Biochem. J. (1970) **118**, 747–753
Printed in Great Britain

Early Steps in the Biosynthesis of Mycobactins P and S

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(*Received* 27 *April* 1970)

1. Lysine is readily incorporated into mycobactins P and S. Incorporation is into the hydroxamic acid moieties only and is equal in the mycobactic acid and cobactin portions of the molecule. 2. 2-Amino-6-hydroxyaminohexanoic acid is not taken up by cells of *Mycobacterium phlei* and is not detectable in extracts of cells actively synthesizing mycobactin. 3. The most abundant material derived from lysine that can be detected in such cell extracts is an N^6-acyl-lysine. Cells grown in the presence of iron contain markedly less of this material than do those grown under conditions of iron deficiency. 4. When added to growing cultures of *M. phlei* the N^6-acyl-lysine is readily incorporated into mycobactin. 5. The hydroxy acid of cobactin P is derivable from propionate.

The mycobactins are a family of compounds produced by mycobacteria, grown under conditions of iron deficiency, that act as growth promoters for *Mycobacterium johnei*, the only mycobacterial species so far tested that does not produce a mycobactin. The mycobactins extracted from different species vary in detail, but the basic structure is very similar in all cases and is unique to the mycobacteria (Snow, 1965*a*,*b*; White & Snow, 1969). All mycobactin molecules possess two hydroxamic acid groups, which are involved in the characteristic chelation with ferric iron. The hydroxamic acid group has been found in materials from microbial and plant sources and most frequently in compounds showing iron-binding properties similar to those of mycobactin (Neilands, 1967). The structures of mycobactins P and S are shown in Scheme 1, with a list of the products of their hydrolysis by acid. The biosynthesis of mycobactin probably involves the derivation of these units by separate biochemical pathways and their subsequent assembly in a sequence that is not yet known. The object of this work was to examine the possible derivation of the hydroxamic acid groups from lysine.

MATERIALS AND METHODS

Chemicals. A.R.-grade chemicals were used whenever possible; other chemicals were of the best available purity or as stated in the text. L-Lysine monohydrochloride was biochemical grade from British Drug Houses Ltd., Poole, Dorset, U.K. 2-Methylpropan-2-ol, reagent grade from British Drug Houses Ltd., was redistilled before use, material of b.p. 81–83°C being used. Butyl-PBD [5-(4-biphenylyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole] scintillator was obtained from Ciba (A.R.L.) Ltd., Duxford, Cambridge, U.K. Naphthalene was purchased from Thorn Electronics (Scintillator Division), Tolworth, Surrey, U.K. N^6-Hydroxylysine was prepared by refluxing cobactin P with 5M-HCl for 6h and extracting the solution repeatedly with an equal volume of ether. The aqueous solution was evaporated to dryness under reduced pressure, redissolved in 0.01M-HCl and stored at −20°C. N^6-Hydroxy[U-^{14}C]lysine was prepared by similar treatment of cobactin prepared from cells grown in the presence of L-[U-^{14}C]lysine. N^2N^6-Diacetyl[U-^{14}C]lysine was prepared by adding an excess of acetic anhydride dropwise over 30min to an ice-cold solution of L-[U-^{14}C]-lysine monohydrochloride.

Radiochemicals. Radiochemicals were from The Radiochemical Centre, Amersham, Bucks., U.K. The L-[U-^{14}C]-lysine monohydrochloride as supplied contained an impurity; to remove this approx. 2mg was applied to a 12cm × 1cm column of Amberlite CG-50 resin equilibrated with 50mM-KH_2PO_4 adjusted to pH7.6 with 2M-NaOH, the column was washed with 25ml of this buffer and the lysine was eluted with M-NH_3. The purified lysine was evaporated to dryness, redissolved in deionized water and stored at −20°C. A Packard Tri-Carb liquid-scintillation spectrometer was employed for radioactivity assays; the phosphor system was: dioxan, 1000ml; naphthalene, 100g; butyl-PBD, 8.0g. Counting efficiencies, determined by use of an internal standard of 10000 d.p.m. of [^{14}C]-hexadecane, were 70–80%.

