Mycobactin, a Growth Factor for *Mycobacterium johnei*

1. ISOLATION FROM *MYCOBACTERIUM PHLEI*

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Mycobactin is the name suggested for a growth factor, present in acid-fast bacteria, which is essential for the growth of *Mycobacterium johnei*. A preliminary account of the work reported here has already been published (Francis, Madinaveitia, Macturk & Snow, 1949). *Myco. johnei* is an organism first observed by John & Frothingham (1895) in cases of chronic enteritis of cattle. It could not be cultivated on ordinary laboratory media. Twort & Ingram (1913) supplemented such media with various extracts of normal cattle tissues, but none produced growth. They postulated that failure to cultivate the bacillus 'must be due to the absence of some necessary foodstuff', and that the missing factor would most likely be found in tubercle bacilli. They showed that when dried, killed, human tubercle bacilli were added to egg medium, a good growth of *Myco. johnei* developed. They were also able to obtain growth by addition of killed cells of other mycobacteria which had been grown on suitable media, and even by addition of cells of *Myco. johnei* which had been acclimatized to grow on beef broth without added growth factor. They made the original suggestion that the specific growth substance was essential to the metabolism of the whole group of acid-fast organisms, *Myco. johnei* differing from the other mycobacteria in lacking the power to initiate synthesis of this factor on an egg medium. This hypothesis has been reinforced by the subsequent discovery of many parallel cases in other families of organisms where some members can synthesize a particular factor while others require the same factor to be supplied from an external source. For example, among the Lactobacilli *Lb. casei*, *Lb. delbrückii* and *Lb. lactis* have been shown to require pyridoxine for growth, whilst *Lb. arabinosus* and *Lb. pentosus* synthesize pyridoxine (Bohonos, Hutchings & Peterson, 1942).

Twort & Ingram prepared extracts of mycobacteria with organic solvents and found that they promoted the growth of *Myco. johnei*. This observation was one of the earliest indications of the existence of a specific organic growth factor for a micro-organism, and was made at a time when even

the concept of vitamins was novel. Although simple extracts of *Myco. phlei* have been used regularly for the purpose of culturing *Myco. johnei*, the literature contains no record of any further attempt to concentrate and isolate the active substance. Woolley & McCarter (1940) claimed that growth of *Myco. johnei* on liquid medium could be promoted by phthiocol or 2-methylnaphthaquinone, but the amount of growth observed was less than that given by addition of extracts of *Myco. phlei*. They gave no evidence of the occurrence of quinones in *Myco. phlei*. Francis *et al.* (1949) found no phthiocol in *Myco. tuberculosis*, though a substance resembling vitamin K₂ was present in considerable amounts. Considering that the growth factor in *Myco. phlei* may well represent an important and specific component in the metabolism of mycobacteria, it is surprising that no further attempt has been made to investigate its constitution. The preparation of chemotherapeutic agents against this group of organisms might well be helped by a knowledge of the chemical structure of the growth factor. The extraction and purification of the factor was therefore undertaken, and the results are presented in this paper. The experiments have led to the isolation of the hitherto undescribed compound, mycobactin, which has the growth-promoting action of the original extracts; its main properties are outlined here.

RESULTS

Extraction and purification of mycobactin

Growth of Myco. phlei for mycobactin extraction. Twort & Ingram (1913) showed that the growth factor was produced by a wide range of mycobacteria grown on glycerol beef broth. The organisms tested included *Myco. phlei*, *Myco. tuberculosis*, *Myco. smegmatis* and *Myco. butyricum*, as well as certain other less well-defined acid-fast bacteria. Of these the first two were regarded as the best sources, and bovine and avian strains of *Myco. tuberculosis* were said to be much inferior to human strains; this distinction was not confirmed by McFadyean, Sheather & Edwards (1912). None of the organisms, however, produced detectable amounts of the growth factor when grown on glycerol-liver broth,

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and, except with human strains, production of growth factor was low or absent on egg medium. Twort & Ingram were therefore fortunate in having chosen cultures of human strains for their original experiments.

In the present investigation the non-pathogenic, readily cultivated *Myco. phlei* was chosen as a source of the growth factor. Large quantities of the bacterial cells were required, and it would have been convenient if these could have been grown on a synthetic medium. Although good growth of *Myco. phlei* was obtained with glycerol-containing media having as a source of nitrogen asparagine (Green, 1946), ammonium malate or peptone, the content of mycobactin in the cells was negligible; addition of yeast extract ('Marmite') did not affect the result. It was therefore necessary to turn to the less convenient beef infusion medium. An infusion containing 4% peptone and 10% glycerol (twice the normal concentrations) gave the best results in bacterial growth and yield of mycobactin, and was used for all the large-scale cultivation of *Myco. phlei*. It may be noted that this more concentrated medium is unsuitable for the growth of tubercle bacilli.

