organ in question. There were scattered deformed nuclei present but no clear cellular outlines. Occasionally somewhat larger irregular masses could be recognized as containing connective tissue and fragments of blood vessels. The homogenates remain stable for considerable periods and can be pipetted with ordinary pipettes without previous filtration; some sedimentation slowly occurs on standing over some hours, but this heavier material can be re-distributed by shaking to give relatively stable homogeneous fluids from which accurate samples can be taken. Lactating mammary tissue also gives good homogenates in 5 min., but these contain small strands of fibrous-connective tissue which should be removed by filtration through glass wool before pipetting. These strands are more prominent in homogenates of involuting mammary gland, which is much more generously interlaced with fibrous-connective stroma, but it is believed that all of the glandular tissue is comminuted. Even with tough tissues like rat's skin, tendon or the cervical region of the uterus, dispersion for 5 min. in this apparatus

produces a surprising degree of comminution. These and many other tissues, which the machine can handle with ease, present a formidable proposition when ground with sand in a pestle and mortar.

The contents of the dispersion vessel tend to warm up during homogenization, and if necessary can be cooled by packing the space between the dispersion vessel and the container with ice. Alternatively, it is easy to maintain the tissue at body temperature from the time of removal from the animal until the start of the enzyme determination.

SUMMARY

A machine for the easy preparation of uniform tissue homogenates for enzyme studies is described.

Our best thanks are due to Mr K. C. Richardson for histological examination of tissue homogenates. We are also indebted to Mr K. W. Gladwell of Messrs Nelco Ltd., Shalford, Guildford, for invaluable help with the production of the machine, and to Mr C. Machin for the drawings.

REFERENCES

- Folley, S. J. & Greenbaum, A. L. (1946). *Biochem. J.* **40**, 46.
Folley, S. J. & Greenbaum, A. L. (1947). *Biochem. J.* **41**, 261.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.

Component Acids of the Lipids of Human Tubercle Bacilli. Part 1

By N. POLGAR, *Dyson Perrins Laboratory, Oxford University*

(Received 10 June 1947)

Investigations by Anderson and his associates on the chemistry of the lipids of various acid-fast bacilli demonstrated the presence in the bacterial lipids of certain new and specific saturated branched-chain fatty acids of high molecular weight. The acids gave ether-soluble lead salts, and this property was utilized for their isolation from the original mixtures; the separation of the individual acids was accomplished by fractional distillation of the corresponding methyl esters.

Tuberculostearic acid and phthioic acid, isolated by the above procedure from the acetone-soluble fat of the human type tubercle bacillus, strain H-37, appeared to be characteristic components of the lipids of human tubercle bacilli, although the composition of the lipids was found to vary from strain to strain (cf. Crowder, Stodola, Pangborn & Anderson, 1936). More recently studies of cell residues from the preparation of the purified tuberculin protein PPD, derived from an unidentified strain of the

human tubercle bacillus, indicated the presence of various branched-chain fatty acids which had not been encountered in previous investigations (Peck & Anderson, 1941; Edens, Creighton & Anderson, 1944; Ginger & Anderson, 1944). According to the last-mentioned paper a dextrorotatory ester fraction, which resembled methyl phthioate (cf. Spielman & Anderson, 1935-6), gave on extended fractionation four fractions varying in composition and in the magnitude of optical rotation.

Investigations in this laboratory (Polgar & Robinson, 1945) led to the synthesis of a number of methyl-substituted long-chain acids, of which 3:13:19-trimethyltricosanoic acid exhibited properties resembling those of phthioic acid. Biological studies by Dr C. E. Coulthard, of the Research Department of Boots Pure Drug Co. Ltd., and Dr J. Ungar, of Glaxo Laboratories Ltd., indicated that the synthetic acid produces in experimental animals lesions similar to the tubercle, the characteristic

lesion of tuberculosis; the activity of the acid in this respect seemed about equal to that reported for phthioic acid.

