Mycobactin. A Growth Factor for Mycobacterium johnei. *Part II.* Degradation, and Identification of Fragments.*

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Mycobactin has been subjected to a number of degradative procedures, and evidence is given of the presence of the following fragments: *trans*-octadec-2-enoic acid, 2-hydroxy-6-methylbenzoic acid, L-serine, and L-2-amino-6-hydroxyaminohexanoic acid. The last compound is a new and unusual type of amino-acid. The presence of an aliphatic acid of equivalent weight about 130 was also indicated.

Mycobactin is a growth factor for *Mycobacterium johnei*, isolated from *Myco. phlei*. Its extraction and general properties were described in Part I * in which evidence was presented that its empirical formula is $C_{47}H_{75}O_{10}N_5$. Among the degradation experiments now described, acid hydrolysis provided the principal information about the general structure. Action of aqueous acid yielded mainly *trans*-octadec-2-enoic acid, *m*-cresol, L-serine, a strongly reducing amino-acid, carbon dioxide, and, apparently an aliphatic acid, which was not obtained pure. Lysine was liberated in small amounts. Other degradations gave products which were apparently related to the fragments isolated from acid hydrolysis.

The ether-soluble products of acid hydrolysis were largely separated by distillation and identified by the usual methods. The water-soluble fraction was chromatographed on cation-exchange resin, and yielded crystalline L-serine and a compound which had the properties of an amino-acid, but also had strong reducing properties. This second substance seemed homogeneous and gave a single spot on paper chromatography, the position of the spot varying according to whether the solution applied to the paper was neutral or acid. The spots were shown in the same positions by either a ninhydrin spray or a spray designed to reveal reducing compounds. Analysis of crystalline salts supported the formula $C_6H_{14}O_3N_2$ for the amino-acid. The reducing action suggested that the compound must contain either a hydroxyamino- or a hydrazine group or possibly a ·C(OH):C(OH)· or ·C(OH):C(NH_2)· group. Among a number of hydrazines tested none was found to reduce alkaline triphenyltetrazolium chloride, whilst in a group of hydroxylamines all resembled the degradation product in giving an immediate red colour with this reagent. Compounds containing the groups ·C(OH):C(OH)· or ·C(OH):C(NH_2)· usually reduce iodine in acid, whereas the product from mycobactin only reduced it in neutral or alkaline solution. The compound could be reduced to lysine either by reaction with hydriodic acid or by catalytic hydrogenation, during which two atoms of hydrogen were taken up per molecule. This suggested strongly that the substance was either 2-amino-6-hydroxyamino- (I) or 6-amino-2-hydroxyamino-hexanoic acid (II). Further evidence of the presence of a hydroxyamino-group was obtained by oxidation with

$HO{\cdot}NH{\cdot}[CH_2]_4{\cdot}CH(NH_2){\cdot}CO_2H$ (I) $NH_2{\cdot}[CH_2]_4{\cdot}CH(NH{\cdot}OH){\cdot}CO_2H$ (II)

periodic acid, requiring 1 mol. of periodic acid per mol. of hydroxyamino-acid. This might be expected to yield an oxime, and in fact, the crude oxidation product on hydrolysis gave a solution which showed a positive reaction for hydroxylamine. Paper chromatography showed the oxidation product to be complex; it is possible that geometrical isomers were formed. Acetylation of the reducing amino-acid gave a non-crystalline non-reducing derivative shown by titration to be mainly the diacetate. It behaved as a typical hydroxamic acid, giving a strong reddish-purple ferric chloride reaction; titration showed it to be dibasic, the stronger acid group (pK 3·7) being presumably the carboxyl and the weaker (pK 9) the hydroxamic acid. Titration of the untreated reducing material also supported its formulation as an amino-hydroxyamino-

* Part I, Francis, Macturk, Madinaveitia, and Snow, *Biochem. J.*, 1953, **55**, 596.

acid. Alkali titrations showed an ionising group having a pK value of approx. 9·6 (the amino-group), and acid titration gave indications of two ionising groups, one with a pK of about 5·2 (probably the hydroxyamino-group) and the other of much lower pK (the carboxyl group). In glacial acetic acid the titration curve showed a single inflection after the addition of 2 equivs. of perchloric acid, corresponding to neutralisation of both basic groups.

In order to decide between structures (I) and (II) reference was made to a number of α-hydroxyamino-acids. These compounds had reducing properties similar to those of the product from mycobactin, though the " reducing values " given on oxidation with alkaline ferricyanide were somewhat lower and more variable. The α-hydroxyamino-acids reacted in a characteristic way with periodic acid, giving 1 mol. of carbon dioxide and forming aldehydes; 2—2·5 mols. of periodic acid were taken up and the hydroxyamino-group appeared to be converted into oxides of nitrogen. This behaviour differed from that of the product from mycobactin, which was oxidised with the uptake of 1 mol. of periodic acid without the production of carbon dioxide or aldehyde. The degradation product has therefore been assigned formula (I). When mycobactin was degraded with hydriodic acid, alanine and lysine were isolated, resulting from the reduction of serine and 2-amino-6-hydroxyaminohexanoic acid, respectively. The L-configuration of the lysine established the L-configuration of the hydroxyamino-acid. It could be shown that the " reducing value " of acid hydrolysates of mycobactin reached a maximum value of 13·0 equivs. per mole after 1·5—3 hours' hydrolysis. The corresponding reducing value of the pure hydroxyamino-acid was 7·25 equivs. per mole, so that each molecule of mycobactin must yield 2 mols. of the hydroxyamino-acid. This was confirmed by the finding that, during the degradation with hydriodic acid, up to 1·5 mols. of lysine were isolated as crystalline hydrochloride. In all acid hydrolyses some lysine could be demonstrated by paper chromatography; the amount was small. It may represent a degradation product of the hydroxyamino-acid. Experiments with varying periods of acid hydrolysis showed that the reducing value rose to a maximum and then slowly declined as the acid treatment was prolonged, and this is evidence that some secondary breakdown of the hydroxyamino-acid does occur. However, it is also possible that the lysine may be derived from a close relative of mycobactin which has not been separated by the purification methods.