Establishment of an assay method. The assay of the growth factor required a method of cultivating and assessing the amount of growth of *Myco. johnei*. Solid egg medium was chosen because irregular results were obtained when attempts were made to obtain growth of pellicle on the surface of fluid media. Egg medium was a more suitable material than liquid medium in which to emulsify the original crude extracts; moreover, many of these extracts would have had a surface-active effect and might have caused the pellicle to sink. To determine the best conditions for the assay of mycobactin, *Myco. johnei* was grown on solid egg medium containing varying amounts of the factor. Fig. 1 shows the average of a large number of such experiments, in which the concentration of mycobactin has been correlated with the mean growth score after various time intervals. This diagram shows the main features of the growth of *Myco. johnei* under the chosen conditions. In the presence of high concentrations of mycobactin there is a 'rapid' growth period during the second to eighth week of incubation, after which the growth rate becomes much less. When a smaller amount of the factor is present the lag phase is slightly longer and subsequent growth occurs more slowly, though it continues for a long period. Experiments were also carried out in which growth was measured up to 36 weeks. These showed that the differential growth response for varying concentrations of mycobactin became less as the time increased, because growth at high concentrations had almost ceased, whilst that at lower concentrations was still continuing. It was thus

evident that the best time to assess the amount of growth was after 4–6 weeks' incubation. Shorter periods did not allow sufficient growth for assessment and longer periods gave no improvement in the ratio of growth response to mycobactin concentration, or even, in very prolonged experiments, a decrease in the ratio.

Fig. 1. Growth of *Myco. johnei* in tubes containing different concentrations of mycobactin. Figures against the curves show period of growth in weeks. Each point represents the average of 150 tubes.

Extraction methods. The work of Twort & Ingram (1913) indicated that the growth factor could be extracted from the bacterial bodies by a variety of organic solvents. Exploratory experiments confirmed these findings and acetone was chosen as the solvent for the early experiments. Acetone extracts were more active than the bacterial bodies from which they originated; this is presumably due to the non-availability of the growth factor present inside the bacterial cells. Presumably it also explains the finding that no great loss of activity resulted when the bacterial bodies were heated with sodium hydroxide solution, although extracts lost their activity when treated with alkali, even at 0°. Light petroleum extracted very little activity from the dried bodies, and addition of light petroleum to the concentrated acetone extracts produced a precipitate which contained the whole of the active materials. This proved to be a most important step because, although it was not realized at the time, the precipitate contained up to 50% of mycobactin. The light-petroleum precipitation was followed by an extraction with water which removed further inactive impurities. At this stage it was found that the growth factor was strongly adsorbed by alumina, from which it could be eluted by hot ethanol. Repeated extractions with hot ethanol removed the active materials. On concentration of these eluates mycobactin separated as a crystalline aluminium complex, the aluminium having been derived from the adsorbent. Table 1 shows the steps in such a

purification and the activities of the fractions obtained, expressed in arbitrary units. The figures show the increase of growth-promoting activity in the initial acetone extraction which has already been mentioned. In later steps the weight of material and growth-promoting activity agree within the limits of the experimental error of the assay. The figure given for the relative weight of the final product does not take into account material in the mother liquors which contained considerable activity.

Table 1. *Direct isolation of crude crystalline aluminium mycobactin from dried Myco. phlei*

Fraction	Relative wt. of fraction	Relative activity by assay	Mycobactin (% by assay)
Dried <i>Myco. phlei</i>	100	1*	0.6*
Acetone-soluble	20	14*	10
Petroleum-insoluble	4	80	50
Water- and petroleum-insoluble	2.3	80	50
Crystalline Al complex	0.4	160	(100)

* Assay figures for the mycobactin content of the dried bacteria are considerably lower than the actual content. The full growth-promoting activity is liberated during acetone extraction.

The crystalline product obtained by this simple method was always reddish brown. The amount of the coloured impurity was not great, but no means could be found of removing it. Methods were therefore devised for obtaining mycobactin in a colourless, metal-free form. One method is described in detail and is summarized in Table 2. Alternative methods are indicated briefly.