PRESENT INVESTIGATION

Characterization of fatty acids through semicarbazones of their acetol esters

In view of the above position, further studies seemed desirable. On searching for derivatives, which would facilitate the characterization and purification of such acids, the acetol esters, $R \cdot \text{CO}_2\text{CH}_2 \cdot \text{CO} \cdot \text{CH}_3$, were found to provide a suitable starting point. Semicarbazones of such esters have been described (Locquin, 1904). In the present investigation, acetol esters of various acids were obtained in almost quantitative yields by refluxing an alkali salt of the acid in ethanolic solution with an excess of chloroacetone. On treatment with semicarbazide, the acetol esters of palmitic and stearic acids gave semicarbazones (m.p. 111.5 and 112.5–113.5°, respectively), which were very sparingly soluble in cold ethanol, whereas the semicarbazones of acetol 13:16-dimethyltricosanoate and acetol 13:17:21-trimethyldocosanoate (m.p. 67–68° and 70–71°, respectively), obtained in the same way, were comparatively soluble in this solvent, and the semicarbazone of acetol 3:13:19-trimethyltricosanoate was an easily soluble oil. A less soluble and higher melting derivative of the last-mentioned ester could be obtained by treatment with 2:4-dinitrophenylsemicarbazide (McVeigh & Rose, 1945); the resulting *dinitrophenylsemicarbazone* (m.p. 137–138°) was a pale yellow substance which also seemed well suited for application to chromatographic analysis.

The acids could be easily recovered from the foregoing derivatives by alkaline or acid hydrolysis. The acids obtained on hydrolysis of the dinitrophenylsemicarbazones were contaminated with nitro compounds derived from the reagent, but the latter could be readily removed by treating the products with tin and hydrochloric acid, the amines formed by the reduction being easily soluble in the aqueous medium.

Fractionation of fatty acids from human tubercle bacilli

These findings suggested that it might be possible to apply the fractional crystallization of such derivatives to the mixed acids of the tuberculolipids as a step towards isolating the individual acids. The lipids of the residues of human tubercle bacilli were extracted by known procedures and hydrolyzed with ethanolic potassium hydroxide. The acidified product, after removing incompletely hydrolyzed waxy substances by precipitation from an ethereal solution with methanol, was subjected to the scheme of separation described below.

As a preliminary to the application of the fractional crystallization processes, the whole mixture of fatty acids was separated, by fractional distillation of the corresponding methyl esters, into three fractions; each fraction was then converted in the manner already described into acetol esters and thence into semicarbazones. Crystallization of the latter from ethanol gave a sharp separation from very sparingly soluble products which were mainly derived from normal-chain acids. The remaining mixtures were further separated by fractional crystallization and the products so obtained eventually subjected to alkaline hydrolysis to regenerate the corresponding acids.

The ethanolic mother liquors remaining from the above crystallizations contained easily soluble substances of low melting points. After regeneration of the acids from the isolated products, the resulting mixture of acids was oxidized with hydrogen peroxide in formic acid (cf. Swern, Billen, Findley & Scanlan, 1945), the purpose of this operation being to convert unsaturated substances into hydroxylated derivatives. The free hydroxy acids were then isolated by treatment with light petroleum in which they were very sparingly soluble. The remaining petroleum solution contained saturated branched-chain acids which were subjected to further separations by conversion into acetol esters, and then into 2:4-dinitrophenylsemicarbazones. The latter were crystallized from various solvents, and eventually the corresponding acids recovered by hydrolysis of the products. On applying the above scheme to the three methyl-ester fractions obtained from the crude mixture of fatty acids, the lowest ester fraction (b.p. 120–130°/0.5 mm.) appeared to consist mainly of methyl palmitate, and has not been investigated further.

The intermediate fraction (b.p. about 140–160°/0.7 mm.) yielded, on conversion of the acids into acetol esters and thence into semicarbazones, among other products a *semicarbazone* melting at 72–73° which seemed to be derived from a C_{19} acid, in the following referred to as acid A. The regenerated and purified acid was a low-melting solid, m.p. 23–24°; for comparison with tuberculostearic acid it has been further characterized as its amide, m.p. 78–79°, and by the preparation of the tribromoanilide, m.p. 97.5–98°. The melting points of these derivatives correspond within a few degrees to those recorded for the amide and tribromoanilide of tuberculostearic acid (76–77 and 93–94°, respectively; cf. Spielman, 1934), but there is a discrepancy of about 12° between the melting point of the free acids (23–24° as against 10–11°). It may be recalled that Spielman, ascribing to tuberculostearic acid the structure of 10-methylstearic acid, found a similar divergency between the melting point of tuberculostearic acid (10–11°) and that of the

synthetic acid (20–21°), whereas the amides and tribromoanilides had identical melting points. Spielman considered the possibility that tuberculostearic acid may contain, in addition to 10-methylstearic acid, another acid of similar structure, such as 9-methylstearic acid, which could have escaped detection. However, he preferred the explanation that the difference in the melting points of the acids was due to the presence of an asymmetric carbon atom.