Organisms. The organisms used were *M. phlei* (N.C.I.B. 8573), *M. smegmatis* (N.C.I.B. 8548) and *M. johnei* (A.T.C.C. 9808). Cultures of *M. phlei* and *M. smegmatis* were maintained on slopes of Lowenstein–Jensen egg medium, subcultured at 3-monthly intervals and stored at 4°C. Cells were grown in stationary culture at 37°C in 100ml volumes of medium adjusted to pH6.8 and containing (per litre): KH_2PO_4, 1g; Na_2HPO_4, 2g;

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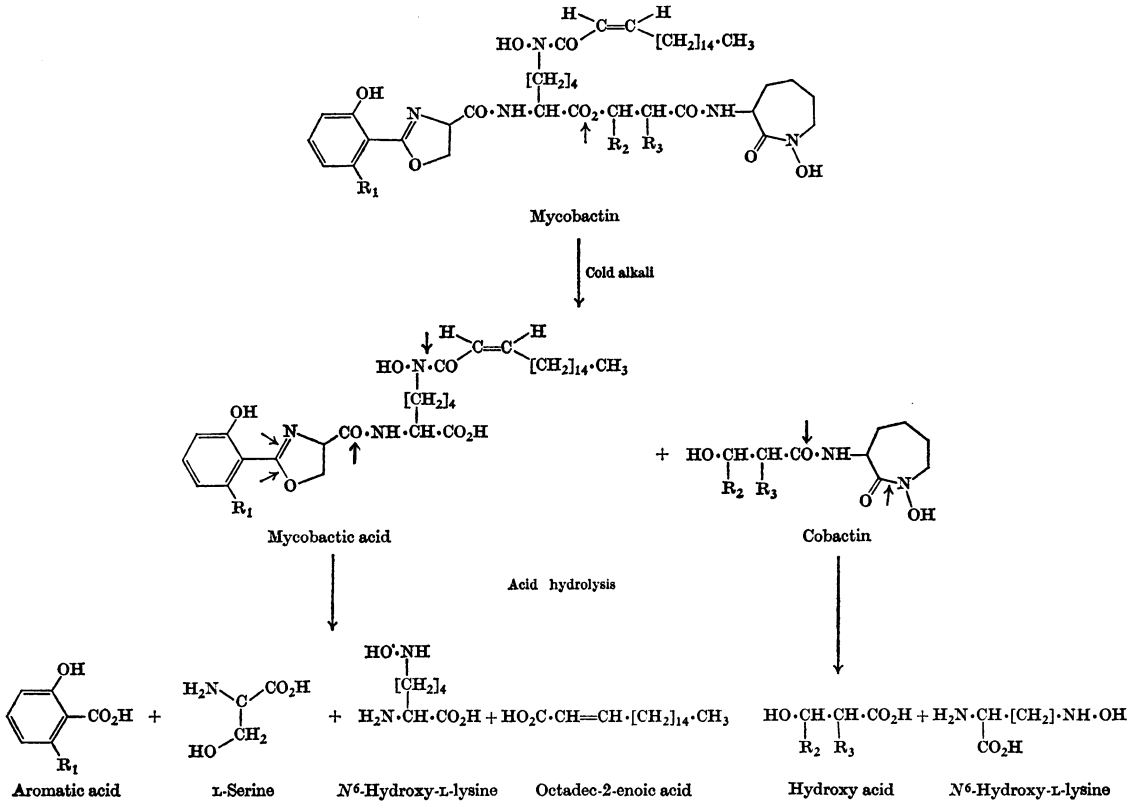

Scheme 1. Structures of mycobactins P and S and their degradation products. The points of scission are indicated by arrows. The side chains for mycobactin P are: R_1, CH_3; R_2, C_2H_5; R_3, CH_3; and for mycobactin S: R_1, H; R_2, CH_3; R_3, H. For both mycobactins the component containing the C_{18} side chain is illustrated.

