accordingly the present investigation was started with the object of securing further information on this subject. Four different cultures of bacilli, recently isolated from human cases of tuberculosis, have been studied, while for comparison a freshly grown lot of H-37 was also examined, identical procedures being used throughout.

In many past investigations it has been shown that the age of the culture, the type of bacilli and

(1) An abstract of this paper was presented before the Division of Biological Chemistry at the meeting of the American Chemical Society, New York, April, 1935. The present report is a part of a coöperative investigation on tuberculosis; it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

(2) Holder of a National Tuberculosis Association Fellowship, Yale University, 1934–35.

(3) Holder of a National Tuberculosis Association Fellowship, Yale University, 1934–36.

(4) Holder of a National Tuberculosis Association Fellowship, Yale University, 1933–34.

(5) R. J. Anderson, *J. Biol. Chem.*, **74**, 525 (1927).

(6) E. R. Long, *Am. Rev. Tuberc.*, **13**, 393 (1926).

the composition of the medium has a profound effect upon the lipid content. Data on this subject have been reported by DeSchweinitz and Dorset,[7] Frouin,[8] Pfannenstiel,[9] Long and Campbell,[10] Terroine and Lobstein,[11] Chargaff[12] and many others. Reviews of earlier work on this subject will be found in the monographs by Calmette[13] and by Long.[14] More recently a differentiation between human, bovine and "BCG" bacilli, based upon the lipid content, has been reported by Chargaff[15] in which only slight variations were noted between different lots of the same strain but pronounced differences were found between the different types of bacilli. The values found in our investigation of five strains of the human tubercle bacillus show much greater variation than those reported by Chargaff.

All of the acid-fast bacteria examined in this Laboratory[16] have been found to contain liquid saturated fatty acids as characteristic lipid constituents. The higher members of these acids such as phthioic acid[17] isolated from the human H-37 bacillus are optically active, whereas analogous acids from other types of the acid-fast group of bacteria are optically inactive. It was a matter of interest therefore to determine whether other cultures of the human tubercle bacillus contained optically active acids identical with phthioic acid. Although we have not had time to examine all the lipid fractions from the new strains, one of them, A-12, was studied in some detail and the acetone-soluble fat was found to contain a mixture of optically active acids very similar to that obtained from H-37.

It will be noticed from the data presented in the experimental part that very great variations were found not only in the per cent. of the various lipid fractions but also in the chemical constants. These findings were surprising because the bacteria were grown under identical conditions and the analytical operations were strictly comparable. The results would indicate that every lot of the tubercle bacillus grown on the Long[6] synthetic medium produces lipids which show individual differences in amount and in composition.

Notwithstanding the great variations, certain characteristic similarities, perhaps a family resemblance, may be noted in all the strains. The phosphatides have a very low percentage of nitrogen and on hydrolysis yield fatty acids, reducing sugars, etc. The acetone-soluble fats contain large amounts of free fatty acids and the neutral esters composing the fats apparently are not glycerides but esters of fatty acids with trehalose.[18] It was found in two of the strains which were particularly examined that the pigment phthiocol[19] was present in about equal amounts in the free and combined state.

In previous papers on the lipids of the tubercle bacillus and other acid-fast bacteria[20] we have stated that none of the lipid fractions or their unsaponifiable matter gave any sterol color reactions. It is claimed in recent papers by Hecht[21] that not only tubercle bacilli but other acid-fast bacteria as well as *B. coli* contain demonstrable quantities of sterols. A careful search for sterols was made in the five strains included in the present investigation but only traces of sterol-like substances giving insoluble digitonides were found in the acetone-soluble fat and in the crude chloroform-soluble wax.[22] The amounts of sterol-like substances precipitable with digitonin were so minute that we can only regard them as accidental impurities and not as metabolic products of the tubercle bacillus.

Experimental

In order to secure as comparable data as possible five strains of tubercle bacilli were grown in 1-liter Pyrex bottles in lots of about 300 cultures at the Mulford Biological Laboratories, Sharp and Dohme, Glenolden, Pa., for a period of eight weeks. The bacilli included the old strain H-37 and four strains recently isolated from human

(7) E. A. DeSchweinitz and M. Dorset, *Central. Bakt. Orig.*, **32**, 186 (1902).