In the present experiments, the substances remaining in the mother liquors of the above semicarbazone derived from acid A were hydrolyzed to recover the corresponding acids, and the latter oxidized with hydrogen peroxide in formic acid; after removing hydroxy acids, apparently mainly consisting of dihydroxystearic acid (formed by oxidation of oleic acid), an oil remained which seemed to contain a saturated branched-chain acid different from acid A. It will be necessary to investigate this substance more closely, in order to clarify the position.

The present work was mainly concentrated upon the higher methyl-ester fraction (b.p. about 170–215°/0.7 mm.). This yielded, by application of the same processes, three saturated branched-chain acids, in the following referred to as B1, B2 and B3; besides, a dihydroxy acid, B4, which was obtained by the use of the hydrogen peroxide-formic acid procedure. Acid B1 was isolated as its *acetol-ester semicarbazone*, m.p. 44–45°. The regenerated acid, which had m.p. 25° and $[\alpha]_D + 4.8^\circ$, was further characterized by a series of derivatives, namely, as its *amide*, m.p. 46°, *tribromoanilide*, m.p. 64–65°, and *p-bromophenacyl ester*, m.p. 33–34°. The analytical values indicate $C_{30}H_{60}O_2$ or its near homologues as possible formulae for the parent acid. It may be mentioned in this connexion that a C_{30} acid, designated mycocerosic acid, has been isolated by Anderson and his associates from the waxes of human tubercle bacilli (cf. Ginger & Anderson, 1945), but this acid was laevorotatory, and its *p-bromophenacyl ester* had m.p. 47–48°.

Acid B2 was obtained as its *acetol-ester semicarbazone*, m.p. 81–82°, from the mother liquors of the semicarbazone derived from acid B1. The acid had m.p. 22° and was also optically active, $[\alpha]_D + 11.7^\circ$. It formed an *amide*, m.p. 44°, and a *tribromoanilide*, m.p. 47°. From the analytical figures for these derivatives, the acid may have the composition $C_{27}H_{54}O_2$ or $C_{28}H_{56}O_2$. The acetol-ester semicarbazone derived from acid B3 was liquid at room temperature and very easily soluble in organic solvents; it remained in the mother liquors which resulted from the fractional crystallizations of the semicarbazones derived from the acids B1 and B2. The recovered acid, after removing an unsaturated component (B4, discussed below) by the hydrogen peroxide-formic acid process, and admixed low-boiling acids by fractionation of the methyl esters,

was converted into its acetol ester, and thence into the 2:4-dinitrophenylsemicarbazone. The latter had m.p. 153–154.5°, and the analytical figures agreed with those required for phthioic acid, $C_{26}H_{52}O_2$, as the parent acid. The regenerated acid solidified at 0° but melted at room temperature; its specific rotation was about +11°. The amide showed very poor crystallizing properties, and the tribromoanilide was obtained as a low-melting solid which liquefied at room temperature. The acid obviously requires further purification. Kuhn-Roth determination of C-methyl gave 3.4 mol. of acetic acid from acids B1 and B3, and 2.9 mol. from acid B2, as compared with 2.1 mol. of acetic acid in case of acid A. Assuming that the last is a monomethylstearic acid, comparison of the above values would indicate the presence of possibly four terminal methyl groups in case of the acids B1 and B3, and three such groups in acid B2.

Preliminary studies of monolayers of the acids B1, B2 and B3 indicate that only acid B3 exhibits properties like those characteristic for phthioic acid (cf. Stenhagen & Stållberg, 1941), collapsing on compression at about 40 Å.²; acids B1 and B2 appear to give unstable films continually collapsing on compression.

It remains to describe briefly the *dihydroxy acid* B4 which has been mentioned above in connexion with the isolation of acid B3. It had m.p. 69–70°, and the analytical data were in agreement with a composition $C_{26}H_{52}O_4$ (or near homologues). Oxidation with chromic acid by the Kuhn-Roth method gave 1.6 mol. acetic acid, indicating the presence of two terminal methyl groups.