$MgSO_4,7H_2O$, 0.2g; L-asparagine, 5g; glucose, 50g; glycerol, 10ml (White & Snow, 1968). When required, iron was added to the medium as a sterile solution to give a final concentration of 5mg of $FeCl_3$/l. *M. johnei* was maintained as described by Wheater & Snow (1966) and grown in Hart's (1958) medium supplemented with 10μg of mycobactin P/ml. Cells were harvested by filtration.

Extraction of cells. The extraction of crude mycobactins and their purification by column chromatography have been described by White & Snow (1969). The 'water-soluble extract' was prepared from cells harvested after 7 days of growth, at which time the mycobactin content of the cells (mg of mycobactin/g dry wt. of cells) was 30–50% that of a 14-day culture. Such cells are thus still synthesizing mycobactin. Cells were soaked overnight in ethanol (5ml/g wet wt.). Water (10 vol.) was then added and the mixture stirred for 24h. The cell residue was removed by filtration, a slight excess of ethanolic $FeCl_3$ was added and the solution was repeatedly extracted with chloroform to remove mycobactin. When necessary, unchanged lysine was removed from the water-soluble extract by passage through an Amberlite CG-50 column as described under 'Radiochemicals' above. Iron could be removed from this solution by adjusting the pH to about 9 and extracting repeatedly with a chloroform solution of 8-hydroxyquinoline. The aqueous layer was washed several times with chloroform and the excess of chloroform was removed by bubbling nitrogen through the warmed solution.

Paper chromatography and electrophoresis. Samples of the water-soluble hydrolysis products and of water-soluble cell extracts were submitted to paper chromatography with 2-methylpropan-2-ol–formic acid–water (14:3:3, by vol.) and to paper electrophoresis at pH2 (1.0M-acetic acid and 0.75M-formic acid; 70V/cm for 45min) and at pH9 (50mM-sodium borate; 80V/cm for 60min). The separated components were detected by ninhydrin or by the triphenyltetrazolium and alkali reagent of Snow (1954).

Alkaline hydrolysis of mycobactin. Mycobactin was dissolved in the minimum volume of warm methanol and 1M-NaOH was added. After 1h at room temperature a slight excess of HCl was added and the creamy precipitate extracted with ether (3×1 vol.). The ether extracts were combined and washed with M-HCl (2×$\frac{1}{4}$ vol.), the washings being added to the aqueous solution of the original hydrolysis. The ether solution of mycobactic acid was dried over Na_2SO_4 and the aqueous solution of cobactin

freed of ether by gently bubbling N_2 through it. For addition to bacterial cultures the cobactin was evaporated to dryness under reduced pressure; the residue was dissolved in water and the pH was adjusted to 7.0. The mycobactic acid solution was shaken with an equal volume of 0.1 M-Na_2HPO_4 and the ether was removed by gently bubbling N_2 through the warmed solution. Solutions were sterilized by filtration through Millipore membrane-filter discs (0.22 μm pore size).

Acid hydrolysis of mycobactic acid and cobactin. Material was refluxed for 6h with 5M-HCl and after cooling the solution was extracted with ether as described above.

Incorporation of lysine. Cultures of *M. phlei* and *M. smegmatis* were grown on low-iron medium containing L-[U-^{14}C]lysine monohydrochloride (40mg, 10μCi/l). After 3 weeks' growth the mycobactin was extracted, purified and assayed for ^{14}C radioactivity; it was subjected to alkaline and acid hydrolysis and the fragments were also assayed for ^{14}C. The results are given in Table 1. The water-soluble fragments of acid hydrolysis were subjected to electrophoresis at pH2 in the presence of lysine, serine and N^6-hydroxylysine, and the ninhydrin-positive spots were cut out, eluted in water (2ml) in a scintillation vial and assayed for ^{14}C. The results are recorded in Table 2.

