Mycobactin. A Growth Factor for Mycobacterium johnei. *Part III.* Degradation and Tentative Structure.*

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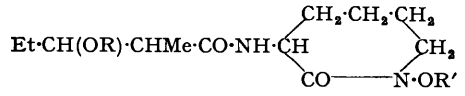
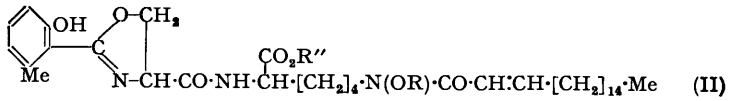
Mycobactin has been cleaved under alkaline conditions into two components which have been named cobactin and mycobactic acid. Hydrolysis of cobactin has yielded (−)-3-hydroxy-2-methylpentanoic acid. Evidence suggests that cobactin is a cyclic hydroxamic acid (I; R = R′ = H). Two alternative structures (IV and V; R = R′ = H) are tentatively proposed as explaining satisfactorily the properties of mycobactin and its derivatives.

Mycobactin, a growth factor for *Mycobacterium johnei* isolated from *Myco phlei*, probably has the empirical formula $C_{47}H_{75}O_{10}N_5$ (Part I); previous degradations (Part II) demonstrated the presence of octadec-2-enoic acid, 2-hydroxy-6-methylbenzoic acid, 2-amino-6-hydroxyaminohexanoic acid, and serine as hydrolytic fragments. It has now been shown that mild alkaline treatment of mycobactin yields a crystalline water-soluble compound having a neutral reaction, and an ether-soluble acidic product; these have been named cobactin and mycobactic acid respectively. Evidence is presented supporting the formula (I; R = R′ = H) as the structure of cobactin. No rigid proof of the structure of mycobactic acid has been achieved, but its properties appear to agree with the alternative formulæ (II and III; R = R″ = H). Consideration of the point of linkage between cobactin and mycobactic acid then leads to (IV and V; R = R′ = H) as tentative structures for mycobactin.



(I)



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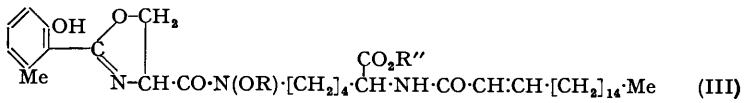


(III)

The nature of the fractions resulting from alkaline cleavage was established by acid hydrolysis under the conditions previously used with mycobactin (Part II). Hydrolysis of the mycobactic acid fragment yielded octadec-2-enoic acid, *m*-cresol (derived from 2-hydroxy-6-methylbenzoic acid), and amino-acids identified by paper chromatography as serine and 2-amino-6-hydroxyaminohexanoic acid; the cobactin fragment gave as the only products 2-amino-6-hydroxyaminohexanoic acid and an acid identified as (—)-3-hydroxy-2-methylpentanoic acid. The hydrolysates of the mycobactic acid and cobactin fragments each accounted for half the total reducing value obtained on hydrolysis of mycobactin. It was thus clear that one of the two 2-amino-6-hydroxyaminohexanoic acid residues was present in each of the two fragments resulting from alkaline cleavage, and that the action of alkali was to split the mycobactin molecule into two clearly defined portions.

Cobactin was obtained relatively pure from the alkali treatment and, after recrystallisation, was shown to have the molecular formula $C_{12}H_{22}O_4N_2$. On acid hydrolysis it gave the non-volatile oily acid which had been observed in the hydrolysis of mycobactin but not

* Part I, Francis, Macturk, Madinaveitia, and Snow, *Biochem. J.*, 1953, **55**, 596; Part II, Snow, *J.*, 1954, 2588.

characterised. The acid was optically active, and analysis of its crystalline *p*-bromophenacyl ester showed its formula to be $C_6H_{12}O_3$. This and the demonstration of the presence of one hydroxyl group by quantitative acetylation showed it to be a hydroxyhexanoic acid. Removal of the hydroxyl group by conversion into the iodo-compound and subsequent reduction gave a volatile acid from which a lævorotatory anilide was prepared. This anilide was compared by infra-red spectroscopy with the anilides of the three isomeric synthetic hexanoic acids containing asymmetric centres, and shown to be identical with 2-methylpentanoanilide. It seemed probable that in the acid obtained from cobactin the hydroxyl group was in the β-position, since the acid lacked properties of an α-hydroxy-acid and did not lactonise or polymerise. Oxidation with chromic acid liberated carbon dioxide and gave diethyl ketone, identified as its dinitrophenylhydrazone. This proved the acid to be (—)-3-hydroxy-2-methylpentanoic acid. The configuration of the asymmetric β-carbon atom was not determined; that of the α-atom may be fixed by reference to the optically active anilide of the deoxy-acid. A synthetic specimen of 3-hydroxy-2-methylpentanoic acid prepared by the Reformatski reaction gave a *p*-bromophenacyl ester differing in melting point and infra-red spectrum from that of the acid derived from cobactin; it was apparently the racemic form of the other epimer.

From consideration of the formula of cobactin it was evident that the hydroxy-acid and the amino-acid residue were combined with elimination of the elements of two water molecules. The only ionising group detectable in cobactin by electrometric titration was a weakly acidic group having pK 9·1. The compound gave an intense reddish-purple colour with ferric chloride, and had no reducing properties. These observations suggested that the hydroxyamino-group of 2-amino-6-hydroxyaminohexanoic acid was combined in cobactin to give a secondary hydroxamic acid. Moreover monomethyl and monoacetyl derivatives were prepared which lacked both the ferric chloride reaction and the weakly acidic group. The position of the methyl group in the methyl derivative was confirmed by acid hydrolysis. The methyl derivative yielded a non-reducing amino-acid which appeared to be *L*-2-amino-6-methoxyaminohexanoic acid.* The presence of a second hydroxyl group in cobactin was shown by the preparation of an acetyl derivative from *O*-methylcobactin. In formulating cobactin the residues must be linked in such a way as to mask both the carboxyl groups and the amino-group, to leave a free hydroxyl group, and to show the hydroxyamino-group combined as a secondary hydroxamic acid. It appeared that these conditions could be met by formula (I; R = R′ = H) (or the corresponding tautomeric form of the hydroxamic acid).

O-Methyl- and *O*-acetyl-cobactin were represented by (I; R = H, R′ = Me or Ac respectively), and *O*-acetyl-*O*-methylcobactin by (I; R = Ac, R′ = Me). It was possible, by gentle acid hydrolysis, to open the cobactin ring without disrupting the amide linkage. The product, $C_{12}H_{24}O_5N_2$, was named cobactinin. It had reducing properties, gave no reaction with ninhydrin or ferric chloride, and was weakly acid in reaction. Electrometric titration showed the presence of an acidic group pK 3·3 (carboxyl) and a very weak basic group pK 6·15 which was evidently the hydroxyamino-group. These properties agree with its formulation as (VI).

$$Et\cdot CH(OH)\cdot CHMe\cdot CO\cdot NH\cdot CH(CO_2H)\cdot [CH_2]_4\cdot NH\cdot OH \quad (VI)$$

The structure of mycobactic acid has been more difficult to approach because of the intractable nature of the acid itself and of its partial degradation products. Mycobactic acid was a gum which was not obtained pure. It was unstable to acids and to alkalis and was apparently always decomposed to some extent in the process of isolation. When mycobactin was treated with an excess of cold alkali the liberation of acid could be shown by back-titration. Within the errors of the experiment the amount of acid liberated was estimated as 1 equivalent per molecule of mycobactin (molecular weight 870), but the titrations did not allow a high degree of accuracy.