(8) A. Frouin, *Compt. rend. soc. biol.*, **84**, 606 (1921).

(9) W. Pfannenstiel, *Z. Hyg. Infektionskrankh.*, **95**, 87 (1922).

(10) E. R. Long and L. K. Campbell, *Am. Rev. Tuberc.*, **6**, 636 (1922).

(11) E. F. Terroine and J. E. Lobstein, *Bull. soc. chim. biol.*, **5**, 182 (1923).

(12) E. Chargaff, *Z. physiol. Chem.*, **201**, 198 (1931); **217**, 115 (1933).

(13) A. Calmette, "Tubercle Bacillus Infection and Tuberculosis in Man and Animals," translated by W. B. Soper and G. H. Smith, Williams and Wilkins Co., Baltimore, 1923.

(14) H. G. Wells and E. R. Long, "The Chemistry of Tuberculosis," 2d ed., The Williams and Wilkins Co., Baltimore, 1932.

(15) E. Chargaff and J. Dieryck, *Biochem. Z.*, **255**, 319 (1932).

(16) R. J. Anderson, *J. Biol. Chem.*, **83**, 169 (1929); R. J. Anderson and E. Chargaff, *ibid.*, **84**, 703 (1929); **85**, 77 (1929); R. J. Anderson and E. G. Roberts, *ibid.*, **85**, 519 (1930); R. J. Anderson and E. Chargaff, *Z. physiol. Chem.*, **191**, 157 (1930); R. J. Anderson and E. G. Roberts, *J. Biol. Chem.*, **89**, 599 (1930); M. L. Burt and R. J. Anderson, *ibid.*, **94**, 451 (1931); M. C. Pangborn and R. J. Anderson, *ibid.*, **94**, 465 (1931); R. J. Anderson and N. Uyei, *ibid.*, **97**, 617 (1932).

(17) R. J. Anderson, *ibid.*, **97**, 639 (1932).

(18) R. J. Anderson and M. S. Newman, *ibid.*, **101**, 499 (1933).

(19) R. J. Anderson and M. S. Newman, *ibid.*, **101**, 773; **103**, 197 (1933).

(20) R. J. Anderson, *Physiol. Rev.*, **12**, 166 (1932).

(21) E. Hecht, *Z. physiol. Chem.*, **231**, 29, 279 (1935).

(22) R. J. Anderson, R. Schoenheimer, J. A. Crowder and F. H. Stodola, *ibid.*, **237**, 40 (1935).

cases of tuberculosis at the Henry Phipps Institute, Philadelphia. The new strains are designated as A-10, A-12, A-13 and A-14. The bacteria were grown one lot at a time under identical conditions.

The bacterial growths were filtered off on Büchner funnels, washed with water, and immediately transferred to 20-liter Pyrex bottles containing 16 liters of alcohol saturated with carbon dioxide. The containers were thoroughly shaken and shipped to the Sterling Chemistry Laboratory. The bacteria were extracted by the methods briefly outlined below, every effort being made to maintain uniform conditions.

All solvents had been freshly distilled. The alcohol and ether had been distilled over potassium hydroxide. The extractions were all made at room temperature and the solvents were saturated with carbon dioxide. Air was displaced from all containers with carbon dioxide before use.

Extraction of the Bacteria with Alcohol and Ether.—The containers when received at the laboratory were shaken and were then allowed to stand for a day or two until the bacterial cells had settled. The supernatant solution was siphoned off under carbon dioxide pressure after which the bacterial mass was further dehydrated by shaking with 4 liters of alcohol. The solvent was siphoned off and the bacteria were extracted three times with 5 liters of equal parts of alcohol and ether by shaking occasionally for two days. After the bacteria had settled the extracts were siphoned off. The bacteria were next extracted in the same manner with 5 liters of ether after which the bacterial cells were filtered on a Büchner funnel and washed with a mixture of equal parts of alcohol and ether.