In the extraction described, water is removed from the cells by successive treatments with cold methanol. This procedure also removes much inactive material. Mycobactin is not removed because of its low solubility in 90% aqueous methanol. The active material is extracted with hot methanol. An essential step in mycobactin extractions is the formation of the copper complex which is characterized by insolubility in ethanol, solubility in chloroform and stability towards dilute acetic acid. The copper complex is purified by chromatography and precipitation with ether from chloroform, before reconversion into metal-free mycobactin. The mycobactin thus obtained is finally purified by precipitation with solvents.

Biological properties

Mycobactin content of organisms. From Table 2 it will be seen that the yield of mycobactin isolated was 0.86% of the dry weight of *Myco. phlei*. The actual content of the growth factor in the cells, therefore, probably exceeds 1%. A similar deduction can be made from the results given in

Table 1. By growth promotion assays, the crude acetone extract representing 20% of the weight of dried organisms was estimated to contain about 90 mg. mycobactin/g., indicating a content in the dried material of about 18 mg./g. The living bacteria may thus contain 3 mg. of mycobactin/g. wet weight when grown on a beef-infusion medium. Growth of *Myco. johnei* is optimum on medium containing 40–80 µg./ml. and 50% of the optimum on medium containing 3–6 µg./ml. It thus appears probable that the production of mycobactin by *Myco. phlei* grown on beef-infusion medium is greatly in excess of its metabolic requirements. When it is grown on synthetic medium, mycobactin may still be produced in amounts adequate for intermediary metabolism, but too small to be detected by the assay procedures used. A similar suggestion was made by Twort & Ingram (1913) in relation to growth in liver broth.

Search for growth-promoting activity in other materials. It is not known whether *Myco. johnei* can synthesize mycobactin when growing in the animal intestine or whether it uses material available in the host, but according to Twort & Ingram (1913) strains adapted to grow on synthetic medium do promote the growth of unadapted strains. They found that aqueous extracts of various organs of cattle did not support the growth of *Myco. johnei*. Twort & Ingram (1914) tested ethanolic extracts of about 130 seeds, fungi, plant extracts and seaweeds for ability to support the growth of *Myco. johnei*. Most of the extracts were inactive, but with figs, oats, linseed, the fungus *Cantharellus auranticus*, and especially the currant grape, good results were sometimes obtained. The amount of growth-promoting activity in these tissues was always low, varying from sample to sample, and being sometimes undetectable.

We have carried out growth-promotion tests on extracts of miscellaneous plant and vegetable products. In the first series of experiments the starting materials were dried and extracted with acetone; some of the acetone extracts were further extracted with light petroleum; the extracts were incorporated in Dunkin's medium. The maximum amount of material added was the quantity that would be obtained from a weight of starting material equal to that of the medium used. Further dilutions down to one-hundredth of this amount were employed. It will be seen from Table 3 that some substances did produce a slight stimulation of growth but it was only about twice that in the control tubes. In later experiments a similar series of starting materials were extracted with methanol without previous drying and fractionated into petroleum-soluble and insoluble portions. Extracts were added in amounts varying from 1000 to 1 mg./100 ml. of media. Again only slight and variable

Table 2. *Extraction of mycobactin*(Letters *a*, *b*, *C*, etc. indicate fractions.)

responses were obtained and concentration of any growth-promoting substances, that may have been present, was not achieved. Currants, which were included in this series because they had been found specially active by Twort & Ingram (1914), gave negative results. It cannot therefore be assumed that there is any wide distribution of mycobactin in natural products. Growth stimulation may possibly result from the presence of substances with a different constitution.

Following up the observations of Woolley & McCarter (1940) we have tested phthiocol, 2-methylnaphthaquinone, and a vitamin K extracted from *Myco tuberculosis* (Francis *et al.* 1949) for their

effect on *Myco. johnei* inoculated on egg medium. No growth stimulation was observed under these conditions.

Chemical properties of mycobactin

Criteria of purity. Considerable difficulty has been experienced in assessing the degree of purity of mycobactin and most of its derivatives owing to their failure to form visible crystals. Attempts at crystallization have invariably led to the separation of the products in gelatinous form. Fortunately, however, mycobactin forms a highly crystalline aluminium complex which may be recrystallized from different solvents without change of pro-

perties. Some confidence may be placed upon this derivative as showing mycobactin to be a definite chemical entity. The status of metal-free mycobactin rests most firmly on its conversion into the crystalline aluminium complex and recovery from it via the copper complex. In the different batches of

Table 3. Tests on growth promotion of *Mycobacterium johnsonii* by miscellaneous extracts

Acetone extracts showing slight activity	
Material	No. of active samples
Crushed oats*	1 of 3
Bovine intestine*	1 of 2
Bovine liver	1
Sheep liver	2 of 3
Sheep lung	2 of 3
Sheep intestine	2 of 3
Rat-leprosy tissue	2 of 3
Lungs of mice infected with <i>Mycobacterium muris</i>	1

* The fractions of these active extracts soluble in light petroleum were also active.