Mycobactic acid samples, when titrated directly with alkali, gave equivalent weights varying from 700 to 900, often increasing on attempted purification. Back-titration after the addition of at least 3 mols. excess of alkali, however, gave an equivalent of about 650,

* The *L*-prefix is used in the sense defined by Linstead *et al.* (*J.*, 1950, 3333).

suggesting that the acid had undergone some reaction akin to partial lactonisation during its isolation. It has proved difficult to establish the formula of mycobactic acid in view of the uncertainty of its equivalent weight, and of the lack of crystalline derivatives. Acetylation, benzoylation, and esterification with various alcohols yielded intractable gums; metallic salts were mostly soap-like; the picrate and perchlorate were also gummy. The hydrochloride and the copper complexes were, however, dry amorphous powders, suitable for analysis. On the assumption that the cleavage of mycobactin into mycobactic acid and cobactin is a hydrolysis involving the addition of the elements of one molecule of water, the formula of mycobactic acid would be $C_{35}H_{55}O_7N_3$. Analysis of the hydrochloride and a copper derivative agree with this formula if they are hydrates. This point is discussed below. It must be emphasised that all experiments on mycobactic acid have been carried out on products which were not more than 90% pure. Some degradations were carried out on methylated mycobactic acid which was also an acidic gum not readily purified.

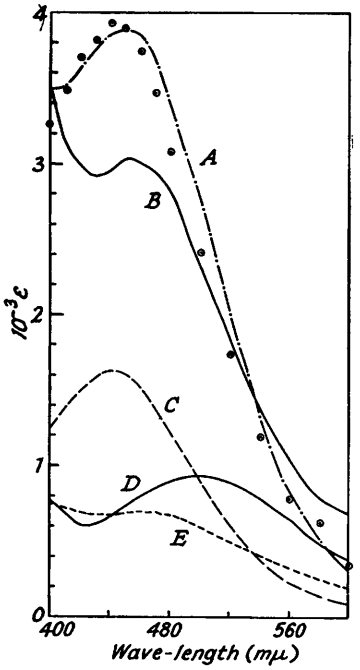


FIG. 1. *Absorption spectra of iron complexes in chloroform. A, Mycobactin. B, Mycobactic acid. C, Ethyl octadec-2-enohydroxamic acid. D, Acetylated mycobactic acid. E, Diacetyl mycobactin.*

⊙—⊙, *Points representing summation of* ε *for curve E with twice* ε *for curve C. Spectra measured in* 1-*cm. cells. Concns.,* 30—60 μM.

Besides the acidic group, mycobactic acid was found to contain a basic group similar to that present in mycobactin (Part I). The presence of this group was shown by the formation of the hydrochloride and by titration in glacial acetic acid. The variable purity of mycobactic acid preparations was also reflected in these titrations which gave equivalent weights from 670 to 800. Quantitative acetylation of mycobactic acid preparations indicated the uptake of somewhat less than one equivalent of acetyl per molecule, under the conditions in which mycobactin takes up two equivalents; the basic group remained unaffected by acetylation. Both crude mycobactic acid and its acetylated product resembled mycobactin in coupling with diazotised sulphanilic acid in alkaline solution to give yellow solutions, and both gave colours with ferric chloride in ethanol. The colour of the iron complex of the acetylated product, however, was violet, in contrast to the red-purple of that of mycobactic acid. For a more precise comparison of the colorations, the iron complexes were extracted into chloroform under acid conditions. A parallel experiment was carried out with the iron complex of ethyl octadec-2-enohydroxamic acid. The spectra (Fig. 1) showed the iron complex of mycobactic acid to have an absorption peak at 450 mμ compared with the peak given by the model hydroxamic acid at 440 mμ. The acetylated product gave a much lower absorption curve, with a maximum at 500 mμ.

The conditions of the experiment were such as to depress the colour given by phenols but to allow for maximum development of colour by hydroxamic acids. These findings suggested that mycobactic acid contained a group capable of acetylation which had the properties of a hydroxamic acid, and that there was also present a free phenolic group. The presence of a hydroxamic acid group has already been demonstrated in the cobactin moiety, where it arises from the hydroxyamino-group of the 2-amino-6-hydroxyamino-hexanoic acid. Since this amino-acid residue was also present in mycobactic acid, it seemed likely that it also existed in combination as a hydroxamic acid. In fact the hydroxy-amino-group could not be free since mycobactic acid had no reducing properties. The product of methylation of mycobactic acid gave no colour with ferric chloride, nor any phenolic reactions, and so was methylated on both the hydroxamic acid group and the phenolic group. Hydrolysis of the methylated product yielded an amino-acid, identical with that given by hydrolysis of *O*-methylcobactin, which was shown above to be probably 2-amino-6-methoxyaminohexanoic acid. The same compound was also detected on hydrolysis of the mycobactic acid moiety derived from incompletely methylated mycobactin, together with some 2-amino-6-hydroxyaminohexanoic acid. It is thus probable that in mycobactic acid, as in cobactin, the hydroxyamino-group is combined with a carboxyl to give a secondary hydroxamic acid.

In attempts to isolate an intermediate hydrolysis product no compound other than the ultimate breakdown products could be separated. However, some information about the structure could be gathered from the rate of liberation of the groupings characteristic of the end products. Table 1 shows that progressive acid hydrolysis yielded first water-

TABLE 1. *Progressive hydrolysis of mycobactic acid with boiling* 5N-*hydrochloric acid.*

Hydrolysis (min.)	H_2O-sol. fraction excluding Cl^- (% of original wt.)	Atoms of Cl^- per mole	NH_4^+ (moles per mole)	"Reducing value"	*m*-Cresol (moles) per mole)	Paper chromatography * S	L	AHH	X
20	34	1·3	—	3·1	0·03	—	Tr	Tr	+
40	45	1·5	—	4·5	0·12	Tr	Tr	+	+
80	54	2·0	—	5·7	0·38	+	Tr	++	—
180	58	2·0	0·16	6·2	0·74	++	+	+++	—
360	55	2·2	0·20	6·0	0·74	++	+	+++	—
720	52	2·5	0·32	5·5	0·76	++	++	+++	—
	R_F values of spots (butanol–acetic acid)					0·17	0·10	0·20	0·94
	Colour of spots					Brownish	←—	—Purple	—→

* S = serine; L = lysine; AHH = 2-amino-6-hydroxyaminohexanoic acid; X = partial hydrolysis product; Tr = trace.

soluble material which gave a reducing reaction under the conditions of Hanes's ferricyanide oxidation method. At the same time octadec-2-enoic acid was liberated and it could be isolated after 40 minutes' hydrolysis. Paper chromatography of the water-soluble components showed that 2-amino-6-hydroxyaminohexanoic acid appeared at an early stage of the hydrolysis, whilst serine appeared somewhat more slowly. There was also evidence of an intermediate hydrolysis product, revealed as a fast-running spot (X) on the chromatogram; this material disappeared as the hydrolysis proceeded. The benzenoid fragment of the molecule appeared entirely as *m*-cresol; 2-hydroxy-6-methylbenzoic acid would be immediately decarboxylated under these conditions. The yield of *m*-cresol rose to a maximum after 3 hours' hydrolysis, and its liberation ran approximately parallel with that of serine. This maximum value fell short of 1 mole per mole of mycobactin, probably owing to side reactions which are known to produce a small amount of unidentified material during the hydrolysis (*e.g.*, fraction B discussed in Part II). Production of ammonia was minute during the first few hours' hydrolysis: its formation later probably indicated some secondary breakdown; some would be formed from serine (Boyd and Logan, *J. Biol. Chem.*, 1942, **146**, 279).