The alcoholic extracts were concentrated *in vacuo* until most of the alcohol was removed. The ether–alcohol extracts and washings were combined and most of the ether was removed by a current of carbon dioxide at a temperature of 40° after which the alcohol was distilled off *in vacuo*. The residues from the extracts, consisting of aqueous suspensions of lipids, were combined and extracted with ether, the aqueous solutions being saved for the isolation of polysaccharide as will be described later. The ethereal extract was passed through a Chamberland filter under carbon dioxide pressure and the perfectly clear filtrate was concentrated to about 500 cc.

The Phosphatide.—The concentrated ethereal extract was mixed with 600 cc. of acetone, whereupon a sticky precipitate of crude phosphatide separated. After the precipitate had settled out the clear supernatant solution was decanted and the flask was rinsed out with acetone. The phosphatide was precipitated ten times from ethereal solution with acetone after which it was treated with ethyl acetate in order to remove a slight admixture of wax-like material. This treatment was followed by three precipitations from etheral solution with acetone. The last precipitation was carried out by pouring the ethereal solution into ice cold acetone. The product thus obtained was a white amorphous powder which when rubbed up with water formed a uniform colloidal solution. The purified phosphatide was distinctly hygroscopic. The material contained in the mother liquors was worked up as will be described below.

The Acetone-Soluble Fat.—The mother liquor from the first precipitation of the phosphatide was concentrated until the ether was removed, after which the solution was cooled in ice water. The wax-like material which separated was filtered off and combined with similar material obtained from the mother liquors in the purification of the phosphatide. The filtrate on concentration to dryness yielded the acetone-soluble fat as a dark reddish-brown salve-like mass having the perfume-like odor characteristic of tubercle bacilli.

Isolation of a Wax-like Substance from the Phosphatide Mother Liquors.—The mother liquors from the phosphatide purification on concentration to dryness left a wax-like residue. It was possible to isolate an additional small amount of phosphatide from this material by treatment with ethyl acetate in which the phosphatide is insoluble and by repeated precipitations with acetone from ethereal solution.

The wax-like substance was again recovered and dissolved in hot acetone. A white precipitate which separated on cooling the solution in ice water was filtered off and washed with cold acetone. The filtrate was evaporated to dryness and left a slight reddish-brown residue of salve-like consistency. This fraction which was soluble in ice cold acetone was combined with the acetone soluble fat.

The substance which separated from the acetone solution on cooling was further purified by precipitation from acetone until a snow white product was obtained. The substance was optically active. It melted at about 40° to an oily liquid which on cooling formed a hard non-crystalline wax-like mass. It may be mentioned that similar fractions had been isolated in our earlier studies and these fractions had always been kept separate although for the sake of simplicity they had been recorded as crude wax. At the present time a special investigation is under way concerning the composition and cleavage products of this fraction.

The Chloroform-Soluble "Wax."—The bacterial residue after extraction with alcohol and ether was twice extracted for two days with four-liter portions of chloroform being filtered and washed with chloroform each time. The chloroform extracts were combined, passed through a Chamberland filter, and evaporated to dryness *in vacuo*. The bacterial residue was further treated with 4 liters of alcohol–ether, equal parts, filtered and washed with alcohol–ether. The filtrate and washings were passed through a Chamberland filter and evaporated to dryness *in vacuo*. The residue was combined with the material obtained from the chloroform extract and will be designated as crude chloroform-soluble wax.

The Bacterial Residue.—The extracted bacterial cells were dried *in vacuo* at 40° and reserved for other experiments.

Extraction of the Bacterial Residue with Petroleum Ether.—The bacterial residues remaining after the treatments described above are practically free from any lipids soluble in neutral solvents as shown by the following experiment: 425 g. of the bacterial residue from H-37 was extracted exhaustively with two portions of carefully purified boiling petroleum ether. The extract was passed through a Chamberland filter and concentrated to dryness. The operations of extraction and concentration were

