*Constituents of the Lipids of Tubercle Bacilli. Part II.**

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The lipid extracts of tubercle bacilli (human type) have been subjected to a two-step hydrolysis, and the resulting dextro- and lævo-rotatory acids studied. The dextrorotatory mixture of acids, liberated by a mild hydrolysis, is shown to exhibit αβ-unsaturation (cf. *Nature*, 1950, **166**, 693). The lævorotatory acid resulting from a more vigorous hydrolysis of the remaining mixture of the lipids is found to be saturated. Some preliminary studies pertaining to the structure of these acids are also described.

PRELIMINARY studies of the component acids of the lipids of tubercle bacilli (human type) (Part I *) resulted in the isolation of three dextrorotatory acids which, as already briefly reported (Chanley and Polgar, *Nature*, 1950, **166**, 693) showed αβ-unsaturation. Two of these acids, having $[\alpha]_D$ +11·7° and +11°, respectively, had molecular weights and optical rotations similar to those of the "phthioic acid," isolated by Anderson and his collaborators (Anderson and Chargaff, *J. Biol. Chem.*, 1929, **85**, 77; Spielman and Anderson, *ibid.*, 1936, **112**, 759) and formulated as $C_{26}H_{52}O_2$. The third acid (approximately C_{30}) having $[\alpha]_D$ +4·8°, was probably a mixture including as a major constituent the lævorotatory acid described below. These acids were isolated by a fractional crystallization of the semicarbazones and the 2 : 4-dinitrophenylsemicarbazones of their acetol esters. Although some separation of the initial mixture was achieved, the acids were obtained in too small amounts for further purification and for detailed structural studies.

In the work now described, which was carried out in 1949—1950, a simplified procedure of isolating the hydrolytic product of the lipids was employed. This was based upon the following observations. (*a*) Mild hydrolysis of the lipid extracts (obtained by Soxhlet-extraction of the bacterial cells with acetone, and then with *iso*propyl ether) liberates mixtures of the dextrorotatory acids with acids of lower molecular weight, from which the dextrorotatory acids can be easily separated by distillation of their methyl esters. (*b*) The dextrorotatory mixture of acids thus liberated is soluble in cold methanol, whereas the unhydrolysed lipids are very sparingly soluble and are thus readily separable from the former. (*c*) Further hydrolysis of the resulting methanol-insoluble lipid fraction under more vigorous conditions releases a lævorotatory acid. (*d*) Both the dextro- and the lævo-rotatory acids yield potassium salts which are sparingly soluble in water, but readily soluble in ether at ordinary temperatures, and are partly deposited from the ethereal solutions at about 10° or below.

Details of the isolation of these acids are described in the Experimental section. It should be noted that we avoided separating the lipid extracts into fat, phosphatide, and wax fractions, since mutual solubility effects usually result in considerable overlap in the composition of such fractions. At the present stage of this investigation directed, in the first instance, towards isolating the constituent acids of the lipids, it seemed preferable to hydrolyze the total lipid extracts.

The dextrorotatory acids were separated into two fractions by the solubility of their potassium salts in ether. The less soluble fraction yielded a solid acid having $[\alpha]_D$ +7·9°, n_D^{36} 1·4600, and the potassium salt which remained in the ethereal solution on cooling gave a liquid acid having $[\alpha]_D$ +10·3°, n_D^{36} 1·4655, after decolorization by chromatography over silica. It appeared that this preliminary separation might facilitate further studies.

The ultra-violet absorption of both the solid and the liquid acid ($\lambda_{max.}$ 2200 Å; log ε 3·94 and 4·04, respectively) was characteristic of an αβ-unsaturated acid. This was confirmed by the infra-red absorption spectra which showed bands at 1646 (conjugated C=C) and at 1694 cm.$^{-1}$ (conjugated CO_2H). The ultra-violet and infra-red absorption spectra of the hydrogenated products showed the absence of unsaturation.

* Part I, *Biochem. J.*, 1948, **42**, 206.

The specific rotations were considerably decreased on hydrogenation, namely, from +7·9° to +1·1° for the solid, and from +10·3° to +1·7° for the liquid acid. This already suggested the presence of an asymmetric centre near the double bond.