Progressive hydrolysis of methylated mycobactic acid showed clearer differences. Table 2 shows that the methylated product yielded water-soluble products as rapidly as mycobactic acid itself; this first stage of hydrolysis was accompanied by liberation of

TABLE 2. *Progressive hydrolysis of methylated mycobactic acid with boiling 5N-hydrochloric acid.*

Hydrolysis (min.)	H_2O-sol. fraction excluding Cl^- (% of original wt.)	Paper chromatography * S	L	AMH	Y	Z
20	45	—	—	+	Tr	+
40	58	—	Tr	++	+	++
90	58	Tr	Tr	++	+	++
180	56	+	+	+++	Tr	+
960	48	++	+	+++	—	—
R_F values of spots (butanol–propanol–diethylamine)		0·09	0·16	0·28	0·65	0·93
Colour of spots		Brownish	Purple	Bright blue	Brownish	Bright blue

* S, L, Tr as in Table 1; AMH = 2-amino-6-methoxyaminohexanoic acid; Y and Z partial hydrolysis products.

octadec-2-enoic acid. Paper chromatography demonstrated the production of two partial hydrolysis products Y and Z giving ninhydrin reactions; their concentration rose to a maximum in 40—90 minutes and then declined. 2-Amino-6-methoxyaminohexanoic acid

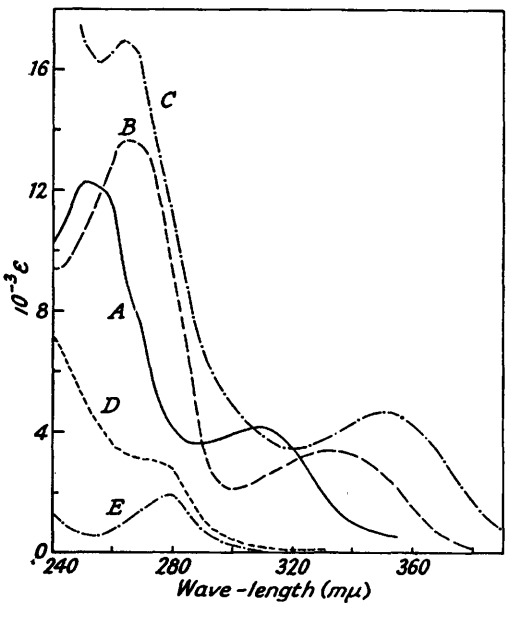


FIG. 2. *Absorption spectra. A, Mycobactic acid,* 0·85 mM *in methanol. B, Mycobactic acid hydrochloride,* 0·72 mM *in chloroform. C, Mycobactic acid copper complex,* 0·52 mM *in chloroform. D, Methylated mycobactic acid,* 0·72 mM *in methanol. E, 2-Methoxy-6-methylbenzoic acid,* 3·3 mM *in methanol. Spectra measured in* 1*-mm. cells.*

appeared at a rate similar to that for 2-amino-6-hydroxyaminohexanoic acid from mycobactic acid. Serine, however, was released very slowly. It had been shown that 2-methoxy-6-methylbenzoic acid was decarboxylated under the conditions of acid treatment used. The benzenoid hydrolysis product to be sought was therefore *m*-methoxytoluene. Solutions from prolonged hydrolysis were shown to contain a steam-volatile compound which resembled *m*-methoxytoluene in odour and in absorption spectrum which had characteristic twin peaks at 274 and 279 mμ. No *m*-cresol could be detected. The rate of liberation of this ether during hydrolysis appeared to be slow.

These observations, taken together, suggested that in the mycobactic acid molecule the serine and 2-hydroxy-6-methylbenzoic acid residues were joined together, since in this way the preferential release of the other components could readily be explained.

The spectrum of mycobactic acid (Fig. 2) was almost identical with that of mycobactin (Fig. 3), except that the molecular extinction at 250 mμ was slightly lower, possibly owing to impurity. The spectra of the hydrochloride and the copper complex (Fig. 2) showed shifts of the peaks similar to those observed on conversion of mycobactin into the corresponding derivatives (Part I). The hydrochlorides of mycobactin and mycobactic acid

had peaks of almost identical intensity. The grouping in mycobactin responsible for the absorption spectrum thus remained unaltered in mycobactic acid. From the nature of the degradation products of mycobactic acid it appears that any absorption maxima above 230 mμ were probably associated with the benzenoid residue. All the other components were aliphatic and their maxima would be at shorter wave-lengths. The spectrum could not, however, be explained on the basis of simple derivatives of 2-hydroxy-6-methylbenzoic acid. Esters and amides of this acid possess maxima below 300 mμ. The existence of a peak at 310 mμ suggested conjugation with the benzene ring. Evidence described above had pointed to the direct attachment of the 2-hydroxy-6-methylbenzoic acid and serine residues. Since the phenolic group appeared to be free in mycobactin the point of attachment of the aromatic residue to the rest of the molecule was presumably through the

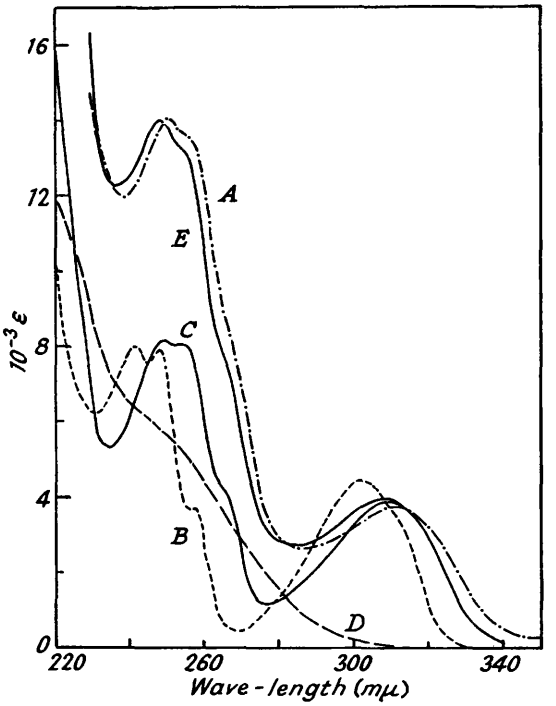
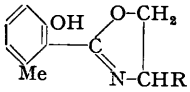


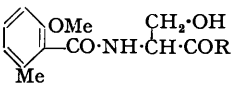
FIG. 3. *Absorption spectra. A, Mycobactin* 0·58 mM. *B,* 2-o-*Hydroxyphenyloxazoline* 0·77 mM. *C,* 2-(2-*Hydroxy*-6-*methylphenyl*)*oxazoline* 0·74 mM. *D,* N-*Ethyloctadec*-2-*enohydroxamic acid* 1·5 mM. *E, Summation of curves C and D. Spectra measured in methanol in* 1-*mm. cells.*

carboxyl group. In Part II pyrolysis or vigorous alkaline hydrolysis of mycobactin was reported to yield 2-hydroxy-6-methylbenzamide. Hence there could well be a nitrogen atom attached to this carboxyl group in the parent compound. A possible explanation of the spectra of mycobactin and mycobactic acid was thought to be the presence of an oxazoline ring, giving the partial structure (VII), the C=N bond being conjugated with the benzene ring. Moreover the cyclic nitrogen atom would account for the basic properties of mycobactic acid and mycobactin, and the shift of the absorption maxima which occurs when these compounds are converted into their hydrochlorides. The oxazoline ring would be expected to open readily, giving a serine derivative linked to the carboxyl group of 2-hydroxy-6-methylbenzoic acid as either an ester or an amide. In order to test the validity of this, 2-(2-hydroxy-6-methylphenyl)oxazoline (VII; R = H) was prepared; its spectrum is shown in Fig. 3. In mycobactic acid or mycobactin the other groups likely to contribute to ultra-violet absorption were the αβ-double bond in octadec-2-enoic acid and the hydroxamic acid group. Accordingly the spectrum of ethyl octadec-2-enohydroxamic acid was measured. Adding the absorptions of this compound to those of the synthetic oxazoline gave a curve extremely close to that of mycobactin (Fig. 3). The resemblance was not only in the height and position of the two main peaks, but in such detail as the shoulder at 258 mμ and the small inflection at 269 mμ. (The significance of these inflections

was shown more clearly in the spectrum of 2-*o*-hydroxyphenyloxazoline where they appeared as distinct peaks.) In fact the two curves were superimposable apart from a shift of 2—3 mμ towards the shorter wave-lengths in the summation curve. The presence of the oxazoline structure in mycobactin was thus highly probable. The instability of this ring to acids and alkalis explains the difficulty of obtaining mycobactic acid in a pure state.