TABLE I

LIPIDS FROM FIVE STRAINS OF TUBERCLE BACILLI

Strain of organism	First lot of H-37		New lot of H-37		A-10		A-12		A-13		A-14	
No. of cultures	2000		265		293		292		285		296	
	G.	%	G.	%	G.	%	G.	%	G.	%	G.	%
Phosphatide	253.1	6.54	4.5	0.84	7.2	1.00	9.3	1.3	4.2	1.2	11.0	1.6
Wax-like material from phosphatide mother liquors			19.6	3.6	5.0	0.7	17.6	2.5	15.5	4.4	18.0	2.7
$CHCl_3$-soluble wax	427.0	11.03	27.2	5.1	63.0	8.9	35.4	5.0	5.7	1.6	29.0	4.3
Acetone-soluble fat	240.0	6.20	53.5	10.1	34.5	4.9	50.1	7.1	12.4	3.5	44.5	6.7
Total lipids soluble in neutral solvents	920.1	23.78	104.8	19.7	109.7	15.5	112.4	16.1	37.8	10.8	102.5	15.5
Lipids soluble in acid alcohol–ether				11.2		17.3		16.1		12.0		13.5
Total lipids				30.9		32.8		32.2		22.8		29.0
Bacterial residues	2902.0	75.01	427.0	80.3	595.0	84.4	585.0	83.9	311.5	89.1	557.0	84.4
Total bacterial mass			531.8		704.7		697.4		349.3		659.5	
Bacterial mass per culture	1.928		2.006		2.405		2.388		1.225		2.228	

performed in all-glass apparatus so that the solvent never came in contact with either cork or rubber stoppers. A wax-like residue weighing only 85 mg. was obtained. This fraction was used for a quantitative determination of sterols as described in a former publication with completely negative results,[22]

Extraction of the Bacterial Residues with Acidified Alcohol and Ether.—As shown in the preceding paragraph extraction of tubercle bacilli with alcohol and ether and with chloroform removes practically all of the lipids which are soluble in neutral solvents. It is a well-known fact, however, that acid-fast bacteria contain a considerable proportion of lipids firmly bound in the cellular structure which can only be removed by treatment with acids or alkali.

In order to determine the amount of bound lipids the following experiments were conducted: 10 g. of bacterial residues of the five strains were refluxed separately for eight hours with 150 cc. of equal parts of alcohol and ether containing 1% of hydrochloric acid, after which the bacterial cells were filtered off and washed with ether. The filtrates were concentrated *in vacuo* to dryness. The material thus obtained varied with the different strains from about 15 to 30% but only about one-half was soluble in ether. The other portion was soluble in water and contained carbohydrates and other undetermined constituents.

The lipid constituents were removed by digesting the extracted material with water and then extracting with ether. The ethereal solution on evaporation to dryness left a wax-like non-crystalline solid.

The bacterial cells, after the acid extraction, were further refluxed for four hours with 150 cc. of equal parts of chloroform and ether, filtered off and washed with chloroform and ether. The filtrates on evaporation *in vacuo* to dryness left only small residues of wax-like material which were combined with the ether-soluble substances mentioned above. The amounts of firmly bound lipids are indicated in Table I.

The total bound lipids obtained from the five strains were combined and examined for sterols as described in a previous paper.[22] The results were completely negative. We have as yet no other data on the nature or composition of the firmly bound lipids.

Alkaline Hydrolysis of the Extracted Bacterial Cells.—The residual bacterial cells after the acid extraction described in the preceding paragraphs were further examined for any additional very firmly bound lipids. Each lot of cell residues was refluxed with 100 cc. of 10% aqueous potassium hydroxide for four hours. The resulting cloudy solutions were allowed to cool and were twice extracted with ether. The extracts were dried with sodium sulfate, filtered and evaporated to dryness and yielded about 10 mg. of solid wax-like substance.

The alkaline solutions were acidified with hydrochloric acid and extracted with ether. The ethereal extracts after being washed with water, dried and evaporated to dryness yielded from 35 to 40 mg. of solid substance. The slight amount of ether-soluble substance recovered after the alkaline hydrolysis gave no sterol color reactions. The material was not further examined.

The Polysaccharide.—The aqueous solutions from the five lots of bacteria from which the lipids had been removed by ether extraction were combined and the polysaccharide was isolated by the method described in a former paper.[5] A nearly white powder was obtained which weighed 17.4 g. The substance which contained 2.14% of nitrogen and 1.68% of phosphorus was saved for future investigation.