No fractions insoluble in light petroleum were active.

Acetone extracts showing no activity: sprouted oats, maize, lentils, sunflower seed, soya meal, linseed, grass meal, peanuts, carrots, egg yolk, *Penicillium mycelium*, bovine lung, bovine muscle, horse lung, horse liver, horse intestine, horse mesenteric gland.

the metal-free material that have been made, repeated purification has always led finally to products having the same melting point and ultraviolet-absorption spectrum, with analyses consistent with those found for the aluminium complex. Measurements of optical rotation, however, have been more variable, and there is a possibility that some strongly rotating impurity may be present in varying amounts in some of our preparations. The value given for the rotation in the experimental section is that found for the most rigorously purified preparations. The possibility of error in rotation measurements is evident when the very high positive rotation of the aluminium complex is considered. The presence of 0.3 % of this derivative would lower the specific rotation of the metal-free product by 1°. In view of the amorphous character of the material and the occasional discrepancy in optical rotation, some caution is necessary in assessing the purity of the preparations of metal-free mycobactin. Degradation studies, which will be described in later papers, favour the view that metal-free mycobactin is substantially a single substance, but do not exclude the possibility that some preparations might contain a few per cent of a closely related product. The diacetyl derivative and copper complex of mycobactin are directly derived from the metal-free product and resemble it in lacking visible crystalline structure; they therefore probably reflect any degree of inhomogeneity which may be present in the mycobactin from

which they are made. Their analyses are consonant with that of the parent compound, and the diacetyl derivative has a melting point reproducible from one preparation to another. Both compounds give reproducible ultraviolet spectra.

General properties. Although mycobactin has yielded no visible crystals, an X-ray powder photograph showed seven or eight rings, though with a general diffuseness. This evidence, together with the sharp melting point, suggests that mycobactin exists in a solid state as very small crystallites. The solid exhibits a pale-green fluorescence, and

Fig. 2. Absorption spectra of mycobactin and its derivatives. A, mycobactin (27 μM) in methanol; B, diacetyl-mycobactin (29 μM) in methanol; C, mycobactin hydrochloride (510 μM) in absolute CHCl_3 (measurement in 1 mm. cells); D, aluminium mycobactin (20 μM) in methanol; E, copper mycobactin (24 μM) in absolute CHCl_3 .

methanol solutions a blue fluorescence. The spectrum (see Fig. 2) has maxima at 250 and 310 $\text{m}\mu$, and in addition shows a characteristic shoulder at 258 $\text{m}\mu$ and a slight inflexion at 270 $\text{m}\mu$. Solubility characteristics have been discussed under the heading of extraction; in addition to the solvents mentioned there, mycobactin is soluble in pyridine, glacial acetic acid and in hot high-boiling solvents such as xylene. It is stable to heat up to 150° and is not oxidized in air. It dissolves in solutions of caustic alkalis but not in sodium bicarbonate solution. Alkaline solutions rapidly decompose, even at 0°, with loss of growth-promoting activity. Mycobactin does not dissolve in dilute mineral acids, but dissolves in concentrated sulphuric acid without immediate decomposition,

and has a slight solubility in concentrated hydrochloric acid. Warming with mineral acids causes inactivation. Elementary analysis of mycobactin gives values agreeing with the formula $C_{47}H_{75}O_{10}N_5$ (mol.wt. 870). Owing to the large molecular size it is not possible to deduce this formula from the analytical figures for mycobactin alone. The formula takes into account the analytical data for all the derivatives of mycobactin, molecular-weight and equivalent-weight determinations, and the degradation products which have been isolated; the latter will be described in a later paper.

This formula differs somewhat from that postulated in our preliminary note (Francis *et al.* 1949). The difference is mainly due to difficulties of nitrogen analysis, the early values being considerably too high. Molecular-weight determinations by the usual physical methods have proved unreliable, but X-ray measurements upon a single crystal of the aluminium complex have indicated a unit-cell weight of $1828 \pm 4\%$. From considerations of crystal structure the molecular weight was considered to be one-half or one-quarter of this figure; this corresponds to a molecular weight of 890 ± 36 or 445 ± 18 for metal-free mycobactin, assuming that the crystalline derivative has one atom of aluminium per molecule of mycobactin. The lower figure is, however, excluded by equivalent-weight determination (see below) and by the degradation products.