(VII)



(VIII)

The change of spectrum occurring during methylation of mycobactic acid is probably due to opening of the oxazoline ring by the alkali used in the methylation, giving an amide linkage between the serine residue and the 2-methoxy-6-methylbenzoic acid residue (VIII) which would be relatively stable to hydrolysis. The spectrum would therefore be expected to lack the characteristic oxazoline absorption and rather to resemble that of 2-methoxy-6-methylbenzoic acid. In fact the spectrum was one showing progressively increasing absorption towards the shorter wave-lengths with a shoulder at 270—280 mμ (Fig. 2). A very similar curve could be produced by summing the absorption curves of 2-methoxy-6-methylbenzoic acid which had a weak absorption maximum at 279 mμ and octadec-2-enoic acid which produced end-absorption similar to that expected from the groupings present in the methylated product.

It was possible at this stage to speculate on the total structure of mycobactic acid. Three residues, namely, 4-carboxy-2-(2-hydroxy-6-methylphenyl)oxazoline, 2-amino-6-hydroxyaminohexanoic acid, and octadec-2-enoic acid, must be combined together to satisfy the following conditions: one of the three carboxyl groups to be free, the others combined; the amino-group to be combined; the phenolic group to remain free; the hydroxyamino-group to be combined with a carboxyl group to produce a hydroxamic acid. If octadec-2-enoic acid occurred as an unsaturated residue in the original compound, it must be attached by its carboxyl group. The point of attachment of the oxazoline residue would presumably also be through the carboxyl group. It therefore appeared that the free carboxyl group of mycobactic acid was that present in the 2-amino-6-hydroxyaminohexanoic acid residue. The simplest formulæ which would meet the required conditions were (II or III; R = R″ = H). These structures have the empirical formula $C_{35}H_{55}O_7N_3$ postulated above from consideration of the formulæ of mycobactin and cobactin. Analysis of the hydrochloride agrees with $C_{35}H_{55}O_7N_3,HCl,H_2O$. The question of the composition of the copper complex is complicated by the variations of copper content which result from different methods of preparation. However, the product obtained by addition of mycobactic acid solution to an excess of ethanolic copper acetate appears to be an entity, $C_{35}H_{51}O_7N_3Cu_2,2H_2O$. It would be unwise to rely too greatly on the analytical figures for these amorphous compounds because of the difficulty of establishing their purity, and, unfortunately, the free acid, owing to its physical form, yields analytical results too variable to be of value; but since both the derivatives are shown as hydrates the question arises whether the formula of mycobactic acid could be $C_{35}H_{57}O_8N_3$. Such an incorporation of the elements of water seems improbable in the hydroxyamino-fragment, or in the oxazoline fragment since the spectra of the hydrochloride and copper complex indicate that the oxazoline ring is still intact. It is conceivable that the fatty acid could be present as a β-hydroxy-acid with conversion into the αβ-unsaturated acid during hydrolysis. However, there was no evidence of such a hydroxy-group in mycobactic acid, and octadec-2-enoic acid was shown to be liberated under gentle conditions of acid hydrolysis which would be unlikely to remove a β-hydroxy-group.

Therefore formulæ (II and III; R = R″ = H) have been tentatively accepted as alternatives for mycobactic acid. An attempt to decide between them by comparison of the infra-red spectrum of mycobactic acid with spectra of compounds containing similar groupings has not so far been successful. Formula (II; R = R″ = H) might be slightly favoured as affording an explanation of the simultaneous rapid liberation of octadec-2-enoic acid and the hydroxyamino-group which marked the earliest stages of acid hydrolysis.

The cleavage of mycobactin into cobactin and mycobactic acid appears to involve the rupture of a bond between the carboxyl group of mycobactic acid and one of the two hydroxyl groups of cobactin. The point of attachment of cobactin was first indicated by the behaviour of mycobactin and cobactin on gentle acetylation. Mycobactin formed a diacetyl derivative (Part I). Only one acetyl group entered the mycobactic acid portion of the molecule, since the phenolic hydroxyl remained free in the diacetyl compound (as shown by the violet ferric chloride reaction and ability to couple with diazonium salts); moreover mycobactic acid itself gave only a monoacetyl derivative. The other acetyl group must therefore be situated in the cobactin portion of the molecule. However when cobactin was acetylated under identical conditions it yielded only a monoacetyl derivative with the acetyl attached to the hydroxamic acid group (I; R = H, R′ = Ac): the alcoholic hydroxyl group resisted acetylation under these conditions. Thus if in acetylation of mycobactin an acetyl group entered the cobactin residue it probably became attached to the hydroxamic acid group of that moiety, and attachment of cobactin to mycobactic acid must be through the alcoholic hydroxyl group. Methylation of mycobactin gave a more direct proof. Because of the lability of mycobactin to alkali, methylation was best effected by use of methyl iodide with two equivalents of thallous hydroxide. This procedure gave a mixed methylated product in which the essential structure of mycobactin was apparently unaltered. A similar product but with a lower degree of methylation was obtained by the use of diazomethane. Methylation occurred both in the mycobactic acid and in the cobactin part of the molecule, but to different extents. Fortunately the free hydroxyl group in the cobactin residue was almost completely methylated and after alkali cleavage of the methylated product *O*-methylcobactin was readily isolated. This was identical with the compound produced by methylation of cobactin, the methyl group being attached to the hydroxamic acid group, as in (I; R = H, R′ = Me).

Combination of the structures suggested for mycobactic acid and cobactin gave (IV or V; R = R′ = H) as tentative formulæ for mycobactin. These accounted for all the degradation products isolated, except the small amount of lysine always detected among the hydrolysis products of mycobactin or mycobactic acid; no lysine was found on hydrolysis of cobactin. Lysine might arise by some side-reaction causing reduction of a part of the 2-amino-6-hydroxyaminohexanoic acid liberated. Alternatively its appearance might indicate the presence in our preparations of mycobactin of a small proportion of an analogue in which the 2-amino-6-hydroxyaminohexanoic acid residue of the mycobactic acid moiety was replaced by a lysine residue: up to 10%, it would not significantly affect the analytical figures. It was found that aluminium mycobactin which had been repeatedly crystallised without change of physical properties still gave some lysine on acid hydrolysis. Even this did not, however, entirely exclude the presence of a proportion of a closely related compound in view of the possibility of mixed crystal formation. The weakly acidic properties of mycobactin were explained by the presence of two secondary hydroxamic acid groups and the phenolic hydroxyl group. Of these the hydroxamic group in the cobactin moiety appeared to be the most acidic; the hydroxamic acid group in mycobactic acid was hardly detectable on electrometric titration in methanol, whilst a weakly acidic group was clearly shown on titration of mycobactin and cobactin. The difference between the two hydroxamic acid groups was also shown by their behaviour on methylation, the cobactin group being more easily methylated. Both hydroxamic acid groups were acetylated under mild conditions, giving *OO*-diacetylmycobactin (IV or V; R = R′ = Ac) in which the acetyl groups were extremely labile to alkali. This derivative still had phenolic properties, the phenolic group being difficult to substitute. The single basic group of mycobactin, which was demonstrated by titration and by the formation of a picrate and a hydrochloride, was represented in the formulæ by the nitrogen atom of the oxazoline ring. The proposed structures showed considerable possibilities of chelation, agreeing with the observed formation of metal complexes by mycobactin. The copper complex, containing one metal atom per molecule of mycobactin, probably involved two of the weakly acidic groups and possibly the nitrogen atom of the oxazoline ring. The aluminium complex which also had only one metal atom per molecule was evidently of unusual structure. It differed from mycobactin and its other derivatives in being easily crystallisable, in its high optical rotation, and in its solubility

in relatively non-polar solvents such as benzene. Possibly with aluminium a tridentate compound is formed in which the molecule is held in a compact folded configuration which facilitates crystallisation. The combination of the metal with all three acidic groups would explain the reduction in polar properties. The aluminium was tenaciously held and could not be displaced by acids. It was, however, displaced by copper in solution, presumably because of the low solubility of the copper complex in ethanol. Aluminium may be specific in its combination with mycobactin; similar compounds were apparently not formed with iron or chromium. The absorption spectrum of the compound produced with ferric chloride could be accounted for as the sum of the absorptions of the iron complexes of the weakly acidic groups. Fig. 1 shows the correspondence of the spectrum of the ferric complex of mycobactin with the summation curve of the spectra of the complexes given by one mol. of *OO*-diacetylmycobactin and two mols. of a hydroxamic acid.