The results obtained with the five strains of tubercle bacilli are summarized in Table I, and for comparison we include the data from the first analysis of H-37 reported in 1927.[5]

Although the bacilli were grown under identical conditions and the analytical operations were strictly comparable it is evident from an inspection of the data in Table I that the different lots of bacilli yielded decidedly varying quantities of lipid fractions. In fact the variations among the five strains of the human tubercle bacillus used in this study are as great as those that have been observed by other investigators between human, bovine, avian or "BCG" bacilli. It would be impossible from our results to differentiate between these types since our values fall into all of these groups.

Analysis of the Phosphatides.—The phosphatides from the different lots of bacilli were all similar in properties and resembled the phosphatide described previously.[5] They were amorphous white powders which formed colloidal solutions when rubbed up with water. Addition of acid to these solutions caused a heavy coagulum and when this suspension was boiled for four or five hours the

TABLE III

CONSTANTS OF THE ACETONE-SOLUBLE FATS

Strain	Iodine no.	Reichert-Meissl no.	Polenske no.	Unsaponifiable matter, %	Neutral fat, %	Saponification no. of neutral fat	The free fatty acids: %	Mol. wt.	Solid, %	Liquid, %	Iodine no.
H-37	35.1	4.6	2.26	9.6	46.7		52.0	340	54.7	34.4	37.9
A-10	48.8	4.03	1.57	17.7	55.7		42.3		41.7	46.4	41.4
A-12	71.1	11.4	1.36	8.02	54.4	180	42.0	344	40.7	49.9	45.5
A-13	50.7	3.36	1.88	16.27	68.1		26.1		34.1	65.1	50.9
A-14	40.6	5.12	1.37	2.47	58.8	185	35.3	338	33.3	52.9	46.9
Earlier H-37 data	52.6	3.96		10.38	72.47		27.53		36.45	60.78	47.4

coagulum was converted gradually into an oily layer which solidified on cooling. The aqueous solution after being neutralized and tested with Fehling's solution gave a heavy reduction. Complete analyses of the phosphatides and their cleavage products have not been made but values for phosphorus and nitrogen were determined and are given in Table II.

TABLE II

ANALYSES OF THE PHOSPHATIDES

Organism	H-37	A-10	A-12	A-13	A-14
N, %	0.44	0.33	0.21	0.56	0.53
P, %	2.95	3.44	3.30	3.76	3.33

Constants of the Acetone-Soluble Fats.—The constants of the acetone-soluble fats were determined according to the methods of the Association of Official Agricultural Chemists[23] and for comparison we include the constants of the acetone-soluble fat of H-37 published previously. The data are given in Table III. This table also includes the values for free fatty acids, neutral fats and the proportions of solid and liquid acids contained in the free fatty acids. The acetone-soluble fats were all soft salve-like masses at room temperature and the melting points were not determined.

All the fats gave negative tests for nitrogen and tests for phosphorus gave negative results except A-13 which showed a trace. All the fats gave no coloration when tested by the Liebermann-Burchard sterol reaction.

The constants of the fats show very great variation. The free fatty acid content varied from 26 to 52% and the iodine number from 35 to 71.

Examination of the Acetone-Soluble Fats.—The acetone-soluble fats from strains H-37 and A-12 were examined in some detail. It was desired to gain some information concerning the following points: (a) is phthiocol present in free or combined form: (b) is trehalose a constant constituent of the neutral fat: (c) are substituted benzoic acids other than anisic acid present in the fat: (d) are optically active acids such as phthioic acid constant metabolic products of the bacillus?

(a) **Determination of Free Phthiocol.**—In order to determine whether free phthiocol was present the fat was dissolved in ether and the solution was extracted with 0.5% sodium bicarbonate solution. The red colored aqueous solution was concentrated *in vacuo*, acidified, and extracted with ether. The ethereal solution on evaporation to dryness left a yellow semi-crystalline mass. It was difficult to isolate the small amount of phthiocol present in this mixture of acids but the following method was found to be most convenient. The mixture was refluxed with absolute methyl alcohol containing dry hydrochloric acid in order to convert the acids to methyl esters. The reaction product was isolated, after the solution had been concentrated and diluted with water, by extraction with ether. The phthiocol was now removed from the ethereal solution by extraction with 0.5% sodium bicarbonate solution. The aqueous solution was acidified and extracted with ether. The crude phthiocol obtained on evaporation of the solvent was recrystallized from methyl alcohol yielding the characteristic yellow prismatic crystals. The substance had the correct melting point and gave no depression when mixed with synthetic phthiocol, m. p. 173–174°.