An early ozonization experiment on the solid acid is recorded in the Experimental section. A discussion is reserved for the following communication (Part III) where the full chemical evidence establishing the structure of the αβ-unsaturated constituent of the solid acid, now named mycolipenic acid, is given.

According to Spielman and Anderson (*loc. cit.*) "phthioic acid," isolated from the lipids of tubercle bacilli by a procedure involving hydrogenation, had $[\alpha]_D$ +12·5°. In view of the fact that the rotatory power of the dextrorotatory acids from tubercle bacilli is considerably decreased on hydrogenation, the high rotatory power given for "phthioic acid" indicates that the hydrogenation carried out by Spielman and Anderson was incomplete. This is confirmed by Cason and Sumrell's recent studies (*J. Biol. Chem.*, 1951, **192**, 405) of a specimen of "phthioic acid" originating from Anderson and his collaborators; ester-fractionation of this product resulted in a variety of fractions including αβ-unsaturated acids.

The lævorotatory acid, exhibiting $[\alpha]_D$ −8·7° on isolation through its potassium salt, was converted into the methyl ester. Distillation afforded, as the main product, an ester fraction having $[\alpha]_D$ −10·3°. This gave on alkaline hydrolysis (see below) the corresponding acid, which was found to be saturated (no change on hydrogenation over platinic oxide; no high-intensity absorption in the ultra-violet; absence of bands characteristic for unsaturation from the infra-red spectra). Titration gave a result in close agreement with the formula $C_{31}H_{62}O_2$.

The acid obtained on hydrolysis of the preceding methyl ester with aqueous ethanolic potassium hydroxide had $[\alpha]_D$ −9·2°, whereas hydrolysis by anhydrous methanolic potassium hydroxide afforded an acid having $[\alpha]_D$ −6·9°. The decrease in rotatory power in the latter reaction suggested partial racemization in respect of an asymmetric centre in the α-position to the carboxyl group, and this was confirmed by a stepwise degradation. This involved oxidation of the derived α-hydroxy-acid (obtained through the α-bromo-acid) by means of lead tetra-acetate. The product was a ketone, thus indicating the presence of one alkyl substituent at the α-carbon atom of the parent acid. Details of further degradative studies, recently reported in a preliminary note (Polgar, *Chem. and Ind.*, 1953, 353) are given in one of the following communications (Part IV).

Anderson and his collaborators have isolated from the wax fractions of human tubercle bacilli lævorotatory acids of similar composition, having $[\alpha]_D$ −6·1°, −9·5°, and −5·7°, severally (Anderson, *J. Biol. Chem.*, 1932, **97**, 639; 1938, **126**, 515; 1945, **157**, 203), of which the last, obtained by purification procedures from a crude acid having $[\alpha]_D$ −7·4°, was named "mycocerosic acid" and characterized as a *p*-bromophenacyl ester of m. p. 47—48°. The acid, $[\alpha]_D$ −6·9°, obtained in the present work on hydrolysis with anhydrous methanolic potassium hydroxide, gave a *p*-bromophenacyl ester, m. p. 31·5°. Since there is no evidence of the presence of a second, closely related lævorotatory acid, the differences in the physical properties are probably due to varying degrees in the purity of the acids, in particular owing to the presence of stereoisomerides arising from partial racemization (see above).

The physiological properties of the acids described in the present communication have been studied by Dr. J. Ungar, of Glaxo Laboratories Ltd., who kindly reported as follows:

"The acids were injected intraperitoneally into groups of four guinea-pigs (of an equal weight of 300 g.) in doses of 5, 10, and 20 mg., suspended in 0·5 c.c. of tragacanth. One guinea-pig from each group was killed at weekly intervals and examined according to the methods described earlier (Ungar, Coulthard, and Dickinson, *Brit. J. Exp. Path.*, 1948, **29**, 322).

"The dextrorotatory solid acid, injected in amounts of 20 mg., produced granulomatous lesions in the omentum and spleen, and only a few isolated nodules in the liver.

"Guinea-pigs injected with the dextrorotatory liquid acid showed a similar picture, but the granulomata were more numerous in the liver and appeared at an earlier date.