Uptake and incorporation of N^6*-hydroxylysine.* Cells of a 7-day culture of *M. phlei* (3.6g wet wt.) were resuspended in 100ml of fresh medium. The suspension was divided into two 50ml volumes, to one of which was added 2.32mg of N^6-hydroxy[U-^{14}C]lysine and to the other 2.60mg of L-[U-^{14}C]lysine monohydrochloride of similar specific radioactivity. The cultures were incubated at 37°C for 5h with occasional shaking. The cells were harvested by centrifugation and washed twice with 50ml of medium, all supernatants being retained for ^{14}C assay. Water-soluble extracts were prepared as described above and samples of mycobactin, water-soluble extract and cell residue were assayed for ^{14}C. The results are given in Table 3.

Decarboxylation of amino acids. The ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) was used. ^{14}C-labelled substrates of known radioactivity were treated with 50mg of ninhydrin in 2ml of citrate buffer (0.5M-citric acid adjusted to pH3.0 with 5M-NaOH). A slow stream of N_2 was bubbled through the reaction mixture into a CO_2 trap [5ml of 10% (v/v) ethanolamine in methanol]. After 10min the contents of the trap were added to phosphor solution for ^{14}C assay. Samples of [U-^{14}C]lysine and N^2N^6-diacetyl[U-^{14}C]lysine were used as controls. The results are recorded in Table 6.

RESULTS AND DISCUSSION

Incorporation of lysine. Lysine is an obvious possible source of the hydroxamic acid groups of the mycobactins and work by Allen, Birch & Jones (1970) has given evidence that lysine is incorporated into mycobactin P. The results of the present work are given in Table 1 and show lysine to be very readily incorporated into mycobactins P and S, the incorporation being approximately equal in the mycobactic acid and cobactin portions of the molecules. Table 2 shows incorporation to be confined almost entirely to the N^6-hydroxylysine moieties. The identity of the radioactive material visible on the chromatogram as a faint yellow spot is unknown, but it may be an oxidation product of N^6-hydroxylysine. Comparison of the specific radioactivities of the mycobactins and the lysine supplement shows that 53% of the N^6-hydroxylysine of mycobactin P was derived from the exogenous lysine and that 36% was so derived in mycobactin S. It should be noted that there are two N^6-hydroxylysine moieties per molecule of mycobactin.

Uptake and incorporation of N^6*-hydroxylysine.* The generation of the hydroxamic acid group from lysine can be considered as involving two stages, hydroxylation and acylation. Mycobactin is somewhat unusual among the sideramines in that its two hydroxamic acid functions carry different acyl groups, and it is tempting to predict that in the interests of metabolic economy hydroxylation, which is common to both hydroxamates, will precede the acylations, which give rise to very different

Table 1. *Incorporation of* L-[U-^{14}C]*lysine into mycobactins P and S*

Mycobactins P and S respectively were extracted from cells of *M. phlei* and *M. smegmatis* grown in medium containing L-[U-^{14}C]lysine. Radioactive measurements were made on the mycobactins and on the products of mild alkaline hydrolysis and subsequent acid hydrolysis. Results for lysine and mycobactins show d.p.m./nmol, and for the fragments d.p.m./nmol of mycobactin from which they were derived.

Material	Organism	*M. phlei*	*M. smegmatis*
L-[U-^{14}C]Lysine		89.3	89.3
Mycobactin		95.0	63.5
Cobactin		43.8	30.6
Hydrolysis fragments	Ether-soluble	0.95	0.62
	Water-soluble	42.3	28.9
Mycobactic acid		49.8	32.1
Hydrolysis fragments	Ether-soluble	0.50	0.56
	Water-soluble	47.8	30.8

Table 2. *Radioactivity of water-soluble products of acid hydrolysis of* [^{14}C]*cobactin and* [^{14}C]*mycobactic acid*

Samples of the products of acid hydrolysis of the cobactins and mycobactic acids derived from [^{14}C]-mycobactins P and S were submitted to paper electrophoresis at pH 2 (1.0 M-acetic acid and 0.75 M-formic acid) at 70 V/cm for 45 min. Ninhydrin-positive areas were cut out, eluted directly in a scintillation vial and assayed for radioactivity. Serine was not present in the products of cobactin hydrolysis. Abbreviation: NHL, N^6-hydroxylysine.