From 40.5 g. of acetone-soluble fat of H-37 we isolated 14 mg. of phthiocol while 37.9 g. of the fat from A-12 gave 11.2 mg. of phthiocol.

Saponification of the Acetone-Soluble Fat. Isolation of the Combined Phthiocol.—The two lots of acetone-soluble fat were saponified separately by refluxing for eight hours with 5% alcoholic potassium hydroxide. The solution was concentrated by distillation, diluted with water and the unsaponifiable matter was extracted with ether after which the solution was acidified with hydrochloric acid and the fatty acids were taken up in ether.

The ethereal solution of the fatty acids was washed with 0.5% sodium bicarbonate for the removal of phthiocol, anisic acid, etc. The red-colored aqueous solution was concentrated *in vacuo*, acidified, extracted with ether and ethereal solution was evaporated to dryness. The residue was esterified by refluxing with absolute methyl alcohol and hydrochloric acid after which the phthiocol was isolated and crystallized as described in the preceding section. The ethereal solution was examined for substituted benzoic acids.

The acetone-soluble fat of H-37 yielded 15.7 mg. of phthiocol while the fat from A-12 gave 16.5 mg. of phthiocol.

The results of these experiments show that the acetone-soluble fats contain nearly equal amounts of free and combined phthiocol. The quantities recorded represent recrystallized pure phthiocol, hence the actual amount of the pigment in the fat is considerably higher than indicated by these figures.

(b) **Search for Substituted Benzoic Acids. Isolation of Anisic Acid.**—In a former analysis of the acetone-soluble fat of H-37 we were able to isolate a small amount of anisic acid[24] and the presence of salicylic acid and phenylacetic acid in tubercle bacilli has been reported by

(23) "Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists," 2d edition, Washington, 1925.

(24) R. J. Anderson and M. S. Newman, *J. Biol. Chem.*, **101**, 773 (1933).

Stendal.[25] If such acids are present in the fat it should be possible to remove them along with phthiocol on extracting the mixed fatty acids with dilute sodium bicarbonate solution. The only acid that we could identify was anisic acid but traces of other acids were present.

The ethereal solution containing the methylation products and from which the phthiocol had been removed as described above was evaporated to dryness. An oily residue was obtained which was distilled with steam. The distillate on extraction with ether yielded a small amount of an oil which was saponified. The free acids after being isolated in the usual manner formed an oil containing some needle-shaped crystals. The crystals were separated and recrystallized from water. Colorless needles were obtained which melted at 183.5–184.5° and did not depress the melting point of pure anisic acid, m. p. 183.5–184.5°.

The fat from H-37 yielded 5 mg. while the fat from A-12 gave 6.6 mg. of the pure anisic acid.

The non-crystalline portion of the acids could not be identified. The material gave no coloration with ferric chloride, thus indicating the absence of salicylic acid.

(c) **Isolation of Trehalose.**—The aqueous solution from which the fatty acids had been extracted was neutralized with potassium hydroxide and evaporated to dryness *in vacuo*. The residue was extracted three times with 70% alcohol. The alcoholic solution was evaporated to dryness and the residue was dissolved in water. The solution was treated with lead acetate and the precipitate which formed was filtered off and discarded. Addition of basic lead acetate and ammonia to the filtrate yielded a precipitate which was filtered off, washed with water and decomposed by hydrogen sulfide. The lead sulfide was filtered off and the filtrate was concentrated to a thick sirup. The latter on trituration with absolute alcohol yielded a nearly white powder which weighed 0.7 g. The substance on acetylation in pyridine solution with acetic anhydride gave an acetate which on recrystallization from methyl alcohol separated in the form of colorless prismatic crystals. The substance melted indefinitely at 78–80°, $[\alpha]_D$ in $CHCl_3$ + 162.3°. The properties of the substance correspond to those of trehalose octaacetate. This preparation was isolated from strain A-12.