The results of this preliminary study indicate that a certain separation of the mixture of acids can be achieved by employing the scheme described. However, it will be necessary, in larger-scale experiments, to supplement the above processes by efficient ester fractionations.

For comparison with the above results, the same scheme of separation has been recently applied by the present author to a mixture of acids from the acetone-soluble fat of *Mycobacterium phlei*, a quantity of which was kindly provided by Imperial Chemical Industries Ltd., Dyestuffs Division. The results of these studies will be published in a later communication, but they may be anticipated by stating that the saturated branched-chain acids of higher molecular weight produced by this non-pathogenic member of the acid-fast group appear to be entirely different from those of the human tubercle bacillus.

EXPERIMENTAL

Analyses, including C-methyl determinations, are by Drs Weiler and Strauss, Oxford.

Acetol esters. The following preparation of *acetol palmitate* is typical: A solution of palmitic acid (0.5 g.) in ethanol

(10 ml.) was neutralized with ethanolic KOH, using phenolphthalein as indicator; chloroacetone (0.5 g.) was then added and the mixture refluxed for 2 hr. Next day, water was added and the product collected by ether. The ethereal extract was washed thoroughly with water, and the residue remaining after evaporation of the ether solution heated at 100° under reduced pressure in order to remove completely any unreacted chloroacetone. The resulting ester (yield, practically quantitative) crystallized from ethanol in the form of needles, m.p. 50°. (Found: C, 73.2; H, 11.6. $C_{19}H_{36}O_3$ requires C, 73.1; H, 11.5%.) On treatment with semicarbazide acetate in ethanolic solution it gave a *semicarbazone*, m.p. 111.5° (from ethanol). (Found: C, 65.3, 65.5; H, 10.2, 10.6. $C_{20}H_{39}O_3N_3$ requires C, 65.0; H, 10.6%.)

The following *semicarbazones* were similarly prepared: *acetol stearate semicarbazone*, m.p. 112.5–113.5° (Found: C, 66.7; H, 10.8. $C_{22}H_{43}O_3N_3$ requires C, 66.5; H, 10.8%); *acetol 13:16-dimethyltricosanoate semicarbazone*, m.p. 67–68° (Found: C, 70.0; H, 11.3. $C_{28}H_{57}O_3N_3$ requires C, 70.3; H, 11.5%); *acetol 13:17:21-trimethyltricosanoate semicarbazone*, m.p. 70–71° (Found: C, 70.25; H, 11.3. $C_{28}H_{57}O_3N_3$ requires C, 70.3; H, 11.5%).

Acetol 3:13:19-trimethyltricosanoate, on treatment with semicarbazide acetate, gave an oil. Its 2:4-dinitrophenyl-*semicarbazone*, obtained by short refluxing of an ethanolic solution of the ester with the calculated amount of 2:4-dinitrophenylsemicarbazide, was a pale yellow powder, m.p. 137–138°, after recrystallization successively from ethanol and benzene-ethanol. (Found: C, 64.2; H, 8.75. $C_{38}H_{61}O_7N_3$ requires C, 64.0; H, 9.0%.)

Extraction of the lipids of tubercle bacilli. The bacterial cells used for this investigation were residues from the manufacture of tuberculin (PPD), kindly provided by the Veterinary Laboratory, Weybridge, of the Ministry of Agriculture and Fisheries. They were derived from three human strains of *Myc. tuberculosis*, designated 'mixed P.N., D.T. and C', which were grown on a synthetic medium (glycerol, glucose, asparagine) for 10 weeks, and then killed by the action of steam. The bacterial cells were extracted by successive treatment with ethanol, ethanol-ether (1:1), and ether-chloroform (1:1) at room temperature. The solutions were decanted, filtered from the bacterial cells, and evaporated under reduced pressure. The residual products were then combined and extracted with ether.