"Extensive changes were noticed after the injection of the lævorotatory acid. After

injection of 5 mg., and more so after 10 mg., the guinea-pigs have shown multiple granulomatous lesions on the omentum, spleen, liver, diaphragm, and lymph glands of the pleural cavity. Undoubtedly the most changes were observed in the guinea-pigs after the injection of the lævorotatory acid."

Experimental

Light petroleum had b. p. 40—60°. Specific rotations were measured in ether, 0·5-dm. tubes being used. Ultra-violet absorption spectra were determined in purified *cyclo*hexane.

Extraction of the Lipids.—The extractions were kindly carried out by Glaxo Laboratories Ltd., Greenford, in the following way:

Moist steam-killed cells (*M. tuberculosis*, human type, obtained from the Veterinary Laboratory, Ministry of Agriculture and Fisheries, Weybridge; 50 lb.) were extracted with acetone (30 l.) for 20 hr. in a Soxhlet apparatus. The acetone extract was then concentrated (to about 6 l.) by heat under reduced pressure, and the residual aqueous mixture extracted with *iso*propyl ether; evaporation of the extract under reduced pressure afforded the product (*A*) (145 g.).

The cells were then extracted (Soxhlet) with *iso*propyl ether (25 l.) for 20 hr. Evaporation of this extract under reduced pressure gave the product (*B*) (430 g.).

Isolation of the Dextrorotatory Mixture of Acids.—The product (*A*) (130 g.) was refluxed with a solution of potassium hydroxide (25 g.) in methanol (500 c.c.) for 5 hr. Half of the methanol was then removed by distillation, and the residual product, after dilution with water and acidification (2N-hydrochloric acid), was extracted with ether. The dried (Na_2SO_4) ethereal extract was evaporated, and the residue boiled with methanol (500 c.c.) for a few minutes. A red tar (i) separated overnight. The remaining methanolic solution was then removed by decantation and filtration, and the residual tar washed with cold methanol (50 c.c.). The combined methanol extracts on evaporation yielded the methanol-soluble substances (ii). A solution of the latter in light petroleum (400 c.c.) was extracted with a solution containing equal volumes (100 c.c. of each) of 5% aqueous potassium hydroxide and methanol and, after repetition of this procedure and re-extraction of the aqueous-methanolic layer with light petroleum (2 × 150 c.c.), the combined petroleum extracts were washed with aqueous methanol (1 : 1; 100 c.c.) (other procedures, *e.g.*, extraction of an ethereal solution with aqueous alkali, led to inseparable emulsions). Evaporation of the dried (Na_2SO_4) petroleum solution gave the non-acid material (iii) (9 g.). Dilution of the aqueous-methanolic extracts with water, followed by acidification (hydrochloric acid), and extraction with ether afforded the acids (iv) (35 g.).

The acids (iv) were converted into the methyl esters by refluxing them with methanol (250 c.c.) and sulphuric acid (8 g.) for 8 hr. After dilution with water, the product was collected with light petroleum, and the petroleum extract washed, successively, with aqueous-methanolic potassium hydroxide and aqueous methanol, then dried (Na_2SO_4) and distilled. The following fractions were collected: (*a*) b. p. <190°/0·2 mm. (23 g.); (*b*) b. p. 190—225°/0·2 mm. (5·8 g.).

Fraction (*b*) (strongly coloured) was chromatographed in light petroleum over silica (The British Drug Houses Ltd.). Elution with light petroleum yielded a pale yellow oil (5·2 g.), $[\alpha]_D^{17}$ +7·4° (*c*, 25·95); further elution with benzene gave a viscous oil (0·5 g.) which had no measurable rotation.

The extract (*B*), hydrolysed and worked up essentially as described for (*A*), afforded on distillation of the methyl esters, after a fore-run (20 g.) of lower-boiling materials, a fraction, b. p. 190—215°/0·2 mm. (1·5 g.), $[\alpha]_D^{18}$ +8·5° (*c*, 30·4).