The lead precipitate obtained from strain H-37 was unfortunately lost through an accident but it has been shown in a former paper[14] that trehalose was present in place of glycerol in the fat from H-37. Since we have now also isolated trehalose from the fat of strain A-12 we may conclude that trehalose functions generally as the alcoholic component of the fats produced by different strains of the tubercle bacillus.

An effort was made to isolate glycerol from the aqueous solution after the trehalose had been removed with basic lead acetate and ammonia but no glycerol could be found.

(d) **Examination of the Fatty Acids.**—The fatty acids, after phthiocol, anisic acid, etc., had been removed by dilute sodium bicarbonate solution, were separated into solid and liquid acids by the lead soap–ether procedure. The solid acids were saved but were not further examined at this time. The liquid acids after treatment with norit were hydrogenated and the reduced acid was removed by repeating the lead soap–ether treatment. From the ether soluble lead salts the characteristic liquid saturated fatty acids were obtained. The data on the analyses of the fats are given in Table IV.

TABLE IV

ANALYSES OF THE ACETONE-SOLUBLE FATS FROM H-37 AND A-12

Strain	H-37	A-12
Weight of fat, g.	40.1	37.90
Unsaponifiable matter, g.	3.84	3.83
Solid acids, g.	11.66	10.15
Liquid acids, g.	18.30	18.56
Iodine no. of liquid acids	...	69
Reduced acids, g.	3.68	4.13
Liquid saturated acids, g.	13.10	10.96
$[\alpha]_D$ of liquid satd. acids	+5.0	+5.86

It will be noticed that the fats from both strains contained liquid saturated fatty acids of nearly the same dextrorotation.

Examination of the Liquid Saturated Fatty Acids.—For purification the two lots of liquid saturated fatty acids were combined, treated with norit and converted into the methyl esters. The esters which weighed 22.9 g. were fractionated in a molecular still into four fractions and a residue.

Isolation of Tuberculostearic Acid.—The first two ester fractions, 9.3 g., which showed no optical rotation, consisted largely of methyl tuberculostearate and on further purification and saponification yielded tuberculostearic acid. The acid was a liquid at room temperature; mol. wt. found, 292; for $C_{19}H_{38}O_2$ calculated mol. wt. 298. The 2,4,6-tribromoanilide was prepared according to Robertson[26] and recrystallized from alcohol. The colorless needle shaped crystals melted at 94–95°. Spielman[27] reported a melting point of 93–94° for the tribromoanilide of tuberculostearic acid.

Anal. Calcd. for $C_{25}H_{40}ONBr_3$(610): C, 49.18; H, 6.61. Found: C, 48.98; H, 6.55.

Isolation of Crude Phthioic Acid.—The ester fractions 3 and 4, 6.0 g., which showed a dextrorotation, were combined and refractionated through a column of the Widmer type. The first two fractions were optically inactive and apparently consisted of methyl tuberculostearate. Fraction 3 had a rotation of +5.85° and fraction 4 had a rotation of +7.65°. These fractions represent typical mixtures containing methyl phthioate such as we have frequently isolated on previous occasions from the acetone-soluble fat of H-37.

The Ester Residue from the First Distillation.—In fractionating the crude mixed esters of the liquid saturated fatty acids we have always noted a considerable quantity of a residue in the distilling flask which did not distil even when the temperature was raised to 360°. No complete investigation has been made of these non-volatile portions of the esters.

In the present case the non-volatile portion of the esters weighed 7.3 g. It was a thick viscous mass, $[\alpha]_D$ +12°, and on saponification gave a mean molecular weight of 559 and an acetyl value of 56. This material evidently

(25) N. Stendal, *Compt. rend.*, **198**, 400 (1934).

(26) P. W. Robertson, *J. Chem. Soc.*, **115**, 1210 (1919).

(27) M. A. Spielman, *J. Biol. Chem.*, **106**, 87 (1934).

represents hydroxy acids of very high molecular weight. The investigation of this material will have to be deferred to a future time.

The Solid Reduced Acids.—The solid reduced acids on recrystallization from acetone were obtained in form of colorless plates and had the properties of stearic acid. The melting point was 68–69° and the molecular weight was 283.7.