Since it has been shown that acid hydrolysis produces 1 mol. of serine and 2 mol. of 2-amino-6-hydroxyaminohexanoic acid, these amino-acid fragments account for all the five nitrogen atoms known to be present in mycobactin.

Acid hydrolysis yields a small fraction insoluble in ether but soluble in butanol. Part of this fraction represents incompletely hydrolysed intermediates, but even after prolonged hydrolysis some unidentified butanol-soluble material remains, which appears to arise from side reactions.

Various other degradation methods have given three related phenolic compounds, *viz.*, *m*-cresol and 2-hydroxy-6-methylbenzoic acid and the corresponding amide. The yields of these compounds suggested that they all represented a single residue in the whole molecule. Experiment showed that 2-hydroxy-6-methylbenzoic acid was readily decarboxylated by boiling 5N-hydrochloric acid, but that in the presence of 20% v/v methanol decarboxylation was lessened and part of the original acid could be recovered. This behaviour paralleled the observation that when mycobactin was hydrolysed with aqueous acid only *m*-cresol was isolated, whilst in the presence of 20% v/v methanol both 2-hydroxy-6-methylbenzoic acid and *m*-cresol were obtained. In the aqueous hydrolysis nearly 1 mole of carbon dioxide was liberated per mole of mycobactin. Both the hydroxy-acid and *m*-cresol were also obtained in alkali fusion. There is thus reason to believe that 2-hydroxy-6-methylbenzoic acid is the fragment existing in the original molecule, decarboxylation occurring during acid hydrolysis. When mycobactin is submitted to drastic alkaline hydrolysis or to pyrolysis, the phenolic fragment isolated is 2-hydroxy-6-methylbenzamide. This amide is exceptionally resistant to acid hydrolysis, much remaining unchanged after prolonged boiling with 50% sulphuric acid. It was not possible to degrade the amide by acid treatment, without simultaneous decarboxylation. It thus seems unlikely that the phenolic acid could be present as a simple amide in mycobactin.

Moreover, it has already been shown that all the nitrogen in the molecule can be accounted for by the serine and 2-amino-6-hydroxyaminohexanoic acid residues. The production of the amide must therefore arise from some structure involving one of these compounds which can give the phenolic acid under conditions of acid hydrolysis. It is likely that one of the nitrogen atoms of the amino-acid fragments is attached to the carboxyl group of the phenolic acid in mycobactin.

The fatty acid fragment obtained by hydrolysis with aqueous acid was *trans*-octadec-2-enoic acid, but in the presence of 20% v/v methanol considerable esterification occurred during the hydrolysis. In alkali fusion, as would be expected, the fatty product isolated was palmitic acid.

Experimental

All melting points are corrected.

Electrometric Titrations.—Titrations were carried out on a semimicro-scale with a glass electrode and saturated calomel reference electrode connected to the titration chamber by a capillary bridge, reagents being delivered from a micrometer syringe. Titrations in glacial acetic acid were performed similarly except that precautions were taken to exclude moisture, and the bridge used in this case was filled with lithium chloride solution in glacial acetic acid; with this assembly the glass electrode was positive to the calomel electrode.

Reducing Values.—A reducing substance was isolated in the course of the degradations. It was conveniently estimated by oxidation with alkaline ferricyanide by Hanes's method (*Biochem. J.*, 1929, **23**, 99) for reducing sugars. The results were expressed as "reducing value" in equivs. of thiosulphate per mole of reducing compound for compounds of known molecular weight, or equivs. of thiosulphate per mole of mycobactin for crude hydrolysates.

Oxidation with Periodic Acid.—For estimation of the loss of periodic acid in reaction with reducing compounds the following method was used. Periodic acid solution (0·5 c.c.; 0·2M) was added to a solution (1—2 c.c.) of the reducing compound, the quantity taken being such that the amount of periodic acid present was at least 1·5 times that required for oxidation, the periodic acid being assumed to be reduced to iodic acid. The mixture was stored for 2 hr. in an ice-bath and then treated with excess of potassium iodide and hydrochloric acid, and the liberated iodine titrated with 0·02N-sodium thiosulphate. For estimation of the evolution of carbon dioxide the reducing compound (about 20 μmole) was dissolved in water (1 c.c.) in one flask of the apparatus described by Van Slyke, MacFadyan, and Hamilton (*J. Biol. Chem.*, 1941, **141**, 671) for the titrimetric estimation of carbon dioxide. A small capsule containing periodic acid solution (0·4 c.c.; 0·2M) was placed in the same flask without mixing. The other flask received saturated barium hydroxide solution (1 c.c.). The apparatus was evacuated (water-pump), the tap closed, and the periodate mixed with the reducing solution. The reaction was allowed to proceed for 1 hr. at room temperature. The contents of the reaction flask were then warmed to 50°, and the water distilled into the barium hydroxide flask which was cooled in ice. Estimation of the precipitated barium carbonate was carried out as in the van Slyke method.