Source of hydrolysis products	Radioactivity applied (d.p.m.)	Radioactivity recovered (d.p.m.)			
		Lysine	Serine	NHL	'Yellow spot'
Cobactin P	2500	90	—	2110	80
Mycobactic acid P	2750	105	<10	2170	110
Cobactin S	2040	80	—	1880	75
Mycobactic acid S	2170	100	<10	1910	105
Distance of migration of components (cm)		21.7	12.6	19.7	10.7

Table 3. *Uptake and incorporation of* [^{14}C]*lysine and* N^6*-hydroxy*[^{14}C]*lysine by* M. phlei

The radioactive supplements indicated were added to concentrated 7-day cultures of *M. phlei* (1.8 g wet wt. of cells/50 ml of medium). After 5 h incubation at 37°C the cells were harvested, washed and extracted overnight with ethanol–water–2 M-HCl (7:2:1, by vol.). Samples of the fractions were assayed for ^{14}C.

	[^{14}C]Lysine		N^6-Hydroxy[^{14}C]lysine	
	d.p.m.	% incorporation	d.p.m.	% incorporation
Radioactivity added	178000		185000	
Recovered in cells				
As mycobactin	6570	3.7	0	
As water-soluble	10650	6.0	340	0.18
In cell residue	4760	2.7	220	0.12
Total	21980	12.4	560	0.3

products. Work on the biosynthesis of ferrichromes (Emery, 1966) and of hadacidin (Stevens & Emery, 1966) suggests that in these cases hydroxylation is the first step on the route of formation of the hydroxamic acid. An attempt was therefore made to demonstrate the incorporation of N^6-hydroxylysine into mycobactin. Table 3 records the result of this experiment. Whereas lysine is readily taken up by *M. phlei* and incorporated into mycobactin and other cell products, there is almost no uptake of N^6-hydroxylysine. This contrasts with the findings of Emery (1966) on the uptake of N^5-hydroxyornithine and its ready incorporation into ferrichrome by *Ustilago sphaerogena*. The very low radioactivity of the water-soluble extract of N^6-hydroxylysine-supplemented cells suggests that exogenous N^6-hydroxylysine does not enter the cell and its failure to be incorporated into mycobactin does not exclude the possibility that endogenous N^6-hydroxylysine may be on the pathway of mycobactin biosynthesis. The detection of N^6-hydroxylysine in cell extracts would support this possibility.

Water-soluble extracts. Cultures of *M. phlei* and *M. smegmatis* were grown in the presence of L-[U-^{14}C]lysine and water-soluble extracts were prepared as described above. Samples of these extracts were examined by paper chromatography. Ninhydrin-positive areas were cut out, eluted in a scintillation vial and assayed for ^{14}C radioactivity. The blank areas between such spots were cut into 1 cm strips and similarly assayed for radioactivity. Two distinct areas of activity were apparent; the larger one corresponded to lysine, whereas the other, much faster-running, material (approx. R_F 0.46) did not coincide with a ninhydrin-positive spot, but occurred between two unlabelled spots. This material was designated compound A. The fast-running materials isolated from *M. phlei* and *M. smegmatis* behaved identically on paper chromatography. The area corresponding to N^6-hydroxylysine was unlabelled. Extracts of cells grown with

Table 4. *Chromatography of water-soluble extracts of cells grown in the presence of* [^{14}C]*lysine with and without added iron*

Paper chromatography was with 2-methylpropan-2-ol–formic acid–water (14:3:3, by vol.). The position of lysine was revealed by ninhydrin treatment; compound A was not itself visible, but was located between the two fastest-running ninhydrin-positive areas. The areas occupied by lysine and compound A were cut out and eluted in a scintillation vial for radioactivity assay. Column chromatography was on Amberlite CG-50 equilibrated with 50mM-potassium phosphate buffer, pH7.6. The first 30ml of eluate contained compound A. Lysine was eluted with M-NH_3.