The loss of biological activity of mycobactin in the presence of alkali under the mildest conditions was explained by the cleavage into cobactin and mycobactic acid, neither of which separately or together had growth-promoting action.

Experimental

M. p.s are corrected. Electrometric titrations, reducing value measurements, paper chromatography, and quantitative acetylation were carried out as in Parts I and II.

Action of Alkali on Mycobactin.—A 2% solution of mycobactin in ethanol was treated with 4—6 equivs. of ethanolic potassium hydroxide and set aside for 6 hours at 20°. The solution was then diluted with three volumes of 90% ethanol, and the excess of alkali titrated with 0·05N-hydrochloric acid in 90% ethanol, a glass–calomel electrode assembly being used. Control titrations were carried out similarly. The acid liberated was 1·03 ± 0·05 equivs. Similar values were obtained when the alkaline mycobactin solution was heated for 15 min. at 100° in a sealed tube before titration.

Alkaline Fission of Mycobactin.—Mycobactin (5 g.) was dissolved in methanol (100 c.c.) and the solution quickly cooled in an ice-bath. Cold N-sodium hydroxide solution (100 c.c.) was added and the mixture kept in the ice-bath for 45 min. N-Sulphuric acid (100 c.c.) was then run in with stirring; a sticky precipitate separated. Water (100 c.c.) was added, and the suspension extracted with ether (3 × 250 c.c.) which dissolved the precipitate. The ether extract was washed with water (2 × 5 c.c.) and evaporated. The residue (3·77 g.; fraction A, crude mycobactic acid) was a transparent yellow resin. The aqueous layer was evaporated to dryness under reduced pressure and the residue extracted with ethanol (3 × 70 c.c.) by grinding in a mortar and filtering. The filtrate was evaporated, the residue taken up in ethanol (30 c.c.), and the solution again filtered to remove a little inorganic material. The filtrate was evaporated, leaving an almost white residue (1·40 g.; fraction B, crude cobactin).

Examination of Fractions A and B from the Alkaline Fission.—Acid hydrolysis. A portion of each fraction (0·2 g.) was refluxed for 8 hr. with 5N-hydrochloric acid (40 c.c.). Fraction A gave a fraction insoluble in water, but extractable by ether, which contained *m*-cresol and octadec-2-enoic acid. The aqueous layer was evaporated under reduced pressure and the residue dissolved in water and submitted to paper chromatography with butanol–acetic acid as a developer. The chromatogram indicated the presence of 2-amino-6-hydroxyaminohexanoic acid, serine, and some lysine. The hydrolysate from fraction B was completely soluble in water; it contained no long-chain fatty acid and gave no reaction for phenols. Repeated extraction with ether removed an acidic product. This remained as a viscous oil on evaporation of the solvent and appeared to be the component detected but not identified in the hydrolysis of mycobactin (Part II). The aqueous layer was evaporated under reduced pressure; the residue, dissolved in water and tested by paper chromatography, gave only one spot, and this corresponded to 2-amino-6-hydroxyaminohexanoic acid. Hydrolysis for 2 hr. with boiling 5N-hydrochloric acid gave water-soluble components having the following reducing values (expressed as equivs. of thiosulphate per mole of mycobactin from which the fraction was derived): fraction A, 6·7; fraction B, 6·5; mycobactin, 12·8.

Cobactin (I; R = R′ = H). Fraction A (1·2 g.) crystallised twice from ethyl methyl ketone with a little carbon gave colourless needles of *cobactin* (0·72 g.), m. p. 152·5—153°, $[\alpha]_D^{20}$ −46·1° ± 0·5° (*c*, 4·9 in MeOH) (Found: C, 55·6, 55·7; H, 8·45, 8·5; N, 10·7. $C_{12}H_{22}O_4N_2$ requires C, 55·8; H, 8·6; N, 10·85%). This was readily soluble in water, giving a solution neutral to

indicator paper, and soluble in the lower alcohols and chloroform but sparingly soluble in less polar solvents. It gave a strong reddish-violet colour with ferric chloride but no reaction with ninhydrin or Fehling's solution and did not couple with diazonium salts. Electrometric titration with alkali showed the presence of one weakly acidic group, pK 9·1. Titration with acid gave no indication of ionising groups. Cobactin showed absorption only in the far ultra-violet with no maxima above 220 mμ.

O-*Methylcobactin* (I; R = H, R′ = Me). Cobactin (0·30 g.) was stirred in methanol (4·5 c.c.) at 55—60° in a water-bath. Methyl sulphate (0·8 c.c.) and 5·5N-sodium hydroxide (1·6 c.c.) were added in portions of 0·05 c.c. and 0·1 c.c. respectively during 1 hr., the solution being weakly alkaline throughout. Then stirring was continued for 10 min., the solution neutralised by addition of a little acid and evaporated to dryness under reduced pressure. The residue was extracted with boiling chloroform (3 × 15 c.c.), and the extracts were filtered and evaporated, leaving a white residue (0·30 g.). Two crystallisations from ethyl acetate gave colourless needles of O-*methylcobactin* (0·12 g.), m. p. 172·5° (Found: C, 57·2; H, 8·5; N, 10·25. $C_{13}H_{24}O_4N_2$ requires C, 57·3; H, 8·9; N, 10·3%). The methoxyl group was not removed under the ordinary Zeisel conditions. The product gave a neutral solution in water; addition of ferric chloride caused no coloration.

O-*Acetyl*-O-*methylcobactin* (I; R = Ac, R′ = Me). *O*-Methylcobactin (52 mg.), powdered fused sodium acetate (100 mg.), and acetic anhydride (0·5 c.c.) were heated for 2 hr. at 100°. The product was evaporated to dryness under reduced pressure and the residue extracted with boiling carbon tetrachloride (3 × 2 c.c.). The extract was evaporated, leaving a white residue (53 mg.) which, crystallised from light petroleum (b. p. 100—120°), yielded O-*acetyl*-O-*methylcobactin* as colourless needles, m. p. 156·5—157° (Found: C, 56·9; H, 8·15; N, 8·8. $C_{15}H_{26}O_5N_2$ requires C, 57·3; H, 8·3; N, 8·8%).

O-*Acetylcobactin* (I; R = H, R′ = Ac). Cobactin (100 mg.), acetic anhydride (100 mg.), and pyridine (2·5 c.c.) were kept in an ice-bath for 30 min. Water (2·5 c.c.) was then added and the solution evaporated to dryness under reduced pressure. The residue (115 mg.) was twice crystallised from light petroleum (b. p. 100—120°), giving colourless fine needles of O-*acetylcobactin*, m. p. 128—128·5° (Found: C, 56·4; H, 7·9; N, 9·7. $C_{14}H_{24}O_5N_2$ requires C, 56·0; H, 8·05; N, 9·3%). The product was soluble in water, giving a neutral solution which did not give a ferric chloride reaction.