Paper Chromatography.—Chromatography was carried out by the descending technique, Whatman No. 54 papers being used. Two-dimensional runs were performed by Dent's method (*Biochem. J.*, 1948, **43**, 169), except that collidine–lutidine was used as the first solvent; phenol with ammonia was used as the second solvent. Other solvents used for one-dimensional chromatography were butanol–acetic acid containing the following proportions by volume: water 25, acetic acid 12, *n*-butanol to 100, and butanol–propanol–diethylamine having the proportions: water 25, propanol 28, diethylamine 2, *n*-butanol to 100. Amino-acids were detected by the conventional ninhydrin spray. Reducing substances were detected by spraying first with 0·1% triphenyltetrazolium chloride in butanol saturated with water; the papers were then dried and sprayed lightly with a solution containing aqueous sodium hydroxide (10 c.c.; 10N), ethanol (40 c.c.), and butanol (50 c.c.). The presence of a reducing compound was shown by the immediate production of a red spot; the sensitivity was comparable to that of ninhydrin.

Hydrolysis of Mycobactin with Aqueous Hydrochloric Acid.—Mycobactin (5 g.) was refluxed for 8 hr. with hydrochloric acid (500 c.c.; 5N), with a slow stream of nitrogen passing through the solution. The issuing gas was passed through bubblers containing saturated aqueous barium hydroxide (200 c.c.) to absorb the carbon dioxide evolved. After neutralisation of excess of barium hydroxide the precipitated barium carbonate was estimated acidimetrically; it was

equivalent to 244 mg. of carbon dioxide (*i.e.*, 0·96 mol.). During the hydrolysis, mycobactin did not pass completely into solution but was transformed into an oil which solidified to a wax on cooling. The solution after hydrolysis was extraced with ether (3 × 250 c.c.), the wax passing into the ether layer. The aqueous layer was evaporated under reduced pressure, and the residue dissolved in hydrochloric acid (20 c.c.; 5N) and re-extracted with ether (5 × 100 c.c.). This ether extract was combined with the first, dried ($MgSO_4$), and evaporated. The residue (2·70 g.) was a yellow, waxy, partly crystalline solid with a phenolic smell (fraction A). The aqueous layer was evaporated under reduced pressure, and the residue dissolved in water (50 c.c.) and extracted with butanol (3 × 50 c.c.). The aqueous layer and butanol extracts were evaporated under reduced pressure; the residue from butanol was a brown gum (0·32 g.; fraction B) and that from the aqueous layer an almost colourless gum (3·17 g.; fraction C).

Fraction A. The product was distilled under reduced pressure, three fractions being collected: A1, a mobile liquid (0·36 g.), b. p. 130—135° (bath temp.)/15 mm.; A2, a viscous oil (0·275 g.), b. p. 120—130° (bath temp.)/0·2 mm.; and A3, a waxy solid (1·36 g.), b. p. 200—210° (bath temp.)/0·2 mm. Fractions A1 and A2 showed some overlap in properties; A1 was predominantly phenolic and A2 contained acidic material. Both fractions were treated with sodium carbonate solution (2 c.c.; 0·5M) and steam-distilled. The distillates (30 c.c.) were extracted with ether (3 × 30 c.c.), the extracts dried ($MgSO_4$), and the solvent removed. The products (306 mg. from A1, 145 mg. from A2) were combined, and the resulting oil was identified as *m*-cresol. It gave with ferric chloride a green colour in ethanol and a violet-blue colour in water; the carboxymethyl ether had m. p. and mixed m. p. 101—103° (Found: C, 65·35; H, 5·95. Calc. for $C_9H_{10}O_3$: C, 65·05; H, 6·05%); the 3 : 5-dinitrobenzoate had m. p. and mixed m. p. 165·5° (Found: C, 55·8; H, 3·4; N, 9·5. Calc. for $C_{14}H_{10}O_6N_2$: C, 55·6; H, 3·3; N, 9·3%); the α-naphthylurethane had m. p. and mixed m. p. 129—130° (Found: C, 77·95; H, 5·5; N, 5·35. Calc. for $C_{18}H_{15}O_2N$: C, 78·0; H, 5·45; N, 5·05%).

The residual solutions from fractions A1 and A2 were acidified, saturated with sodium chloride, and extracted three times with an equal volume of ether. The extract was dried ($MgSO_4$), and the solvent removed. The combined residues (129 mg. from A1, 146 mg. from A2) formed a viscous oil which appeared to contain an aliphatic acid (equiv., *ca.* 130). This hydrolysis did not, however, give a fragment sufficiently pure for identification; the acid was better obtained from a partial degradation product of mycobactin and will be discussed in a later paper.