The Unsaponifiable Matter.—The unsaponifiable matter from H-37 was a dark-colored viscous mass. The iodine number was 194. On combustion it gave the following values: found, C, 81.63; H, 10.76.

An attempt to hydrogenate the substance with platinum oxide as catalyst failed and no hydrogen was absorbed. Reduction with the Raney nickel catalyst[28] at a pressure of 2000 pounds for four hours also failed to yield a saturated compound.[29] The substance recovered after this treatment had an iodine number of 136.

The unsaponifiable matter from A-12 was a dark-colored thick mass with an iodine number of 226. It was possible to isolate 1.2 mg. of a digitonide from 1.51 g.[30] of this unsaponifiable matter as described in a former paper.[22] The digitonide gave a typical Liebermann–Burchard reaction.

Further investigations of the unsaponifiable matter will be carried out in the future.

The Wax Fractions.—The so-called chloroform-soluble wax has not been studied systematically except that the material extracted from strain H-37 was examined for sterols. As already published[22] the unsaponifiable matter from the wax yielded 2.1 mg. of digitonide corresponding to about 0.5 mg. of sterol.

(28) L. W. Covert and H. Adkins, THIS JOURNAL, **54**, 4116 (1932).

(29) We are indebted to Dr. J. M. Sprague of this Laboratory for carrying out this reduction.

(30) In the paper published in *Z. physiol. Chem.*, **237**, 40 (1935), through a typographical error the weight of the unsaponifiable matter used in this determination is given as 1.15 g. instead of 1.51 g.

Summary

A study has been made of the lipids isolated from four recently isolated cultures of the human tubercle bacillus in comparison with a freshly cultivated lot of strain H-37.

Great variation in yields of the different lipid fractions as well as in their chemical constants were found.

The pigment phthiocol occurs in the acetone-soluble fats both in the free and combined state.

Optically active acids similar to or identical with phthioic acid were isolated from the acetone-soluble fats.

The acetone-soluble fats from all the strains contain large proportions of free fatty acids while the neutral esters contain trehalose in place of glycerol.

Minimal traces of sterol-like compounds were detected in the acetone-soluble fats and in the crude chloroform-soluble waxes but the quantities are so small that they can be regarded only as accidental impurities and not as metabolic products of the bacillus.

NEW HAVEN, CONN. RECEIVED FEBRUARY 4, 1936

[CONTRIBUTION FROM THE RESEARCH LABORATORY OF ORGANIC CHEMISTRY, NO. 132, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Studies in the Diphenic Acid Series. V

BY H. W. UNDERWOOD, JR.,[1] AND GEORGE BARKER

In an earlier paper[2] of this series it was reported that phenoldiphenein, the principal product of the reaction of phenol with diphenic anhydride in the presence of fuming stannic chloride, dissolves in aqueous sodium hydroxide to yield a yellow-colored solution. Phenolphthalein, as is well known, gives a deep red solution under similar circumstances. In order to learn more concerning this remarkable difference between substances of presumably analogous structure,[3] we have prepared tetrabromo-, diiodo-, and dinitrophenoldiphenein, *o*-cresoldiphenein, dibromo-, diiodo-, and dinitro-*o*-cresoldiphenein, resorcinoldiphenein, tetrabromo-, and tetraiodoresorcinoldiphenein. While the corresponding derivatives of phenolphthalein dissolve in dilute sodium hydroxide solution to yield bright colored solutions, the substituted dipheneins yield yellowish solutions, indistinguishable from one another, in dilute alkali.

The absorption spectra of these compounds will be published subsequently.

(1) Professor Underwood died on March 20, 1934.

(2) Underwood and Kochmann, THIS JOURNAL, **45**, 3071 (1923); **46**, 2069 (1924).

(3) Underwood and Barker, *ibid.*, **52**, 4082 (1930).

Experimental

Tetrabromophenoldiphenein.—To 2 g. of a hot alcoholic solution of phenoldiphenein is added during one-half hour 4 g. of bromine and then one-half of the solvent is distilled off, a product separates which, purified from glacial acetic acid, gives a 75% yield of colorless crystalline tetrabromophenoldiphenein melting at 213–214°.