Acidic and basic properties. Mycobactin is a weakly acid substance. Electrometric titration in methanol gives the curve shown in Fig. 3; a curve for the titration of *p*-chlorophenol is given for comparison. The titration data show that mycobactin has one or possibly two acidic groups dissociated in methanol to an extent comparable with the phenols. No basic groups can be detected by titration with hydrochloric acid in methanol, but by use of perchloric acid in glacial acetic acid the presence of one weakly basic group can be demonstrated. The equivalent weight of mycobactin as a base can thus be determined; the value found, 870 ± 4 , also represents the molecular weight. The basic properties of mycobactin are further shown by the formation under anhydrous conditions of a hydrochloride and a picrate. The slightly impure hydrochloride has an absorption spectrum similar in shape to that of the parent compound, but with the peaks shifted approximately $20 m\mu$. towards the longer wavelength (see Fig. 2). This shift indicates that the basic group is probably in conjugation with the system responsible for the absorption.

Metal complexes. Mycobactin is characterized by a tendency to chelation with metals. Complexes have been isolated with aluminium and with copper. Both have one metal atom per molecule of mycobactin. The aluminium complex is of particular

importance as being the only derivative of undergraded mycobactin which has been obtained in a visibly crystalline form. It may be crystallized from the lower alcohols or acetone, giving hexagonal leaflets. Both solid and solutions exhibit an intense violet fluorescence in ultraviolet light and a slight fluorescence in visible light. The aluminium complex is considerably less polar than free mycobactin since it dissolves in solvents such as ether and benzene; it is, however, insoluble in light petroleum. It also differs from mycobactin in having a high, positive optical rotation. The aluminium atom is firmly held and cannot be displaced by extraction with acid or by passage through a base-exchange resin in the H^+ form. It can be exchanged for copper in ethanol solution probably owing to the insolubility of the copper complex.

Fig. 3. Electrometric titration of mycobactin and its diacetyl derivative in methanol containing 20% (v/v) $CHCl_3$. Electrodes; glass, Hg-HgCl-LiCl (methanol). Concentration of solutes: 30 mM. Titration solution, 0.25 N-KOH in methanol. Curve A, diacetylmycobactin; B, *p*-chlorophenol; C, mycobactin. Readings are on pH scale of instrument, but are arbitrary owing to the nature of the solvent.

Copper mycobactin is almost insoluble in cold ethanol; it is formed from mycobactin by precipitation with ethanolic cupric acetate. It has little solubility in most solvents at room temperature, except chloroform in which it dissolves to the extent of more than 5% giving a deep-green solution. Copper can be removed from the complex by treating the chloroform solution with hydrogen sulphide, by extracting it with N-hydrochloric acid, or by passage through cation exchange resin in the acid form.

Both copper and aluminium complexes have ultraviolet-absorption spectra resembling that of the parent compound except that the peaks are raised and displaced to longer wavelengths (see Fig. 2). Ferric salts react with mycobactin to give an intense reddish purple colour.

Hydroxyl groups. Two acetyl groups can be introduced into mycobactin under gentle conditions of acetylation. No degradation of the molecule appears to occur as a result of this treatment since the spectrum is not significantly changed (see Fig. 2), and the product still possesses growth-promoting properties. The diacetate is an amorphous powder resembling mycobactin in its fluorescence and other physical properties; it has a reproducible melting point. It differs from mycobactin in forming neither a crystalline aluminium complex nor an ethanol-insoluble copper complex. Ferric chloride in ethanol produces an intense violet colour, noticeably bluer than that given by mycobactin. It has weakly acidic properties shown by its solubility in sodium hydroxide solution, but the acidic group is too weak to be demonstrable by electrometric titration in methanol (see Fig. 3). The weak basic group found in mycobactin is also present in the diacetate. Since the titration of mycobactin has shown the absence of any basic groups strong enough to form a hydrochloride in polar solvents, it may be inferred that acetylation takes place in two hydroxyl groups. At least one of these groups is acidic and is involved in metal complex formation. In addition to these two hydroxyl groups there is probably a third having phenolic properties and not readily acetylated. This is suggested by the ferric chloride reaction and the weakly acidic properties of the diacetate. Further, both mycobactin and its diacetate couple in alkaline solution with diazonium salts to give yellow solutions.

Vigorous acetylation leads to the uptake of acetic anhydride corresponding to three acetyl groups per molecule, but the reaction brings about a great change of properties and probably involves degradation.