A solution of fraction A3 in light petroleum was clarified by filtration, and solvent evaporated. The residue, crystallised successively from acetone and methanol, formed rhombic leaflets or needles, m. p. 58·5°, of *trans*-octadec-2-enoic acid. It reduced permanganate in acetone, but did not reduce bromine in carbon tetrachloride (Found: C, 76·6; H, 12·1%; equiv., by titration, 282. Calc. for $C_{18}H_{34}O_2$: C, 76·55; H, 12·1%; equiv., 282). It did not depress the m. p. of the synthetic acid (Ponzio, *Gazzetta*, 1904, **34**, II, 77; 1905, **35**, II, 569). Derivatives were also prepared from the acid obtained by degradation and from the synthetic acid: p-*bromophenacyl* ester, m. p. and mixed m. p. 88·5—89·5° from light petroleum (Found: C, 64·9; H, 7·8; Br, 15·9. $C_{26}H_{39}O_3Br$ requires C, 65·1; H, 8·2; Br, 16·7%); *benzylthiuronium* salt, m. p. and mixed m. p. 152—153°, from acetone (Found: C, 69·5; H, 9·8; S, 7·3. $C_{18}H_{34}O_2,C_8H_{10}N_2S$ requires C, 69·6; H, 9·9; S, 7·2%). The infra-red spectra of the acids, natural and synthetic, were identical and exhibited bands characteristic of an αβ-unsaturated acid.

Fraction C. The crude fraction (3·17 g.) contained ionisable chlorine equivalent to 0·95 g. of hydrogen chloride, had reducing properties, and gave a ninhydrin reaction. An aqueous solution was submitted to two-dimensional paper chromatography; reaction with ninhydrin revealed three principal spots, of which two corresponded with serine and lysine. The third did not correspond with a known amino-acid and its position would be represented on Dent's map (*loc. cit.*) as R_F 0·73 (phenol–ammonia), 0·23 (collidine).

No reducing substance was detectable after paper chromatography with neutral or basic solvents. A spot representing the reducing material could, however, be detected when butanol–acetic acid was used for development. The R_F values of the reducing material and of compounds reacting with ninhydrin were:

ninhydrin spray, 0·09 (lysine), 0·14 (serine), 0·21; triphenyltetrazolium spray, 0·21

The third spot obtained by conventional two-dimensional paper chromatography was evidently an artefact produced by oxidation of the reducing substance.

Separation of the components of fraction C was achieved by use of Partridge and Westall's method (*Biochem. J.*, 1949, **44**, 418, 521). Fraction C (3·10 g.), dissolved in water (65 c.c.),

was applied slowly to a column (85 × 0·67 cm.) of Zeo-Karb 215 (15·5 g.; 80—120 mesh); all the water-soluble material was held by the resin. A small conductivity cell, registering with a drum recorder, was attached to the end of the column, and the basic compounds were then displaced by slow passage of aqueous ammonia (0·15N), 4-c.c. fractions of the effluent being collected. The pH and reducing value of each fraction were measured and one-dimensional paper chromatograms were run, with butanol–acetic acid as solvent. The paper chromatograms indicated the presence of the two main well-separated components. The first component (fraction C1, tubes 49—54) corresponded chromatographically with serine. The emergence of the second component (fraction C2, tubes 57—63) was accompanied by a sharp rise in conductivity and pH, and the appearance of reducing properties. These properties then remained almost constant until a further rise in conductivity indicated the appearance of the ammonia front. Beyond this point the emergent solution gave no reducing reaction, but showed the presence of a little ninhydrin-reacting substance corresponding chromatographically to lysine; this portion was not further examined.

The aqueous solution containing fraction C1 was evaporated, giving a partly crystalline residue (0·40 g.). This was crystallised from aqueous solution by addition of ethanol, and identified as L-serine, $[\alpha]_D^{23}$ −6·4° (*c*, 6·5 in H_2O), m. p. 226—227° (decomp.) (Found: C, 34·4; H, 6·75; N, 13·15. Calc. for $C_3H_7O_3N$: C, 34·3; H, 6·7; N, 13·3%). It gave a single spot on two-dimensional paper chromatography alone or admixed with authentic serine; after treatment with hydriodic acid (9 hr.' refluxing with constant-boiling acid) the serine spot was replaced by a spot characteristic of alanine. With *p*-hydroxyazobenzene-*p'*-sulphonic acid it formed a salt which could be crystallised from water (Found: C, 44·85; H, 4·85; N, 10·5, 10·6. Calc. for $C_3H_7O_3N,C_{12}H_{10}O_4N_2S,H_2O$: C, 44·9; H, 4·8; N, 10·5%). In some separations the serine was incompletely held by the resin, and part passed into the effluent as the hydrochloride during application of the crude hydrolysate to the column. This did not however interfere with the separation.