Isolation of the mixed fatty acids. The above ethereal extract was evaporated, and the residue (about 270 g.) hydrolyzed by refluxing for 2.5 hr. with a solution of KOH (40 g.) in water (40 ml.) and ethanol (120 ml.). Under these conditions the fats are hydrolyzed, but the waxes incompletely or only to a slight extent. After addition of water and acidification, the product was collected by means of ether, and waxy substances separated by precipitation of the ethereal solution with methanol. The solution remaining after removal of the precipitate was evaporated and the residue (80 g.) converted into methyl esters by refluxing for 3 hr. with 10% (w/v) methanolic H_2SO_4 .

Fractionation of the methyl esters. The following fractions were collected: a low-boiling fraction, b.p. 120–130°/0.5 mm. (7.4 g.); a middle fraction, b.p. 140–160°/0.7 mm. (41 g.); and a high-boiling fraction, b.p. 170–215°/0.7 mm. (8.3 g.).

The low-boiling fraction. This was only tentatively tested. A small sample was hydrolyzed and the acid converted into its acetol ester, and thence into the semicarbazone in the

manner already described. The semicarbazone was obtained as a white crystalline powder, m.p. 110–110.5° after crystallization from ethanol. (Found: C, 64.5; H, 10.5. $C_{20}H_{39}O_3N_3$ requires C, 65.0; H, 10.6%.) There was no depression on admixture with the semicarbazone of acetol palmitate; however, this is not of great significance, as the melting points of several acetol-ester semicarbazones derived from normal-chain acids lie close together and are not depressed on admixture. Hydrolysis of the above semicarbazone gave an acid, m.p. 61° alone or mixed with palmitic acid.

The middle fraction. The ester (41 g.) was hydrolyzed by refluxing with ethanolic KOH for 3 hr. Water was then added, and the alkaline solution extracted with ether; a small quantity (0.4 g.) of a non-acidic product was thus obtained. Acidification of the alkaline solution gave a mixture of acids which were isolated by ether extraction. After removal of the ether, the product was warmed with ethanol (50 ml.) and the solution kept for 3 hr. It deposited 5 g. of a solid, m.p. 52–53°, probably mainly a mixture of normal-chain acids.

The remaining solution was neutralized with ethanolic KOH; a large excess of chloroacetone (17 g.) was then added and the mixture refluxed for 3 hr. The product, isolated in the manner already described, was dissolved in ethanol (200 ml.) and brought to reaction with semicarbazide acetate (from 16 g. of semicarbazide hydrochloride and 24 g. of sodium acetate in 20 ml. of water). Next day the precipitated mixture of semicarbazones was filtered off; recrystallization from ethanol gave a sharp separation from a very sparingly soluble product (12 g.), m.p. 98–100°, probably mainly derived from normal-chain acids. On addition of water to the mother liquors a semicarbazone, m.p. 68° (sintering at 63°), separated; after two further crystallizations from aqueous ethanol the product was obtained as minute needles (5 g.), m.p. 72–73°. (Found: C, 66.8, 66.7; H, 10.7, 10.8. $C_{23}H_{45}O_3N_3$ requires C, 67.2; H, 10.95%.) The corresponding acid, regenerated from the semicarbazone by hydrolysis with ethanolic KOH, was a mobile oil. To remove admixed unsaturated substances, the product was heated with formic acid (98%; 10 ml.) and hydrogen peroxide (30%; 2.5 g.) at 60° (bath temperature) with stirring for 1 hr. Next day water was added and the product, isolated by ether extraction, refluxed with ethanolic KOH, in order to hydrolyze the formoxy compounds which were formed in the foregoing process. On keeping the free acid with light petroleum (b.p. 40–60°) a small quantity of a substance, m.p. 78°, was deposited. The remaining solution was then evaporated and the residual product esterified by means of methanolic H_2SO_4 . The methyl ester distilled as a colourless liquid (2.5 g.), b.p. 157–160°/1 mm., $n_D^{25} 1.4480$. Hydrolysis of the ester gave the corresponding acid (A), m.p. 23–24°, C-methyl, 10.5%. It gave an amide, m.p. 78–79° (from methanol). (Found: C, 76.5; H, 12.8. Calc. for $C_{19}H_{39}ON$: C, 76.8; H, 13.1%.) The tribromoanilide crystallized from ethanol in needles, m.p. 97.5–98°. (Found: C, 48.8; H, 6.3. Calc. for $C_{28}H_{46}ONBr_3$: C, 49.2; H, 6.6%.)