EXPERIMENTAL

Large-scale production of *Myco. phlei*. A beef-infusion broth was prepared containing 4% peptone and 10% glycerol. 600 ml. portions were transferred to a series of plugged Winchester bottles and sterilized. The bottles were stacked on their sides and pellicles from an established culture floated on to the surface with a wire loop, and then incubated for 2 weeks at 32–37°. The pellicles were harvested by filtration on a large stoneware filter, well washed with water and sucked as dry as possible. The yields obtained were usually just over 2 g. dried organisms/100 ml. of medium. In all, 50–60 kg. of dried bacterial bodies have been produced.

Mycobactin formation by *Myco. phlei* grown on various media. The media contained K_2HPO_4 1.8 g., $MgSO_4 \cdot 7H_2O$ 0.5 g., sodium citrate 0.9 g., $Fe_2(SO_4)_3 \cdot 9H_2O$ 0.2 g., glucose 10 g. and glycerol 100 g. To this 14 g. asparagine or 10 g. peptone, or 12.52 g. malic acid neutralized with NH_3 was added. The volume was made up to 1 l. in each case and the pH adjusted to 6.8. Inocula of *Myco. phlei* from cultures adapted to these media by five passages were floated in 10 ml. medium placed in wide tubes and incubated at 37°. The amount of

bacterial nitrogen was estimated every other day. After 12 days it had reached a maximum in all media. Bacteria from all these media failed to support the growth of *Myco. johnei*, whereas bacteria grown in a parallel experiment in meat-infusion broth with 5% glycerol were active in this respect. The amounts of 'bacterial nitrogen' produced (in mg./ml. medium) in the various media were: asparagine (1.68), ammonium malate (1.15), peptone (0.80) and glycerol broth (0.62).

Assay of growth-promoting activity. Egg-liver medium was prepared according to the directions of Dunkin (1928), the standard glycerol-water extract of *Myco. phlei* being replaced by the extract which was being tested. The active extracts were insoluble in water; they were dissolved in the least possible amount of acetone, methanol or $CHCl_3$ and an appropriate amount put in the first tube. Egg medium was pipetted vigorously on to the fraction and the mixture shaken. A satisfactory dispersion resulted and falling 2.5-fold dilutions were made; five tubes were prepared from each dilution. Other tubes contained known amounts of a standard concentrate, or, in later experiments, known amounts of mycobactin; tubes containing no growth factor were also included in every test. The medium was inspissated to give steep slopes, during which process any solvent used to dissolve the growth factor was driven off. The tubes were inoculated with a pinhead inoculum of strain 129, which had been supplied by Prof. R. E. Glover as a strain of *Myco. johnei* which could not easily be adapted to grow in the absence of growth factor. Cotton-wool plugs were dipped in wax, pushed into the tubes and covered with a layer of wax. This precaution was needed to prevent loss of moisture. The tubes were incubated at 37° and growth estimated visually after 4 and 6 weeks. An optimum quantity of growth factor (equivalent to 40–80 μ g. mycobactin/ml. of medium) produced a raised, irregular mass of bacteria roughly circular and up to 6 mm. in diameter. The minimum recognizable growth promotion was produced by a concentration of mycobactin of about 0.5 to 1 μ g./ml. of medium; 1000 μ g./ml. did not inhibit growth. A method of scoring was adopted which recognized six stages between absence of growth and optimum growth, and these were given values of 3, 2.5, 2.0, ..., 0. Some extracts of *Myco. phlei* produced inhibition of growth at concentrations above the optimum. From the scores at different dilutions an estimate of the mycobactin content of fractions could be made, and a statistical analysis by Dr O. L. Davies showed that twofold differences in growth-promoting activity could usually be detected.

Tests on naphthaquinone derivatives and other agents as possible growth-promoters for *Myco. johnei*. Phthiocol was tested at concentrations of 50 to 0.05 μ g./ml. and 2-methylnaphthaquinone at concentrations of 100 to 1 μ g./ml. but neither produced any growth, whereas full growth was produced by an acetone extract of *Myco. phlei* in the same tests. Various mixtures of riboflavin, pantothenic acid, biotin, nicotinamide, pyridoxine, inositol and thiamine also failed to support growth when added to Dunkin's medium.

Isolation of Mycobactin

The following method is probably the most satisfactory for general use, though various modifications have been used successfully. Throughout the description the various fractions are designated by letters; fractions carrying the main bulk of the active material are shown by capital

letters. Yields are recorded in Table 2. Unless otherwise specified, the CHCl_3 used was of B.P. grade containing about 1% of ethanol. The method involved a preliminary extraction of each batch of moist mycobacteria as it was grown. In Table 2 the total yields for about twenty such batches are recorded, but in describing the preliminary treatment below, yields are quoted to indicate the quantities to be expected in working up a single batch of moist organisms.