The aqueous solution containing fraction C2 was evaporated under reduced pressure, leaving an almost colourless solid, m. p. 223—225° (decomp.). The substance could be precipitated from aqueous solution by addition of ethanol or methanol, but no crystallisation could be achieved. It appeared to be homogeneous, however. Paper chromatography with butanol–acetic acid as solvent yielded a single spot (R_F 0·30) detectable with ninhydrin. The hydrochloride of this substance, obtained by use of excess of hydrochloric acid and evaporation, gave a single spot with a lower R_F value (0·21), corresponding to the third spot in the paper chromatograms on the unseparated fraction C. When chromatograms of C2 and its hydrochloride were sprayed with triphenyltetrazolium spray, single spots were revealed at positions identical with those of the ninhydrin spots. The properties of C2 described below led to its formulation as L-2-*amino*-6-*hydroxyaminohexanoic acid*. It had $[\alpha]_D^{20}$ +6·3° ± 0·5° (*c*, 5·0 in H_2O), $[\alpha]_D^{18}$ +23·9° ± 0·5° (*c*, 5·1 in N-HCl) (Found: C, 44·1; H, 8·1; N, 16·9. $C_6H_{14}O_3N_2$ requires C, 44·4; H, 8·7; N, 17·3%). The crystalline *5-nitrobarbiturate* was prepared by dissolving fraction C2 (0·13 g.) in warm water (5 c.c.) with 5-nitrobarbituric acid trihydrate (0·17 g.), allowing the solution to cool slowly, and recrystallising the product (0·21 g.) twice from water; the rhombic prisms charred above 200° without a definite m. p. (Found: C, 34·35; H, 5·5; N, 19·74; loss of wt. when dried at 120°, 5·5. $C_6H_{14}O_3N_2,C_4H_3O_5N_3,H_2O$ requires C, 34·0; H, 5·4; N, 19·8; H_2O, 5·1%). A second salt was obtained when a solution of fraction C2 (0·05 g.) and 2-nitroindane-1 : 3-dione (0·05 g.) in water (3 c.c.) cooled slowly. The product (0·06 g.) was recrystallised twice from water yielding the *salt* of 2-nitroindane-1 : 3-dione as pale yellow crystals, m. p. 217—217·5° (decomp.) (Found: C, 51·0; H, 5·2; N, 11·9. $C_6H_{14}O_3N_2,C_9H_5O_4N$ requires C, 51·0; H, 5·4; N, 11·9%). Fraction C2 was very soluble in water but insoluble in neutral organic solvents; the aqueous solution had pH *ca.* 6·8. Neutral solutions absorbed oxygen from the air, but acid solutions were more stable. The solid material was best kept *in vacuo* over a desiccant. It showed the typical properties of an α-amino-acid: reaction with ninhydrin gave a blue colour; the carbon dioxide evolved was estimated by van Slyke's method (*loc. cit.*), giving a value of 0·93 mol. of carbon dioxide (mol. wt. 162 being assumed). A cold aqueous solution shaken with cupric carbonate gave a deep blue solution, together with some slight reduction to cuprous oxide. It gave no precipitate with the common precipitants for organic bases except phosphotungstic acid which yielded a sticky intractable product. It had strong reducing properties, reducing Fehling's solution or silver nitrate solution on gentle warming, iodine in neutral or alkaline, but not acid solution, and alkaline triphenyltetrazolium chloride. The aqueous solution shaken with lead peroxide gave no colour; 2 : 4-dinitrophenylhydrazine in acid solution did not yield a precipitate or change

colour; tests for sugars were negative. Reducing values of fraction C2 and its salts in equivs. per mole were: fraction C2, 7·25; 5-nitrobarbiturate, 7·22; 2-nitroindane-1 : 3-dione salt, 7·45. The absorption spectrum of fraction C2 showed a maximum at 219 mμ (ε, 2700 in water) with no absorption bands at longer wave-lengths. Electrometric titrations of 0·2M- and 0·1M-solutions with, respectively, N-hydrochloric acid and 0·03N-barium hydroxide, and of a 0·02M-solution in glacial acetic acid with 0·5N-perchloric acid were carried out. Fraction C2 (45 mg.), hydrogenated with Adams's catalyst (25 mg.) in water (3 c.c.), took up 1·05 mol. of hydrogen. The alkaline product was identified as lysine by paper chromatography and as picrate and dihydrochloride, m. p. 194—196° (Found: C, 32·95; H, 7·5; N, 12·75. Calc. for $C_6H_{12}O_2N_2,2HCl$: C, 32·9; H, 7·4; N, 12·8%). Fraction C2 could also be converted into lysine by refluxing constant-boiling hydriodic acid.

Fraction C2 (85 mg.) was acetylated in water (2 c.c.) at 0° by gradual simultaneous addition of acetic anhydride (0·4 c.c.) and 10N-sodium hydroxide in equivalent amount. The addition was made with constant stirring during 30 min.; a slow stream of nitrogen was bubbled through the solution during the acetylation, and any excess of alkali was avoided. Stirring was continued for a further 30 min., then sufficient alkali was added to make the solution 0·2N, and the solution kept at 0° for 30 min. It was acidified with 10N-sulphuric acid and extracted with 17% v/v butanol in chloroform (6 × 2 vols.). The extract was filtered through a dry filter-paper and evaporated under reduced pressure, leaving a colourless gum (114 mg.) which could not be crystallised. The equivalent weights represented by the two steps in the electrometric titration curve suggested that the product consisted of about 80% diacetate (dibasic acid) and 20% triacetate (monobasic acid).