Hydrolysis of Cobactin to Cobactinin (VI).—Cobactin (0·8 g.) was refluxed for 6 hr. with 0·1N-hydrochloric acid (100 c.c.), and the solution then evaporated to dryness under reduced pressure. The residue was dissolved in water (30 c.c.) and applied at 4—5 c.c. per hour to a 70-cm. column of Zeo-Karb 215 (3·5 g.; 80—120 mesh). The column was washed with water, and treated with 0·15N-ammonia. Reducing material, collected in effluent solution at pH 5—5·5, gave, on evaporation under reduced pressure, 0·21 g. of residue. Two crystallisations from water yielded colourless needles of *cobactinin*. A further 0·26 g. of reducing material from effluent solution having a pH of 6·5—7·5 failed to crystallise. Cobactinin had m. p. 174—175·5° (decomp.), varying somewhat with the rate of heating (Found: C, 52·1; H, 8·55; N, 10·4. $C_{12}H_{24}O_5N_2$ requires C, 52·2; H, 8·7; N, 10·15%). It had properties characteristic of the hydroxyamino-group, reducing Fehling's solution, neutral silver nitrate solution, and alkaline triphenyltetrazolium chloride. It gave no colour with ferric chloride or ninhydrin. On paper chromatography (butanol–acetic acid; Part II) it gave a single spot R_F 0·81 shown by the tetrazolium spray. It was soluble in water, giving a solution of pH 4·6. Ionising groups were demonstrated by electrometric titration of 0·1M-solutions with 0·3N-barium hydroxide and N-hydrochloric acid.

Hydrolysis of Cobactin and Isolation of (−)-3-*Hydroxy*-2-*methylpentanoic Acid.*—Cobactin (0·80 g.) was refluxed for 8 hr. with 5N-hydrochloric acid (10 c.c.), then cooled and extracted with a large volume of ether (5 × 50 c.c.). The aqueous layer was evaporated under reduced pressure, leaving a gummy residue (0·74 g.) having strong reducing properties. This was a hydrochloride from which the free base was obtained by absorption on a column of Zeo-Karb 215 and displacement with ammonia. The properties of the base coincided with those described for 2-amino-6-hydroxyaminohexanoic acid isolated from hydrolysis of mycobactin. The ether extract was washed with a minimum of water, dried ($MgSO_4$), and evaporated. The residue (0·39 g.), a viscous oil, distilled at 90—100° (bath-temp.)/0·1 mm., giving (−)-3-*hydroxy*-2-*methylpentanoic acid* as a colourless oil. Analysis indicated that the acid as isolated probably contained about 2% of water. It had $[\alpha]_D^{25}$ −14·8° (*c*, 4·3 in MeOH) (Found: equiv., by titration, 135. $C_6H_{12}O_3$ requires equiv., 132). It was a monobasic acid, pK 4·4. The crystalline p-*bromophenacyl* ester, m. p. 89·5—90° (from light petroleum, b. p. 60—80°), had $[\alpha]_D^{18}$ −15° (*c*, 2·5 in MeOH)

(Found: C, 51·2; H, 5·25. $C_{14}H_{17}O_4Br$ requires C, 51·1; H, 5·2%). Quantitative acetylation of the free acid led to the uptake of 0·89 equiv. of acetyl.

Identification of the Hydroxy-acid from Cobactin.—(*A*) The hydroxy-acid (200 mg.) was mixed with red phosphorus (28 mg.) and powdered iodine (250 mg.) and heated 2·5 hr. at 100—110°; some hydrogen iodide was evolved. The product was cooled, the residue dissolved in ether and filtered, and the solution evaporated. The crude iodo-compound (330 mg.) remaining was refluxed with zinc dust (0·5 g.) and 2N-hydrochloric acid (2 × 4 c.c. added during 1 hr.). The solution was decanted from undissolved zinc, further 2N-hydrochloric acid (2 c.c.) added, and the solution steam-distilled. The distillate (40 c.c.) was neutralised with sodium hydroxide, extracted with ether (2 × 15 c.c.) to remove a little non-acidic material, and evaporated under reduced pressure. The residue was dissolved in ethanol (2 c.c.), and the solution centrifuged and evaporated. From the sodium salt (120 mg.) an anilide was prepared. The crude derivative (75 mg.) contained a little of the anilide of the unsaturated acid. It was treated in acetone (3 c.c.) with potassium permanganate in slight excess. The solution was set aside for 2 hr. at 20°, filtered, diluted with ether, and washed with dilute sodium hydrogen sulphite solution and water. The ether solution was dried and evaporated and the residue distilled under reduced pressure. The main product, distilling at 120° (bath-temp.)/0·2 mm., was crystallised from hexane. It had $[\alpha]_D^{18}$ −19° (*c*, 2·7 in MeOH), m.p. 89·5—90·5° and was identified as (−)-2-*methylpentanoanilide*. For identification it was compared with specimens of the anilides from isomeric hexanoic acids prepared synthetically from substituted malonic esters (see Table).

Compound	Found (%) * C	H	N	M. p.	Mixed m. p. with (X)	Infra-red spectrum compared with (X)
Degradation compound (X)	74·9	8·6	7·6	89·5—90·5°	—	—
(±)-2-*Methylpentanoanilide*	75·3	8·6	7·4	94·5—95	92—93·5°	Identical
(±)-3-*Methylpentanoanilide*	75·0	8·7	7·45	84—85·5	<70	Different
(±)-2-*Methyliso*pentanoanilide	75·5	8·95	7·6	76—76·5	84—87·5	Different
* $C_{12}H_{17}ON$ requires	75·3	8·95	7·3			

(*B*) The hydroxy-acid (0·5 g.) in water (3 c.c.) was treated with sodium dichromate dihydrate (0·6 g.) and 30% sulphuric acid (3·6 c.c.). The mixture was at once steam-distilled. The distillate (30 c.c.) was mixed with 2 : 4-dinitrophenylhydrazine (0·6 g.) in hot 2N-hydrochloric acid (100 c.c.), and the hydrazone (0·28 g.) was later filtered off. After crystallisation from ethanol it had m. p. 155—156° undepressed on admixture with diethyl ketone 2 : 4-dinitrophenylhydrazone (Found: C, 49·7; H, 5·4; N, 20·9. Calc. for $C_{11}H_{14}O_4N_4$: C, 49·6; H, 5·3; N, 21·0%).

Synthetic 3-Hydroxy-2-methylpentanoic Acid.—The method of Astachow and Reformatski (*J. Russ. Phys. Chem. Soc.*, 1901, **33**, 209) gave the acid from which the p-*bromophenacyl* ester was prepared, having m. p. 62·5—63° (Found: C, 50·7; H, 5·4; Br, 24·5. $C_{14}H_{17}O_4Br$ requires C, 51·06; H, 5·2; Br, 24·3%). The infra-red spectrum differed from that of the *p*-bromophenacyl ester of the degradative hydroxy-acid.

Hydrolysis of O-Methylcobactin giving 2-Amino-6-methoxyaminohexanoic Acid.—Methylcobactin (0·66 g.) was refluxed for 8 hr. with 5N-hydrochloric acid (7·5 c.c.). The solution was extracted with ether (5 × 8 c.c.). The ether extract was dried ($MgSO_4$) and evaporated, leaving 3-hydroxy-2-methylpentanoic acid as a viscous oil (0·24 g.). The aqueous solution after ether-extraction was evaporated under reduced pressure. The gummy residue (0·58 g.) was dissolved in water (20 c.c.) and extracted three times with 5% trioctylamine in chloroform. The aqueous layer was washed with ether and evaporated under reduced pressure, leaving a residue (0·39 g.) of the base. This was partly purified by dissolution in water (2 c.c.) and precipitation with acetone (15 c.c.). The amino-acid was not obtained crystalline. The crude amino-acid (47 mg.) and 5-nitrobarbituric acid (59 mg.) were each dissolved in hot water (1 c.c.) and mixed. The solution on cooling gave crystals. Two further crystallisations from water gave 2-*amino*-6-*methoxyaminohexanoic acid di*-5-*nitrobarbiturate* as cream-coloured rhombic plates without definite m. p. (Found: C, 33·5; H, 4·5; N, 21·4. $C_7H_{16}O_3N_2,2C_4H_3O_5N_3,H_2O$ requires C, 33·3; H, 4·5; N, 20·8%). The crude amino-acid had no reducing properties or ferric chloride reaction. It gave a single spot on paper chromatography (R_F values: butanol–propanol–diethylamine 0·33, butanol–acetic acid 0·23, collidine–lutidine 0·35; compositions as in Part II).