Separation method	Growth conditions	Radioactivity applied (d.p.m.)	Radioactivity recovered (d.p.m.)	
			Lysine	Compound A
Paper chromatography	Iron-deficient	3620	2590	910
	Iron-supplemented	2840	2670	205
Column chromatography	Iron-deficient	151000	112000	33400
	Iron-supplemented	122000	110000	8200

Table 5. *Incorporation into mycobactin P of compound A in the presence and absence of lysine*

^{14}C-labelled compound A from *M. phlei* (39000 d.p.m.) was added to 50ml portions of medium inoculated with *M. phlei*. After 14 days' growth the cells were harvested and the mycobactin P was extracted and assayed for radioactivity; it was then hydrolysed and the products were assayed for radioactivity.

	Lysine (4mg) added	No lysine added
Mycobactin P produced (mg)	1.19	0.97
Mycobactin radioactivity (d.p.m./mg)	19800	18400
Radioactivity of the products of mild alkaline hydrolysis (% of that present in mycobactin)		
Cobactin	42	73
Mycobactic acid	58	27

a plentiful supply of iron showed markedly less radioactivity corresponding to compound A (see Table 4). Larger samples of lysine and compound A could be separated by chromatography on an Amberlite CG-50 column. The repression of production of compound A by iron suggested it might be involved in mycobactin production.

Support for this came from incorporation studies in which ^{14}C-labelled compound A was readily incorporated by *M. phlei* into mycobactin (see Table 5). The failure of unlabelled lysine to dilute this incorporation suggests that the effect was not the result of degradation yielding [^{14}C]lysine. The unequal distribution of radioactivity between the cobactin and mycobactic acid of the mycobactin extracted from cells grown in the absence of lysine deserves comment. It is possible that compound A is a precursor to cobactin only, the incorporation of label into mycobactic acid being the result of degradation of compound A to yield lysine. In this case, however, one would expect a supply of unlabelled lysine to dilute incorporation into mycobactic acid, whereas the cells grown in the presence of lysine show higher incorporation in this portion of the molecule. These observations, if verified, could provide a starting point for further investigation of the pathway and possibly some aspects of its control.

Some properties of compound A. Vigorous acid hydrolysis of radioactive material liberated [^{14}C]-lysine without detectable trace of N^6-hydroxy[^{14}C]-lysine. This would suggest that lysine undergoes substitution before hydroxylation. Paper electrophoresis showed the material to behave as a cation at pH2 and as a weak anion at pH9, indicating a mono-*N*-substituted lysine. The position of substitution was investigated by treating the material with ninhydrin. Amino acids with a free carboxyl group and an unsubstituted α-amino group are decarboxylated under these conditions, whereas substitution at the α-nitrogen renders the carboxyl group resistant to removal (Van Slyke *et al.* 1941). As compound A was derived from [U-^{14}C]lysine the carboxyl group represents 16.7% of the total radioactivity. Table 6 shows that, although this value was not attained, considerable amounts of radioactivity were released from the material as $^{14}CO_2$ under these conditions. This indicates the presence of a free α-amino group and thus substitution must be at the 6-amino group. Samples of

Table 6. *Release of $^{14}CO_2$ by ninhydrin treatment of amino acids*

Samples of ^{14}C-labelled substrates of known radioactivity were heated with an excess of ninhydrin in 2ml of citrate buffer, pH3. N_2 was bubbled through the reaction mixture and into a CO_2 trap [5ml of 10% (v/v) ethanolamine in methanol]. After reaction for 10min the contents of the trap were assayed for radioactivity.