A solution of fraction C2 (146 mg.) in water (5 c.c.) was cooled in ice, periodic acid (0·2M; 6 c.c.) was added, and the solution was kept for 2 hr. in an ice-bath. It was then diluted to 25 c.c.; titration of an aliquot portion (0·5 c.c.) indicated the reduction of 2·08 equivs. per mole of C2. Barium hydroxide (0·3M; 6·1 c.c.) was added to the solution to bring the pH to 7·0, and the solution boiled, cooled, and set aside. The precipitated salts were filtered off, the filtrate evaporated, and the residue dissolved in the minimum of water; this solution was filtered and again evaporated. The residue (170 mg.) still contained a little inorganic material. Paper chromatography with phenol–ammonia gave one spot, R_F 0·71, and with butanol–propanol–diethylamine two main spots, R_F 0·05 and 0·16. The product was not further separated. The crude oxidation product gave a strongly positive test for the oxime group (Feigl, " Spot Tests," 1939, 2nd. edn., p. 290).

In order to establish the position of the hydroxyamino-group in fraction C2 its reactions were compared with those of the following α-hydroxyamino-acids, kindly supplied by Dr. G. Swain: (*a*) hydroxyaminoacetic acid, (*b*) 2-hydroxyaminohexanoic acid, (*c*) α-hydroxy-amino-β-phenylpropionic acid. All three resembled C2 in immediately reducing alkaline triphenyltetrazolium chloride. They also reacted with ninhydrin to produce 1 mol. of carbon dioxide with the formation of aldehydes. Other properties were as follows:

		α-Hydroxyamino-acids		
Compound:	C2	(*a*)	(*b*)	(*c*)
Ninhydrin colour	Intense blue	Weak yellow or orange		
Aqueous solution with $CuCO_3$	Blue	Blue	Blue	Colourless
		(all showed reduction on being warmed)		
Reducing values	7·25	4·1 *	5·2 *	5·8 *
Periodate oxidation:				
(*a*) Mol. consumed	1·0	2·45	2·2	2·4
(*b*) Mol. CO_2 liberated	0·0		1·0	

* These figures varied somewhat according to concentration.

A compound believed to be DL-6-amino-2-hydroxyaminohexanoic acid was strongly reducing but gave a pale colour with ninhydrin, and no blue colour with copper carbonate; the crystals of the 5-nitrobarbiturate were of a different form from those of the corresponding derivative of fraction C2.

Fraction B. This fraction appeared to be a complex mixture and was thought likely to contain some incompletely hydrolysed material. The fraction (0·32 g.) was further hydrolysed by refluxing it for 16 hr. with hydrochloric acid (5N; 50 c.c.). The product was separated into three fractions corresponding to the three main fractions isolated in the original hydrolysis: ether-soluble (0·04 g.), butanol-soluble (0·13 g.), and water-soluble (0·16 g.). The water-soluble

fraction had reducing properties, and paper chromatography indicated the presence of serine, lysine, and 2-amino-6-hydroxyaminohexanoic acid. The ether-soluble fraction gave a violet colour with ferric chloride.

Progressive Acid Hydrolysis of Mycobactin.—Portions of mycobactin (80 mg.) were refluxed with hydrochloric acid (5N; 8 c.c.) for varying periods. The resultant solutions were cooled, diluted, and extracted with ether, and the aqueous solutions evaporated under reduced pressure. The residues were dissolved in water and estimations of the reducing compounds present carried out upon aliquot portions:

Time of hydrolysis (hr.)	½	¾	1·5	3	6	12	24	96
Reducing value (equiv. per mole of mycobactin)	9·3	11·0	13·0	12·5	12·1	11·7	11·3	8·5

Synthesis of Hydroxyamino-acids (By G. SWAIN).—Traube's method (*Ber.*, 1895, **28**, 2300) gave *2-hydroxyaminohexanoic acid*, m. p. 168—170° (decomp.), from 50% aqueous ethanol (Found: C, 48·9; H, 8·8; N, 9·95. $C_6H_{13}O_3N$ requires C, 49·0; H, 8·9; N, 9·5%). Diethyl 4-phthalimidobutylmalonate, by the same procedure, yielded a crystalline product, m. p. 168—170° (sinters 160°), which was probably mainly 6-amino-2-hydroxyaminohexanoic acid, though the analysis was unsatisfactory. It gave a *5-nitrobarbiturate* as colourless rhombs without definite m. p. (charred at 220—240°) (Found: C, 31·4; H, 4·85; N, 19·3. $C_6H_{14}O_3N_2,2C_4H_3O_5N_3,2H_2O$ requires C, 30·9; H, 4·4; N, 20·6%).

Reactions of Hydrazines and Hydroxylamines with Triphenyltetrazolium Chloride.—Triphenyltetrazolium chloride (about 1 mg.) was dissolved in a drop of an aqueous solution of the test compound, on a tile, and a drop of 2N-sodium hydroxide added. Immediate development of a deep red colour was taken as a positive reaction. Positive reactions were given by the following hydroxylamines: hydroxylamine, *N*-ethylhydroxylamine, α-hydroxyamino*iso*butyl cyanide, *N*-hydroxymorpholine, and a series of α-hydroxyamino-fatty acids. The following hydrazines gave negative reactions: methylhydrazine, phenylhydrazine, 1 : 1′-dicarboxy-*NN*′-di*iso*propylhydrazine.