The Solid Dextrorotatory Acid.—The above methyl ester, $[\alpha]_D^{17}$ +7·4° (5 g.), was hydrolysed by refluxing it with 5% methanolic potassium hydroxide (50 c.c.) for 5 hr. Most of the methanol was then removed by distillation, and the residue diluted with water. When the mixture was shaken with ether, the entire hydrolysed material was taken up by the ethereal extract (on washing of the ethereal layer with water, followed by the addition of some methanol, the potassium salts were found to pass partly into the aqueous phase from which they were precipitated by potassium ions, *e.g.*, by the addition of potassium hydroxide). Cooling the ethereal solution to about 10° or below precipitated a part of the potassium salts and this property was utilized for separating the potassium salts into two fractions by the following procedure.

The above ethereal extract was shaken with dilute hydrochloric acid, dried (Na_2SO_4), and

evaporated. The residual acidic material was converted into the potassium salts by the addition of the calculated amount of 5% methanolic potassium hydroxide; the methanol was then removed under reduced pressure. To the residue (dried in a desiccator) ether (50 c.c.) was added, and the solution evaporated on a steam-bath under reduced pressure. The remaining dry potassium salts were dissolved in ether (100 c.c.) by heat under reflux, and the solution was set aside at 0° overnight. The precipitated potassium salts were filtered off by gravity, washed with ice-cold ether, and then dissolved in warm ether (50 c.c.). This solution was kept at 0° for 3 hr.; the precipitated potassium salts were then collected as described above and converted into the free acids by shaking them with ether and dilute hydrochloric acid. The product (2·6 g.) was a solid, having $[\alpha]_D^{20}$ +7·9° (*c*, 25·2), n_D^{36} 1·4600, $\lambda_{max.}$ 2200 Å (log ε 3·94) (Found: C, 78·8; H, 12·5. Calc. for $C_{27}H_{52}O_2$: C, 79·4; H, 12·75%).

The Liquid Dextrorotatory Acid.—The combined ethereal mother-liquors from the potassium salts isolated according to the preceding section were evaporated, and the residue was shaken with ether and dilute hydrochloric acid. Evaporation of the dried (Na_2SO_4) ethereal extract gave a viscous liquid (1·9 g.). This was passed in light petroleum (30 c.c.) through a column of silica (10 × 1 cm.), made up with the same solvent. Elution with light petroleum and evaporation of the solvent gave a pale yellow oil (1·3 g.), b. p. 192—197° (bath)/0·01 mm., $[\alpha]_D^{16}$ +10·3° (*c*, 25·4), n_D^{36} 1·4655, $\lambda_{max.}$ 2200 Å (log ε 4·04) (Found: C, 79·2; H, 12·8%).

Hydrogenation of the Solid Dextrorotatory Acid.—A solution of the acid (0·5 g.) in glacial acetic acid (50 c.c.) was shaken under hydrogen (2 atm.) in the presence of platinic oxide (0·1 g.) at room temperature overnight. The filtered solution was distilled, to give the saturated *acid*, $[\alpha]_D^{20}$ +1·1° (*c*, 11·2), n_D^{36} 1·4516, and exhibiting no absorption at $\lambda > 2100$ Å (Found: C, 78·9; H, 13·3. $C_{27}H_{54}O_2$ requires C, 79·1; H, 13·2%).

Hydrogenation of the Liquid Dextrorotatory Acid.—The product, obtained as above, was an oil, $[\alpha]_D^{18}$ +1·7° (*c*, 9·4), n_D^{36} 1·4563 (Found: C, 79·2; H, 13·2%), no absorption at $\lambda > 2100$ Å.