On concentrating the mother liquors of the semicarbazones described in the preceding paragraph an oil separated. Water was added, and the product isolated with ether. It was hydrolyzed by means of ethanolic KOH, and the acidified product extracted with ether. The product remaining after evaporation of the ethereal extract was then oxidized by hydrogen peroxide (30%; 7 g.) in formic acid (98%; 25 ml.) at 50° (bath temperature) for 1 hr., while

stirring the mixture. Next day water was added and the product, isolated by ether extraction, refluxed with ethanolic KOH for 2 hr. After isolation of the acidified product (ether extraction), it was treated with light petroleum (b.p. 40–60°). The substance (5 g.), which separated from the petroleum solution, had m.p. 78°, raised to 93.5° after two crystallizations from 95% ethanol. (Found: C, 68.3; H, 11.05. Calc. for $C_{18}H_{36}O_4$: C, 68.4; H, 11.4%.) From its melting point and analytical values this substance appears to be 9:10-dihydroxystearic acid (m.p. 95°), arising from the oxidation of oleic acid.

The remaining petroleum solution seemed to contain a saturated branched-chain acid; this will be investigated more closely when opportunity permits.

The high-boiling fraction. The ester (8.3 g.) was hydrolyzed with ethanolic KOH. On attempting to remove non-acidic products, by ether extraction of the alkaline solution, emulsions resulted; therefore, the solution was acidified and the product collected with ether. The ethereal solution was then evaporated and the residue kept with ethanol (20 ml.); a crystalline product (0.5 g.) of m.p. 82–83° separated, probably mainly normal-chain acid. (Found: C, 78.0; H, 12.7. Calc. for $C_{24}H_{48}O_2$: C, 78.3; H, 13.0%.) The remaining ethanolic solution was neutralized with sodium ethoxide (0.5 g. of sodium in 10 ml. of ethanol); an excess of calcium chloride in ethanolic solution was then added and after keeping the mixture for several hours, the precipitated calcium salt filtered off. This was decomposed with HCl, and the liberated acid isolated with ether (weight of substance, 4.8 g.). The ethanolic mother liquors of the calcium salt were evaporated and the residue, after treatment with HCl, isolated by ether extraction. The ethereal solution, after washing it first with aqueous KOH and then thoroughly with water, yielded on evaporation 0.8 g. of non-acidic material. The combined alkaline and aqueous washings were acidified and extracted with ether; evaporation of the ether left 1.7 g. of acidic substance which was combined with the material obtained from the precipitated calcium salt.

The above acid (6.5 g.) was neutralized with a solution of sodium ethoxide in ethanol (from 0.5 g. of sodium in 10 ml. of ethanol), and the product brought to reaction with an excess of chloroacetone (7 g.) by refluxing the mixture for 5 hr. The resulting acetol ester was then treated in ethanolic solution with semicarbazide acetate in the usual manner. On crystallization of the crude semicarbazone from ethanol a very sparingly soluble substance (0.22 g.) having m.p. 103–104.5°, probably derived from normal-chain acids, separated first. From the mother liquors two additional *semicarbazones* were obtained by fractional crystallization; one of these (B1, 1 g.) had m.p. 44–45°. (Found: C, 72.4, 72.1; H, 11.6, 11.7. $C_{34}H_{67}O_3N_3$ requires C, 72.2; H, 11.9%), and the other (B2, 0.45 g.), which separated on concentration of the combined ethanolic mother liquors of the foregoing semicarbazone, melted at 81–82°. (Found: C, 71.55; H, 11.4. $C_{32}H_{63}O_3N_3$ requires C, 71.5; H, 11.7%.) Further concentration of the mother liquors of this product gave an oil which was isolated by the addition of water, followed by ether extraction. It was then hydrolyzed with ethanolic KOH and the recovered acid (about 4.5 g.) subjected to a repetition of the above processes (conversion into acetol esters, and thence into semicarbazones). A small quantity (0.3 g.) of solid semicarbazones was obtained which on crystallization from ethanol yielded further

amounts of the semicarbazones B1 and B2; the main product was again an oil. The latter was subjected to the following processes.