Acid Hydrolysis of Mycobactin in the Presence of Methanol.—Mycobactin (2 g.) was dissolved in methanol (40 c.c.), the solution poured with stirring into hydrochloric acid (5N; 160 c.c.), and the resulting suspension refluxed for 12 hr., then cooled, diluted with water (200 c.c.), and extracted with ether (3 × 200 c.c.). The aqueous layer appeared to be similar to that obtained by hydrolysis in the absence of methanol; it was separated as described above for fraction C. The ether layer was extracted first with aqueous sodium carbonate (3 × 300 c.c.) and then with aqueous sodium hydroxide (3 × 300 c.c.). The ether solution was washed with water and dried ($MgSO_4$), and the solvent removed, leaving an oily residue (0·39 g.; fraction D). The sodium hydroxide extract was acidified with hydrochloric acid and steam-distilled. The distillate was extracted with ether, the extract dried, and the solvent removed, leaving an oily residue (0·11 g.) which was distilled and identified as *m*-cresol. The solution after steam-distillation was also extracted with ether; the extract, when dried and evaporated, left a residue containing a little acidic material (0·08 g.) which was combined with fraction E (see below). The sodium carbonate extract was acidified with hydrochloric acid and extracted with ether. The ether solution was dried and evaporated, leaving a brown, waxy, partly crystalline residue (0·32 g.; fraction E).

Fraction D. The product was distilled at 140—160°/0·04 mm. (oil-bath temp.), yielding an oil, m. p. 13—13·5°, apparently slightly impure methyl octadec-2-enoate. It was neutral, free from nitrogen, and reduced permanganate in acetone but not bromine in carbon tetrachloride; there was no reaction for carbonyl groups. Saponification with 2N-sodium hydroxide gave methanol, detected by Boos's method (*Analyt. Chem.*, 1948, **20**, 964), and an acid, m. p. 58·5° after crystallisation, identified as *trans*-octadec-2-enoic acid.

Fraction E. The waxy product (0·40 g.), yielded a crystalline sublimate (0·12 g.) at 100—130°/0·04 mm. (oil-bath temp.) and a waxy solid (0·15 g.) at 160—180°/0·04 mm.; the latter gave octadec-2-enoic acid on recrystallisation. The sublimate, crystallised from water and chloroform, had m. p. 170—170·5°, and was shown to be 2-hydroxy-6-methylbenzoic acid (Found: C, 63·4; H, 5·7%; equiv., by titration, 152. Calc. for $C_8H_8O_3$: C, 63·2; H, 5·3%; equiv., 152). There was no depression of m. p. with a specimen (m. p. 169·5—170·5°) kindly supplied by Professor H. Raistrick; ultra-violet spectra measured in acid and alkaline solutions and at pH 8·0 were identical in every detail for the two specimens.

Degradation of Mycobactin with Hydriodic Acid.—Mycobactin (2 g.) was refluxed for 9 hr. with constant-boiling hydriodic acid (40 c.c.), and the solution poured into water (120 c.c.). It was extracted with ether (4 × 80 c.c.), and the aqueous layer evaporated under reduced

pressure. The ether-soluble fraction contained fatty acid and phenolic products containing iodine; it was not further examined. The residue from the aqueous layer was dissolved in water (40 c.c.), and iodide removed by shaking with an excess of freshly-precipitated silver chloride. The silver halides were filtered off, and the filtrate evaporated under reduced pressure, leaving a clear gum (1·13 g.) which tended to crystallise. Slow crystallisation from methanol (25 c.c.) by progressive addition of ether gave first a product (5 mg.), m. p. 252—253°, which was probably lysine monohydrochloride (Found: C, 36·2; H, 8·7. Calc. for $C_6H_{14}O_2N_2$,HCl,H_2O: C, 35·9; H, 8·5%), then crystalline L-lysine dihydrochloride (0·69 g.), m. p. 194—196° (decomp.) after recrystallisation, $[\alpha]_D^{21}$ +16·5° in N-hydrochloric acid (*c*, 3·0) (Found: C, 33·1; H, 7·3; N, 12·95. Calc. for $C_6H_{14}O_2N_2$,2HCl: C, 32·9; H, 7·35; N, 12·8%). The picrate had m. p. 266° (explodes) (Found: C, 38·3; H, 4·6; N, 18·65. Calc. for $C_6H_{14}O_2N_2$,$C_6H_3O_7N_3$: C, 38·4; H, 4·55; N, 18·65%).

Paper chromatography of the residue after crystallisation of the lysine indicated the presence of much alanine together with a small amount of residual lysine.