Ozonization of the Solid Dextrorotatory Acid.—Ozonized oxygen was passed through an ice-cold solution of the acid (0·6 g.) in carbon tetrachloride (10 c.c.) for 3·5 hr., and then through saturated aqueous barium hydroxide (for determination of carbon dioxide). The solvent was then removed at room temperature by passage of a rapid stream of nitrogen; water (10 c.c.) was added, and the mixture heated slowly to 100° (bath) and kept there for 30 min. During these procedures the ozonization vessel was connected in series with (1) a trap cooled with solid carbon dioxide–acetone, (2) a solution of 2 : 4-dinitrophenylhydrazine in aqueous hydrochloric acid, (3) a trap, (4) saturated aqueous barium hydroxide, and (5) a soda-lime tube; a slow current of nitrogen was maintained. The aqueous ozonization mixture was then cooled with ice and filtered, and the filtrate (*a*) put aside. The carbon tetrachloride solution which collected in the cooled trap was washed with aqueous 5% potassium hydroxide (3 × 3 c.c.), and the alkaline extract distilled under a current of nitrogen into a solution of 2 : 4-dinitrophenylhydrazine in aqueous hydrochloric acid until onlya few c.c. remained (no precipitate of 2 : 4-dinitrophenylhydrazones was obtained). The residual alkaline solution was then acidified (sulphuric acid) and distilled. The distillate was combined with the above filtrate (*a*), and neutralized with 0·1N-sodium hydroxide (phenolphthalein) (this titration indicated a yield of about 0·7 mol. of acetic acid). The neutralized solution was concentrated to a small bulk, *p*-bromophenacyl bromide (0·27 g.) in ethanol (5 c.c.) added, and the mixture refluxed for 2 hr. The product, after crystallization from methanol, had m. p. 81—83°, undepressed on admixture of an authentic specimen of *p*-bromophenacyl acetate, m. p. 84—85° (Found: Br, 31·0. Calc. for $C_{10}H_9O_3Br$: Br, 31·1%). The amount of carbon dioxide collected during the ozonization and the decomposition of the ozonide corresponded to about 0·3 mol. During the decomposition of the ozonide a slight precipitate, identified as acetaldehyde 2 : 4-dinitrophenylhydrazone, was formed. No oxalic acid was found among the ozonization products.

Isolation of the Lævorotatory Acid.—From the hydrolysis of the extract (*A*) already described a methanol-insoluble product (i) resulted (see above). This was combined with similar material from earlier work, and the product (about 250 g.) refluxed with potassium hydroxide (65 g.) in methanol (320 c.c.) and benzene (1280 c.c.) for 215 hr. (a much shorter period is probably sufficient). On dilution with water and acidification (hydrochloric acid) troublesome emulsions were formed. These were shaken with several portions of ether and, after being kept overnight, as much as possible of the clear organic layers was removed. The remaining emulsified portion was poured on sodium sulphate, and again extracted with ether. The combined benzene–ether extracts were dried (Na_2SO_4) (attempts to wash them with water until free from hydrochloric acid had to be abandoned owing to the formation of persistent emulsions), and concentrated (to about 700 c.c.), and to the concentrate methanol (1700 c.c.) was added. Next

morning the precipitated tar was removed and the remaining solution evaporated. The residue was refluxed with 15% ethanolic potassium hydroxide (80 c.c.) for 4 hr. (to hydrolyze any esters formed in the previous stage), and the product obtained on acidification (hydrochloric acid) was collected by means of ether. The solvent was then evaporated, and water removed from the residue azeotropically by the addition of benzene, followed by evaporation to dryness under reduced pressure on the steam-bath. The product was dissolved in hot light petroleum (250 c.c.), and the solution kept overnight at about 10°. The precipitated material, removed by filtration, had after recrystallization from ethyl acetate, m. p. 72—73·5°, and corresponded in its properties to those given by Ginger and Anderson (*J. Biol. Chem.*, 1945, **157**, 213) for the alcohol "phthiocerol." The remaining light petroleum solution was evaporated, and the residue extracted with several portions of hot methanol (altogether 500 c.c.). After removal of the methanol, the residue was taken up in ether, and then shaken with 5% aqueous potassium hydroxide (200 c.c.). The ethereal layer which contained the potassium salts was separated (pouring a few c.c. of methanol along the walls of the separating-funnel facilitated the separation of the layers) and evaporated, and the residue dried (desiccator). The product was then dissolved in ether (150 c.c.) by heat under reflux in a few minutes, and kept overnight at 10°. The fine, hard precipitate (more concentrated ethereal solutions gave gelatinous products) was collected, and this process was repeated with fresh quantities of ether. The product, which was very sparingly soluble in cold ether, was then converted into the free acid by the addition of dilute hydrochloric acid (steam-bath). The acid (10·5 g.), isolated by means of ether, had $[\alpha]_D^{18}$ −8·7° (*c*, 20·9). A further quantity (2·5 g.) of the acid showing the same specific rotation (*c*, 25·2) was obtained from the *iso*propyl ether extract (*B*) of the bacterial cells essentially by the same procedure as described above for the acetone extract (*A*). These products were combined and converted into the methyl esters by refluxing methanol (150 c.c.) and concentrated sulphuric acid (3 c.c.) during 18 hr. (a shorter period is sufficient). The product, isolated in the usual manner, afforded on distillation the fractions: (1) 195—210°/0·4 mm. (0·6 g.), $[\alpha]_D^{13}$ −8·1° (*c*, 12·6); (2) 210—222°/0·3 mm. (2·2 g.), $[\alpha]_D^{13}$ −8·8° (*c*, 11·35); and (3) 223—226°/0·3 mm. (8·9 g.), $[\alpha]_D^{13}$ −10·3° (*c*, 26·2).