Preliminary treatment of micro-organisms. Myco. phlei, filtered from the medium and washed with water, was spun in a basket centrifuge to remove as much water as possible, yielding a crumbly product containing about 80% of water. This material (2 kg.) was stirred with methanol (2 l.) for 2-3 hr., allowed to stand, decanted and the residue again spun on the basket centrifuge. The extract was evaporated to dryness under reduced pressure, giving a dark sticky residue (a). The extracted bacterial cake (approx. 1.04 kg.) contained about 680 g. liquid (56% (v/v), aqueous methanol), i.e. 315 ml. water; it was stirred for 2-3 hr. with a further 3 l. methanol and filtered. This second extract was also evaporated under reduced pressure leaving a red-brown waxy residue (b). The twice-extracted bacterial cells were spread out in a thin layer on absorbent paper and allowed to dry in air at room temperature. A dry, light-brown material (c) resulted which was powdered in a mortar and stored until sufficient had been accumulated for the main extraction.

Extraction. Extraction of 7.2 kg. fraction C was carried out in 1.5 kg. batches, each batch being mechanically stirred and refluxed with methanol (6 l.) on a steam bath. After 30 min. the hot suspension was transferred to a 30 cm. stoneware filter and filtered through cloth with suction. The transfer was conveniently effected through a wide syphon tube by means of gentle air pressure. The extracted material was returned to the flask for re-extraction, three extractions in all being performed on each batch. White waxy material (d) deposited from the methanolic extracts on cooling was filtered off. The clear brown extracts from the various batches were pooled and evaporated, leaving a red-brown waxy residue (E). This residue was treated with warm ethanol (15 l.), containing glacial acetic acid (76 ml.), cooled, and filtered from undissolved waxy material (f). The filtrate was then treated with copper acetate (226 g., monohydrate) dissolved in ethanol (11.2 l.) and glacial acetic acid (56 ml.) and allowed to stand until the copper complex had coagulated and settled. The crude complex (g) was filtered off, washed with ether and dried. Cu was removed from the filtrate by treatment with H_2S . After filtration, solvent and acetic acid were removed by evaporation leaving a brown waxy residue (h). Fraction g was dissolved in warm CHCl_3 (6 l.) and the solution decanted from some insoluble green gum. (This gum after removal of copper with H_2S gave fraction j.) The CHCl_3 solution of the Cu complex was shaken three times with an equal volume of 2% (v/v) aqueous acetic acid and then twice with water. Some Cu passed into the aqueous layer. The washed CHCl_3 solution was evaporated to approx. 1.5 l. and precipitated by addition of ether (6 l.). The precipitate was filtered, washed with ether and dried (fraction K), and the filtrate was evaporated leaving a brownish green, waxy residue (i).

Purification of the copper complex

The copper complex was purified by chromatography. Two different adsorbents were used, either as alternatives or for successive treatments. Decalso (Permutit Ltd.) was

more generally satisfactory than silica gel owing to its greater loading capacity and more reproducible physical properties. On neither adsorbent did copper mycobactin form a band with a distinct rear boundary; the green colour spread over a large part of the column in a broad zone. However, strongly adsorbed impurities were left in a band at the top of the column, and other impurities formed true chromatographic bands in front of the zone containing copper mycobactin. The procedures with the two adsorbents were as follows:

Using silica gel. Silica gel prepared according to the directions of Martin & Synge (1941) was milled and passed through a 200-mesh sieve. It was then stirred with water several times and the finest particles washed away. After filtration and drying at 200° the adsorbent remained as a uniform, free-flowing powder. The physical form of the silica gel varies unpredictably from one batch to another, some being quite useless for chromatography of copper mycobactin. A small pilot experiment must therefore be carried out on each new batch to determine its suitability. Purified silica gel (500 g.) was suspended in CHCl_3 in a tube (10 cm. diameter) giving a column 22 cm. in length. Crude copper mycobactin (fraction K, 8 g.) dissolved in CHCl_3 (160 ml.) was applied to the column and the chromatogram developed with CHCl_3 (3 l.). The appearance of the column was as shown in Table 4. It was allowed to drain and the bands separately removed. Band 1 was eluted with 50% (v/v) ethanol in CHCl_3 , and the other bands with 5% (v/v) ethanol in CHCl_3 . Band 2 yielded the purified product.

Table 4. *Chromatography of crude copper mycobactin on silica gel*

(Copper mycobactin (8 g.) on 500 g. silica gel developed with 3 l. B.P. CHCl_3 .)