Alkali Fusion.—Mycobactin (1·5 g.) was added slowly (30 min.) to a melt containing sodium hydroxide (7·5 g.) and potassium hydroxide (4·5 g.) at 250°. The temperature of the melt was then raised to 290° for a further 1½ hr. An alkaline gas identified as ammonia was evolved and was trapped in bubblers containing N-hydrochloric acid. The melt was cooled and dissolved in water (30 c.c.), and the solution treated with sodium chloride solution (5M; 60 c.c.). The precipitate was filtered off (fraction F) and washed with brine. Carbon dioxide was passed through the filtrate until the pH was 8—9 and the latter was steam-distilled. The distillate was extracted with ether, the extract dried, and the solvent removed, leaving *m*-cresol (0·06 g.). The solution remaining after steam-distillation was acidified with hydrochloric acid and steam-distilled, until no more acid was evolved. The distillate was neutralised with sodium hydroxide and evaporated under reduced pressure, leaving a salt (0·83 g.; fraction G).

Fraction F. The sodium salt was dissolved in hot water (30 c.c.), and the solution acidified, cooled, and extracted with ether. The extract was dried and the solvent removed, leaving a waxy crystalline residue (0·36 g.). This was repeatedly crystallised from acetone and gave a product which appeared to be substantially palmitic acid although the m. p. could not be raised above 59°. It had equiv. 255 by titration (Calc.: 256), and gave a benzylthiuronium salt, m. p. 141° (Found: C, 68·2; H, 9·85; S, 7·6. Calc. for $C_{16}H_{32}O_2$,$C_8H_{10}N_2S$: C, 68·2; H, 10·0; S, 7·6%). The acid was saturated, and its surface-spreading properties were identical with those of palmitic acid.

Fraction G. The sodium salt was dissolved in water (2 c.c.) and acidified with dilute sulphuric acid. A crystalline precipitate separated out. This was filtered off (0·05 g.), crystallised from chloroform, and identified as 2-hydroxy-6-methylbenzoic acid. The filtrate contained a mixture of volatile aliphatic acids, from which acetic acid was isolated as its *p*-bromophenacyl ester, m. p. and mixed m. p. 85—86° (Found: C, 46·85; H, 3·5; Br, 30·8. Calc. for $C_{10}H_9O_3Br$: C, 46·7; H, 3·5; Br, 31·1%). Another acid having a smell resembling that of valeric or hexanoic acid was not identified.

Hydrolysis of Mycobactin in Alkaline Solution.—Mycobactin (1 g.) was refluxed for 6 hr. with potassium hydroxide (10 g.) in 90% ethanol (30 c.c.) in a slow stream of nitrogen. The issuing gas was passed through traps containing hydrochloric acid. The volatile basic gas was estimated by titration and found to be equivalent to 0·11 g.-atom of nitrogen per mole of mycobactin. The alkaline hydrolysate was cooled and diluted with water (40 c.c.), and carbon dioxide was passed through until the pH was 8—9. A slimy precipitate separated and was centrifuged off, leaving a turbid solution. The remainder of the insoluble product separated as an interface on subsequent ether extraction. The combined insoluble product, washed and dried in a desiccator (0·23 g.), was a white, apparently polymeric, powder. The sodium carbonate solution extracted with ether (8 × 100 c.c.) contained a complex mixture of substances from which no pure compound has been separated. The ethereal extract was dried ($MgSO_4$), and the solvent removed. The residue (0·17 g.) was stirred with light petroleum and filtered; the filtrate was not further examined. The petroleum-insoluble product (0·05 g.) gave hexagonal plates (m. p. 199—200°), from water or xylene, identified as *2-hydroxy-6-methylbenzamide* after comparison with a synthetic specimen (see below). The two specimens showed no depression of m. p. when mixed, and their ultra-violet and infra-red spectra were identical. The solution in water was neutral and gave a deep violet colour with ferric chloride. Quantitative acetylation (method as in Part I, *loc. cit.*) gave 1·01 hydroxyl groups per molecule (Found: C, 63·4; H, 5·7; N, 9·45. $C_8H_9O_2N$ requires C, 63·5; H, 6·0; N, 9·3%).

Synthesis of 2-Hydroxy-6-methylbenzamide (with M. A. T. Rogers).—2 : 3 : 5-Trimethyl-

1 : 4-benzopyrone (Robertson, Waters, and Jones, *J.*, 1932, 1681) (3·0 g.) in chloroform (50 c.c.) was saturated with ozonised oxygen at 0° for 3 hr. 2N-Sodium hydroxide (25 c.c.) was added and the chloroform removed by a stream of air. The suspension was warmed for 30 min. at 50° and then to 100° with a further 10 c.c. of sodium hydroxide. Filtration and acidification yielded 2-hydroxy-6-methylbenzoic acid (1·58 g.), m. p. 166°. The recrystallised acid, m. p. 171—172° (0·92 g.), was treated in ether with a slight excess of diazomethane, giving the methyl ester, which was mixed with saturated methanolic ammonia (20 c.c.) and heated at 100° for 12 hr. in a sealed tube. Evaporation and crystallisation of the residue from water (charcoal) gave plates, m. p. 201° (Found: C, 63·45; H, 5·9; N, 9·15%).

Pyrolysis of Mycobactin.—Mycobactin (0·5 g.) was heated under reduced pressure in a small retort immersed in a metal-bath. Over a temperature range 190—250° at 0·07—0·12 mm. a crystalline product sublimed together with some waxy material and a dark oil. The crystals (23 mg.), washed with chloroform, were crystallised from water and identified as 2-hydroxy-6-methylbenzamide.

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