Fraction (3) (Found: C, 80·1; H, 13·2. Calc. for $C_{32}H_{64}O_2$: C, 80·0; H, 13·3%) exhibited no high-intensity absorption of ultra-violet light at $\lambda > 2100$ Å. A sample (2 g.) was hydrolysed by potassium hydroxide (2 g.) in anhydrous refluxing methanol (20 c.c.) for 6 hr. After the mixture had been kept overnight at 10° the resulting potassium salt was separated from the solution. The free acid, obtained by treatment with dilute hydrochloric acid and extraction with ether, was a white solid, m. p. 30° (microscope hot-stage), $[\alpha]_D^{21}$ −6·9° (*c*, 16·8), n_D^{35} 1·4541 (Found: C, 80·1; H, 13·2%; equiv., 464. Calc. for $C_{31}H_{62}O_2$: C, 79·8; H, 13·3%; equiv., 466). The m. p., specific rotation, and refractive index showed no change after the acid (0·5 g.) had been shaken in glacial acetic acid (50 c.c.) under hydrogen (2 atm.) in the presence of platinic oxide for 24 hr. The acid formed a *p*-bromophenacyl ester, m. p. 31·5° (microscope hot-stage) (Found: C, 70·7; H, 10·1. Calc. for $C_{39}H_{67}O_3Br$: C, 70·6; H, 10·1%).

Stepwise Degradation of the Lævorotatory Acid.—The above acid (3·3 g.) was heated with bromine (5·8 g.) in the presence of red phosphorus (0·25 g.) on a steam-bath for 8 hr. After removal of the excess of bromine under diminished pressure, the product was shaken with water, and the mixture set aside at the room temperature for 3 hr. The product, collected by means of ether, was then heated with a solution of potassium hydroxide (3 g.) in water (120 c.c.) at 100° for 24 hr. Acidification with dilute hydrochloric acid, followed by extraction with ether, afforded the crude hydroxy-acid. This was dissolved in dry benzene (100 c.c.) and oxidized by means of lead tetra-acetate (3·2 g.; added in two portions) at 60° (bath) for 3 hr. After decomposition of the excess of lead tetra-acetate by addition of a solution of glycerol in glacial acetic acid, the solution was filtered, washed with dilute hydrochloric acid, then with water, and dried (Na_2SO_4). The benzene was distilled off, and the residue, dissolved in ether, was shaken with 5% aqueous potassium hydroxide. The ethereal layer was separated and kept overnight at about 10°, and the precipitated potassium salts were removed. The remaining solution was evaporated, and the residue heated with formic acid (98%; 10 c.c.) and hydrogen peroxide (30%; 3 c.c.) at 60° (bath) for 2 hr. (to oxidize any aldehydes present). After dilution with water, the mixture was extracted with ether, and the ethereal extract washed with 5% aqueous potassium hydroxide, then with water, and dried (Na_2SO_4). Removal of the solvent left a *ketone* (0·8 g.), b. p. 190—200° (bath)/0·02 mm., $[\alpha]_D^{18}$ +2·1° (*c*, 7·8) (Found: C, 81·9; H, 13·8. $C_{30}H_{60}O$ requires C, 82·6; H, 13·8%). It formed a *semicarbazone*, m. p. 69—70° after crystallization from ethanol (Found: C, 75·0; H, 13·1; N, 8·8. $C_{31}H_{63}ON_3$ requires C, 75·5; H, 12·8; N, 8·5%).

One of us (J. D. C.) participated in the work during leave of absence from the Mount Sinai Hospital, New York.

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