Substrate	Total radioactivity (d.p.m.)	Radioactivity released (d.p.m.)	Radioactivity released (%)
^{14}C-labelled compound A from *M. phlei*	6240	720	11.6
	26300	3530	13.4
^{14}C-labelled compound A from *M. smegmatis*	15760	2030	12.9
[U-^{14}C]Lysine	27900	4480	16.1
N^2N^6-Diacetyl[U-^{14}C]lysine	24600	180	<1

Table 7. *Incorporation of [^{14}C]propionate and [^{14}C]acetate into mycobactins P and S*

Mycobactins P and S respectively were extracted from cells of *M. phlei* grown in the presence of [2-^{14}C]-propionate and *M. smegmatis* grown in the presence of [1-^{14}C]acetate. Radioactivity measurements were made on the mycobactins and on the products of mild alkaline hydrolysis and subsequent acid hydrolysis. Results for mycobactins show d.p.m./μg of mycobactin and for the fragments d.p.m./μg of mycobactin from which they were derived.

Organism		*M. phlei*	*M. smegmatis*
Additions per litre of medium		[2-^{14}C]Propionate (1mmol, 20μCi)	[1-^{14}C]Acetate (1mmol, 20μCi)
Material			
Mycobactin		642	49.0
Cobactin		555	22.3
Hydrolysis fragments	Ether-soluble	528	21.6
	Water-soluble	<2	<1
Mycobactic acid			
Hydrolysis fragments	Ether-soluble	88.1	25.9
	Water-soluble	<2	<1

[U-^{14}C]lysine and N^2N^6-diacetyl[U-^{14}C]lysine were also treated to test the efficacy of the method.

Incorporation of propionate and acetate into mycobactins P and S. The mycobactins extracted from *M. phlei* and *M. smegmatis* grown in media containing [2-^{14}C]propionate and [1-^{14}C]acetate respectively were heavily labelled. The distribution of activity is shown in Table 7. The very heavy labelling of the hydroxy acid portion of cobactin P provides experimental support for the suggestion that 3-hydroxy-2-methylpentanoic acid could be derived by the condensation of two propionic acid molecules (Asselineau, 1962). The incorporation of acetate into mycobactin S was more general, but the relatively high proportion of radioactivity in cobactin S suggests the derivation of the 3-hydroxybutyrate moiety from the condensation of two acetate units. Water-soluble extracts of these cells showed radioactivity in the fast-running material, the radioactivity being released as an acidic compound on acid hydrolysis. Attempts to identify this compound have been unsuccessful.

Water-soluble extracts of M. johnei. A fast-running radioactive compound has been detected in water-soluble extracts of *M. johnei* grown in the presence of L-[U-^{14}C]lysine. On paper chromatography this material (approx. R_F 0.59) differed from those extracted from *M. phlei* and *M. smegmatis*, but like those compounds it yielded [^{14}C]-lysine on acid hydrolysis. The presence of this material in *M. johnei* is of particular interest as this organism, when grown on defined media, is unable to synthesize mycobactin. The site of the metabolic block is not known, but it may be that *M. johnei* can perform one or more of the early steps and could thus be a useful organism for the further study of this pathway.

Concluding remarks. The possibility that N^6-hydroxylysine is on the pathway of mycobactin biosynthesis has not been excluded. The failure to detect N^6-hydroxylysine in cell extracts could be due to its very low concentration in such cells. Exogenous N^6-hydroxylysine is not taken up by the cell and is thus not available for incorporation into mycobactin. The results showing the existence and properties of the N^6-acyl-lysine do, however, provide evidence that acylation may be the first step in the incorporation of lysine into mycobactin,

hydroxylation occurring later. This is in contrast with results obtained on the biosynthesis of ferrichrome (Emery, 1966) and hadacidin (Stevens & Emery, 1966), in which the hydroxyamino acids were readily incorporated, indicating hydroxylation as the first step. It thus seems possible that more than one pathway exists for the biogenesis of the hydroxamic acid group.

I thank Dr G. A. Snow for his interest and encouragement throughout this work and Mr P. J. Bailey for valuable technical assistance.

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