The recovered acid was treated with hydrogen peroxide (30%; 2 g.) in formic acid (98%; 12 ml.) at 50° for 1.5 hr. in the manner previously described. After hydrolyzing the resulting formoxy compounds by means of ethanolic KOH, the free acid (isolated by ether extraction) was refluxed with light petroleum (b.p. 40–60°) for a few minutes, and the mixture allowed to stand overnight. It deposited a *substance* (acid B4, about 0.1 g.), m.p. 69–70° (after recrystallization from aqueous ethanol). (Found: C, 72.7; H, 11.9. $C_{26}H_{52}O_4$ requires C, 72.9; H, 12.15%); C-methyl, 5.5%.

The petroleum solution was then evaporated, and the residual product converted into its methyl ester by refluxing with 10% (w/v) methanolic H_2SO_4 for 3 hr. On fractionation of the ester a fore run (0.85 g.) distilled below 165°/0.7 mm.; the remainder distilled mainly at about 190–210°/0.7 mm. as a viscous oil (2.05 g.), $n_D^{17.5}$ 1.4620, $[\alpha]_D + 8.2^\circ$ (in ether; $c=11$). The product was hydrolyzed with ethanolic KOH, and the resulting acid converted by the usual procedure into its acetol ester. A solution of this ester in ethanol (40 ml.) was refluxed with 2,4-dinitrophenylsemicarbazide (1.4 g.) for about 10 min., and the solution then filtered hot. Next day the precipitated product was collected and crystallized successively from ethanol and benzene-ethanol. A *dinitrophenylsemicarbazone*, m.p. 153–154.5° was thus obtained as the main product (1 g.) which on hydrolysis, as will be described below, gave acid B3. (Found: C, 63.7; H, 8.6; N, 10.4. $C_{36}H_{61}O_7N_5$ requires C, 64.0; H, 9.0; N, 10.4%.)

Acid B1. This was obtained from the above semicarbazone, m.p. 44–45°, by hydrolysis with ethanolic KOH. The acid distilled at 210–220°/0.05 mm. as a viscous oil (0.76 g.) which quickly solidified and had m.p. 25°; $[\alpha]_D + 4.8^\circ$ (in ether; $c=15.2$); C-methyl, 11.7%. The acid was saturated, and gave an *amide*, m.p. 46° (from methanol) (Found: N, 3.1. $C_{30}H_{61}ON$ requires N, 3.1%). Its *tribromoanilide*, after three crystallizations from ethanol, melted at 64–65° (sintering at about 60°). (Found: C, 57.0; H, 7.9. $C_{38}H_{62}ONBr_3$ requires C, 56.6; H, 8.1%.) The *p-bromophenacyl* ester had m.p. 33–34° (sintering at 30°) after repeated crystallizations from ethanol. (Found: C, 69.9; H, 9.6. $C_{38}H_{65}O_3Br$ requires C, 70.3; H, 10.0%.)

Acid B2. Hydrolysis of the semicarbazone previously described, m.p. 81–82°, in the usual manner afforded the crude acid which distilled from a bath at 210–220°/0.05 mm. as a viscous oil (0.32 g.). It quickly solidified and had m.p. 22°, $[\alpha]_D + 11.7^\circ$ (in ether; $c=6.5$), C-methyl, 10.5%; the acid was saturated. It gave an *amide*, m.p. 44° (from methanol). (Found: N, 3.25. $C_{28}H_{57}ON$ requires N, 3.3%.) The *tribromoanilide* crystallized from ethanol as short needles, m.p. 47°. (Found: C, 55.0, 55.3; H, 7.6, 7.5. $C_{34}H_{56}ONBr_3$ requires C, 54.9; H, 7.8%. $C_{34}H_{58}ONBr_3$ requires C, 55.5; H, 7.9%.)

Acid B3. The isolation of the acetol-ester dinitrophenylsemicarbazone derived from this acid has been outlined above. Hydrolysis with a mixture of sulphuric acid, acetic acid and water (5 ml. of each), by refluxing the mixture for 3 hr., afforded the crude acid which was contaminated with nitro compounds. After isolating the product by ether extraction, it was heated with tin (1.2 g.) and HCl (10 ml. of conc. acid and 5 ml. of water) on a steam bath for 1 hr. Water was then added, and the acid isolated by ether. It

distilled at 200–210°/0.05 mm. as a pale yellow oil which solidified on cooling with ice, and melted at about 20°; $[\alpha]_D + 11.1^\circ$ (in ether; $c=11.1$); C-methyl, 12.8%. The amide was obtained as a low-melting solid which was very soluble in organic solvents; the tribromoanilide separated from aqueous methanol as an oil.