Partial Methylation of Mycobactin.—(*A*) Mycobactin (2 g.) was warmed with methanol (10 c.c.) and methyl iodide (5 c.c.). It swelled and partly dissolved, giving a gelatinous suspen-

sion on cooling. Freshly prepared thallous hydroxide (1·03 g.) was added in portions with stirring during 15 min. The solution first became clear and then deposited thallous iodide. The mixture was refluxed for 1 hr., cooled, and filtered. The filtrate was evaporated, and the residue dissolved in chloroform, filtered again, evaporated to 2—3 c.c., and treated with excess of ether. The gelatinous precipitate was filtered and dried, giving partly methylated mycobactin (1·88 g.). The product was a pale cream non-crystalline powder, m. p. 144—144·5°, having a pale green fluorescence like that of mycobactin. It gave a red-violet ferric chloride reaction (bluer than that given by mycobactin) and formed no precipitate with ethanolic cupric acetate.

(*B*) Mycobactin (0·2 g.) was dissolved in methanol (7 c.c.). Ether (3 c.c.) was added and the solution cooled in an ice-bath. Ethereal diazomethane (4 c.c. containing approx. 4 equivs.) was added. After 5 min. excess of diazomethane was removed by shaking with dilute acetic acid. Water (20 c.c.) was added and the product extracted with chloroform. Evaporation of the chloroform gave the partially methylated material (0·19 g.) having properties similar to that obtained by the use of methyl iodide.

Alkaline Fission of Partly Methylated Mycobactin.—The methylated product from the methyl iodide preparation (1·4 g.) was dissolved in methanol (20 c.c.), cooled in ice, and treated with N-sodium hydroxide (20 c.c.), as for the alkaline fission of mycobactin. It yielded an ether-soluble (1·01 g.) and a water-soluble fraction (0·39 g.). The latter which gave only a slight colour reaction with ferric chloride crystallised from carbon tetrachloride, leaving a little insoluble material. Further crystallisation from ethyl acetate gave *O*-methylcobactin, m. p. 172—173°. The ether-soluble fraction was a yellowish acidic resin resembling mycobactic acid. Portions of this fraction, the crude water-soluble fraction, and the partly methylated mycobactin from which they were derived, were refluxed for 3 hr. with 5N-hydrochloric acid. The aqueous layers after extraction with ether were evaporated under reduced pressure and the residues tested for reducing value and by paper chromatography. A parallel experiment was carried out with mycobactin partly methylated with diazomethane. Results are given in Table 3.

TABLE 3. *Hydrolysis of partly methylated mycobactin and its alkaline fission products.*

Fraction hydrolysed	Reducing value	Amino-acids shown by paper chromatography * Serine	AHH		AMH	Lysine
Mycobactin methylated with MeI–TlOH						
Crude methylation product		5·0	++	++	+++	+
Water-sol. alkali-fission product		v.l.	—	—	+++	—
Ether-sol. alkali-fission product		4·4	++	++	++	+
Mycobactin methylated with CH_2N_2						
Crude methylation product		5·5	++	++	+++	+
Water-sol. alkali-fission product		1·5	—	+	+++	—
Ether-sol. alkali-fission product		3·9	++	++	++	+
Untreated mycobactin		13·0	++	+++	—	+

v.l., very little. * AHH, AMH as in Tables 1 and 2.

Mycobactic Acid.—Fraction B from the alkaline fission of mycobactin is crude mycobactic acid. To obtain a rather less degraded specimen, mycobactin (2 g.) was dissolved in chloroform (40 c.c.), shaken mechanically with N-sodium hydroxide (20 c.c.) in an ice-bath for 40 min., and the emulsion separated by centrifugation at 0°. The aqueous layer was acidified with 5N-hydrochloric acid (5 c.c.) and extracted with benzene (3 × 30 c.c.). The extract was washed with water, and part of the benzene distilled off to remove dissolved water. The solution was diluted to 100 c.c. with dry benzene and dried from the frozen state. The white powder (1·35 g.) remained dry if stored below 0° but soon became sticky at room temperature. The mycobactic acid produced in this way was suitable for many purposes. It could, however, be further purified by conversion into a copper complex (see below). The purified complex (0·5 g.) was dissolved in chloroform (20 c.c.). Hydrogen sulphide was passed through the solution, methanol (40 c.c.) was added, and the copper sulphide filtered off. The resultant solution was evaporated under reduced pressure to small bulk, treated with benzene, and again evaporated. Finally benzene was added and the solution dried from the frozen state, giving a product (0·40 g.) which was completely colourless in the solid state or in solution. Mycobactic acid preparations were titrated electrometrically in methanol. Only one acid group was detectable. The equivs. of typical specimens were 711, 860, 820 by direct titration. Back-titration was carried out by treating a methanolic solution of mycobactic acid with more than 4 equivs. of methanolic potassium hydroxide. After 18 hr. at 20° the solution was back-titrated electrometrically with methanolic hydrochloric acid, giving an equiv. of 640 ± 20. Ethanolic solutions of mycobactic

acid gave a reddish-purple ferric chloride reaction. The coloured product could be extracted into chloroform, giving a crimson solution (the corresponding extract of the iron complex of mycobactin was orange). Alkaline solutions coupled with diazotised sulphanilic acid to give a yellow solution. The ultra-violet absorption spectrum (Fig. 2) resembled that of mycobactin. The specimen used for this spectrum had an equiv. of 665 by perchloric acid titration. Specimens with higher equivs. gave lower absorption maxima.

Mycobactic Acid Copper Complexes.—Mycobactic acid in ethanol with cupric acetate gave a precipitate, the composition of which depended upon the conditions of precipitation; the copper content varied from 9 to 16% and the complete analyses suggested that the insoluble products contained between 1 and 2 atoms of copper per mol. of mycobactic acid.

To prepare a complex having the maximum copper content 1 g. of mycobactin was treated with sodium hydroxide under the conditions described above. The mycobactic acid fraction was extracted under the mildest conditions and immediately dissolved in ethanol (10 c.c.). This solution was added slowly to a solution of cupric acetate (0·6 g. of hydrate) in ethanol (30 c.c.). The precipitate was separated at the centrifuge, washed repeatedly with ethanol, then with ether, and dried. The grey-green amorphous *copper* complex (0·45 g.) had no m. p. (charred above 250°) (Found: C, 53·0; H, 6·5; N, 5·5; Cu, 16·0. $C_{35}H_{51}O_7N_3Cu_2,2H_2O$ requires C, 53·3; H, 6·8; N, 5·3; Cu, 16·1%). This and other complexes could be repeatedly precipitated from chloroform solution by addition of ethanol or ether without significant change of composition. Complexes required for regeneration of mycobactic acid were purified by absorption chromatography on silica gel, development being carried out with 5% v/v ethanol in chloroform. This treatment removed traces of coloured impurities. The solubility properties of copper mycobactic acid complexes resembled those of copper mycobactin (Part I).

Mycobactic Acid Hydrochloride.—A specimen of mycobactic acid (0·20 g.) of low equiv. weight was dissolved in dry ether (5 c.c.), centrifuged to remove a trace of insoluble matter, cooled in ice, and treated with ethereal hydrogen chloride (1 c.c.). The precipitate was centrifuged off, washed with dry ether, and dried in a vacuum-desiccator. *Mycobactic acid hydrochloride* was obtained as a pale cream-coloured powder, m. p. 96—98° (after softening) (Found: C, 61·5; H, 8·3; N, 6·4; Cl, 5·1. $C_{35}H_{55}O_7N_3,HCl,H_2O$ requires C, 61·5; H, 8·55; N, 6·15; Cl, 5·2%). The ultra-violet absorption spectrum (Fig. 2) resembled that of mycobactin hydrochloride (Part I). The compound was soluble in chloroform and the lower alcohols. When a chloroform solution was shaken with water, hydrochloric acid passed into the aqueous layer leaving mycobactic acid in the chloroform. The solid showed a light blue fluorescence in ultra-violet light.

Methylation of Mycobactic Acid.—Mycobactic acid (2·6 g.) in methanol (30 c.c.) was warmed to 55—60° with stirring. Methyl sulphate (4 c.c.) and 5·5N-sodium hydroxide (8 c.c.) were added simultaneously in increments of 0·2 c.c. and 0·4 c.c. respectively during 1 hr., the solution remaining weakly alkaline. Stirring was continued for a further 15 min., then the solution was cooled, diluted with water (60 c.c.), and acidified. The gummy precipitate was extracted with ether (3 × 30 c.c.), the extract washed with water and evaporated, and the residue freed from water by evaporation with chloroform. The residue was dissolved in methanol and the methylation repeated with half the quantities of methyl sulphate and sodium hydroxide. The residue from the second methylation (2·30 g.) was a clear yellow gum, with ultra-violet absorption spectrum shown in Fig. 2. It did not react with ferric chloride, ethanolic cupric acetate, or diazotised sulphanilic acid. Electrometric titration in methanol showed it to be a monobasic acid. The equiv. wt. (800—850) was higher than the theoretical for *OO*-dimethylmycobactic acid.

Acetylation of Mycobactic Acid.—Mycobactic acid (0·63 g.) was dissolved in dry pyridine (2 c.c.), and acetic anhydride (0·12 c.c.) added. The mixture was kept at room temperature for 1 hr., diluted with water (5 c.c.), and acidified with hydrochloric acid. The sticky precipitate was extracted with ether, and the extract washed with water, and evaporated to dryness. The residue was dissolved in benzene (25 c.c.) and evaporated from the frozen state, giving a creamy-white powder (0·54 g.) which became sticky at room temperature. The product gave no precipitate with cupric acetate in ethanol. Ethanolic ferric chloride gave a violet colour, much bluer than that given by mycobactic acid. Titration in methanol showed it to be a monobasic acid. It gave a yellow colour in alkaline solution with diazotised sulphanilic acid. Quantitative acetylation of a specimen of mycobactic acid showed the uptake of 0·82 OAc per mol.

Acid Hydrolysis of Methylated Mycobactic Acid.—Methylated mycobactic acid (2·31 g.) was refluxed for 40 min. with 5N-hydrochloric acid (100 c.c.). The solution was cooled, diluted with water, and extracted with ether. The residue from the dried ether extract was treated with light petroleum. The petroleum-soluble wax (0·77 g.) was recrystallised and identified as

octadec-2-enoic acid. The aqueous solution after ether-extraction was evaporated under reduced pressure, and the residue dissolved in water (20 c.c.) and extracted with butanol (3 × 20 c.c.). The residue after evaporation of the aqueous layer (0·49 g.) was shown by paper chromatography to be mainly the hydrochloride of 2-amino-6-methoxyaminohexanoic acid, and contained no detectable serine. The portion (0·86 g.) of the ether-extract insoluble in light petroleum, and the residue (0·42 g.) from the butanol extract of the aqueous layer, were gums containing mixtures of partial hydrolysis products.

Progressive Acid Hydrolysis of Mycobactic Acid and its Methylation Product.—Portions (100 mg.) of mycobactic acid and its methylation product were refluxed with 5N-hydrochloric acid for varying times. The solutions were diluted with water, extracted with ether, and evaporated under reduced pressure. Estimations on the residues (Table 1) were carried out as follows: for chloride, an aliquot was ignited with a slight excess of sodium carbonate, the residue was dissolved in water, neutralised, and titrated with silver nitrate (chromate indicator); ammonia was estimated by steam-distillation from pH 11 buffer in a micro-Kjeldahl apparatus. *m*-Cresol was collected by steam-distillation of the whole hydrolysis solutions without ether-extraction and estimated in the distillate by Miller and Urbain's method (*Ind. Eng. Chem. Anal.*, 1930, **2**, 123).

Colorimetric Estimation of the Iron Complexes of Mycobactin Derivatives and Synthetic Compounds.—Solutions of the test substances were made in chloroform and suitable aliquots (*e.g.*, equiv. to 0·5 mg. of mycobactin) were diluted to 10 c.c. with chloroform. Ethanolic ferric chloride solution (0·1 c.c.; 0·5M) was added and the solution mixed. It was then shaken with 0·01N-hydrochloric acid (10 c.c.), and the lower layer run off into a 25-c.c. graduated flask. The aqueous layer was extracted with a further 10 c.c. of chloroform and the contents of the flask were made up to volume with the same solvent. The chloroform solution was filtered through two thicknesses of dry filter paper to give a clear solution, and the spectrum at once plotted by using a spectrophotometer. The curves are shown in Fig. 1.

Synthetic Oxazolines.—*N*-2′-Hydroxyethylsalicylamide (20 g.; m. p. 116—117°) was converted into the oxazoline by a method similar to that of Pfister, Robinson, Shabica, and Tishler (*J. Amer. Chem. Soc.*, 1949, **71**, 1101). The crude base (14·5 g.) was distilled (b. p. 87—89°/22 mm.) and crystallised from light petroleum, giving 2-o-*hydroxyphenyloxazoline*, m. p. 39—40° (Found: C, 66·65; H, 5·3; N, 8·9. $C_9H_9O_2N$ requires C, 66·3; H, 5·55; N, 8·6%). Reaction of methyl 2-hydroxy-6-methylbenzoate with ethanolamine gave *2-hydroxy*-N-*2′-hydroxyethyl-6-methylbenzamide*, m. p. 139·5—140° (from acetone) (Found: C, 61·5; H, 6·3; N, 7·0. $C_{10}H_{13}O_3N$ requires C, 61·5; H, 6·7; N, 7·15%). This material (1·3 g.) was converted into the oxazoline, giving first the hydrochloride (0·80 g.), m. p. 121·5—122°, from which the base was prepared and purified by sublimation under reduced pressure (yield 0·55 g.). Crystallisation from light petroleum gave 2-(*2-hydroxy-6-methylphenyl*)*oxazoline*, m. p. 102—102·5° (Found: C, 67·8; H, 6·8; N, 7·8. $C_{10}H_{11}O_2N$ requires C, 67·8; H, 6·3; N, 7·9%). The spectra of the oxazolines are given in Fig. 3.

Synthetic Hydroxamic Acids.—Stearoyl chloride (10 g.) in benzene (10 c.c.) was shaken for 1½ hr. with *N*-ethylhydroxylamine oxalate (9·5 g.) dissolved in N-sodium hydroxide (88 c.c.). The mixture was acidified and filtered. The solvent layer was separated from the filtrate and evaporated, and the residue combined with the solid which had been filtered off. The combined product crystallised from ethanol (55 c.c.) gave N-*ethylstearohydroxamic acid* as square plates, m. p. 73·5—74° (Found: C, 73·3; H, 12·4; N, 4·1. $C_{20}H_{41}O_2N$ requires C, 73·3; H, 12·6; N, 4·3%). A similar preparation gave N-*ethyloctadec-2-enohydroxamic acid*, m. p. 67·5° (from acetone) (Found: C, 73·6; H, 11·9; N, 4·3. $C_{20}H_{39}O_2N$ requires C, 73·8; H, 12·1; N, 4·3%).

Aluminium Mycobactin.—This compound was made by the following improved method. Mycobactin (0·50 g.) was dissolved in warm butanol (15 c.c.), and a solution of hydrated aluminium chloride (0·5 g.) in ethanol (3 c.c.) was added. The mixed solution was washed with water until free from acid and evaporated to dryness under reduced pressure. The residue (0·50 g.) gave an absorption spectrum in methanol which indicated a quantitative conversion into the aluminium complex. The product was dissolved in ethanol and allowed to crystallise, yielding 0·31 g. of aluminium mycobactin, m. p. 211—213°, which could be recrystallised as before. Iron-free reagents were necessary to avoid colour.

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