SUMMARY

1. A scheme of separating mixtures of the component fatty acids from the lipids of human tubercle bacilli is described. The scheme, which may be found of value in other connexions, depends upon the fractional crystallization of derivatives, obtainable by converting the acids into acetol esters, $R.CO_2CH_2.CO.CH_3$, and treating the latter with an appropriate reagent for ketones, such as semicarbazide or 2:4-dinitrophenylsemicarbazide.

2. The acids isolated by this process include a saturated C_{19} -acid, resembling tuberculostearic acid in respect of the melting points of derivatives; how-

ever, the melting points of the free acids are found to differ by about 12°, and the available evidence seems to indicate the presence of an additional acid of similar composition, having a lower melting-point.

3. From a mixture of the acids of higher molecular weight three dextrorotatory products are obtained by the above procedure; of these, two appear to contain four terminal methyl groups, and the third, three such groups.

4. The presence of an unsaturated branched-chain acid of high molecular weight is indicated by the isolation of its oxidation product; for the oxidation hydrogen peroxide in formic acid has been employed.

The author wishes to thank Prof. Sir Robert Robinson, P.R.S., for his interest, and the Therapeutic Research Corporation of Great Britain, Ltd., for a grant. A part of the work was carried out during the tenure of an I.C.I. Research Fellowship.

REFERENCES

- Crowder, J. A., Stodola, F. H., Pangborn, M. C. & Anderson, R. J. (1936). *J. Amer. chem. Soc.* **58**, 636.
 Edens, C. O., Creighton, M. M. & Anderson, R. J. (1944). *J. biol. Chem.* **154**, 587.
 Ginger, L. G. & Anderson, R. J. (1944). *J. biol. Chem.* **156**, 443.
 Ginger, L. G. & Anderson, R. J. (1945). *J. biol. Chem.* **157**, 203.
 Locquin, R. (1904). *C.R. Acad. Sci., Paris*, **138**, 1274.
 McVeigh, J. L. & Rose, J. D. (1945). *J. chem. Soc.* p. 713.
 Peck, R. L. & Anderson, R. J. (1941). *J. biol. Chem.* **138**, 135.
 Polgar, N. & Robinson, R. (1945). *J. chem. Soc.* p. 389.
 Spielman, M. A. (1934). *J. biol. Chem.* **106**, 87.
 Spielman, M. A. & Anderson, R. J. (1935–6). *J. biol. Chem.* **112**, 759.
 Stenhagen, E. & Stållberg, S. (1941). *J. biol. Chem.* **139**, 345.
 Swern, D., Billen, G. N., Findley, T. W. & Scanlan, J. T. (1945). *J. Amer. chem. Soc.* **67**, 1786.

The Determination of Glutathione and other Substances Contained in Yeast

By T. ASTRUP AND V. ØHLENSCHLÄGER, *Biological Institute, Carlsberg Foundation, Copenhagen*

(Received 16 June 1947)

In the course of an investigation of the action of glutathione on the growth of tissue cells *in vitro* (Astrup & Fischer, 1946) we made some determinations of the amount of glutathione present in Danish yeasts. During these determinations some difficulties were encountered which are of importance also for the determination of other substances contained in yeast and which, as far as we could find, have not previously been described.

METHODS

Glutathione was measured colorimetrically with nitroprusside (Fujita & Numata, 1939; Numata, 1940), using, however, $2N-NH_3$ instead of a N -solution for developing

the colour, and a standard curve made under identical conditions for comparison. Reduction of GSSG to GSH was carried out electrolytically after Dohan & Woodward (1939). Phosphorus was estimated according to Fiske & Subbarow (1925), using a Pulfrich photometer (Tørell, 1931). Glutathione was prepared by the method of Pirie (1930).

RESULTS

When metaphosphoric acid was used for extracting GSH from yeast no definite results could be reached. Thus, Fig. 1 shows a curve *A* the values of which were obtained by grinding different 1 g. lots of baker's yeast with fine sand in a mortar until a paste was formed, then 4 ml. of 5% (w/v) freshly prepared metaphosphoric acid solution were added and