Band no.	Distance from top of column (cm.)	Appearance of band	Wt. of eluate (g.)
1	0-2	Brownish green	0.12
2	2-15	Bright-green zone	6.32
3	15-18	Colourless	0.40*
4	18-20	Rust-red band	0.72

* Containing some material from bands 2 and 4.

Using Decalso. Coarse Decalso was milled to pass a 200-mesh sieve and then boiled four times with 50% (v/v) ethanol in CHCl_3 (4 l./kg.) to remove coloured impurities. The adsorbent was filtered off and dried, first at room temperature and then at 150° . Variations in Decalso from batch to batch have been observed, and a small trial column should be run with each new batch. Purified Decalso (430 g.) was suspended in CHCl_3 and allowed to settle in a tube (diameter 7.2 cm.) giving a column 20 cm. in length. Crude copper complex (fraction K, 30 g.) in CHCl_3 (600 ml.) was applied to the column and developed with CHCl_3 (500 ml.). A reddish brown band separated from the front of the main green zone, and was eventually expelled from the column. As development proceeded the main zone filled the whole of the column except for a small darker band at the top, but with CHCl_3 no significant elution of the copper complex occurred. Development was continued with 5% (v/v) ethanol in CHCl_3 (700 ml.) which removed most of the complex, leaving a brownish green band 2-3 cm. wide at the top of the column.

Below this the whole column retained a light-green colour. The top band was then scraped out and the residual copper complex eluted from the lower part of the column with 20% (v/v) ethanol in CHCl_3 (1 l.). This last effluent was collected separately, combined with a small fraction representing the overlap between the main band and the lower brown band, and evaporated. The residue was dissolved in CHCl_3 and applied to a smaller Decalso column treated exactly as before. Fractions from this second column were bulked with corresponding fractions from the larger column. These fractions were then evaporated. Another small fraction was obtained by eluting the dark band from the top of the Decalso columns with 50% (v/v) ethanol in CHCl_3 . The overall result is shown in Table 5.

Both silica gel and Decalso may be recovered for further use provided that the top 2-3 cm. containing strongly adsorbed material is discarded. The adsorbent should be well washed with 50% (v/v) CHCl_3 in ethanol and dried at 150° before using again.

Table 5. *Chromatography of crude copper mycobactin on Decalso*

(Copper mycobactin (30 g.) on 430 g. Decalso developed with B.P. CHCl_3 .)

Band no.	Appearance of band	Residue from eluate	
		Wt. (g.)	Appearance
1	Brownish green, adsorbed at top of column	2.3	Almost-black wax
2	Broad, green zone	22.5	Bright-green resinous solid
3	Brick-red band, immediately below band 2	1.5	Dark-red-brown wax

Final purification of mycobactin. The Cu complex purified by chromatography (fraction *N*) was dissolved in CHCl_3 (20 ml./g.), and dry ether (3 vol.) added slowly with stirring. The precipitated Cu complex was filtered, redissolved in CHCl_3 and the precipitation repeated. It was then washed three times with ether by resuspension and finally filtered and dried in dry air. This precipitation achieved a further slight purification, and yielded Cu mycobactin (*P*) as a grey-green powder convenient to handle. The combined filtrates on evaporation gave fraction *o*, a green, resinous solid.

The complex (50 g.) was dissolved in CHCl_3 (1 l.) and redistilled methanol (2.5 l.) added. Washed H_2S was passed through the solution until all Cu had been converted into sulphide; air was then blown through to remove excess H_2S . The solution was filtered and the precipitate well washed with CHCl_3 .

The pale-yellow filtrate usually contained a little colloidal CuS. It was evaporated and the residue dissolved in CHCl_3 (1 l.). The solution was applied to a short column made by suspending pure anhydrous MgSO_4 (40 g.) in CHCl_3 in a 5 cm. diameter tube. The column was then washed with CHCl_3 (250 ml.). The CuS was removed as a thin black layer on top of the column and there was also a faint-brown band of some other impurity below.

The solution passing through the column was filtered, evaporated to about 700 ml., and the mycobactin precipitated by the addition of redistilled ether (1.8 l.). The

precipitate was filtered off, washed with dry ether and dried in air. The product *R* from this method of extraction was usually faintly yellow but was suitable for most purposes. Evaporation of the filtrate gave a fraction *q*. Additional purification of fraction *R* could be achieved by repeated precipitation with ether, and the following method was found useful for removing traces of colour. Mycobactin (2 g.) was dissolved in dry methanol (100 ml.) and water (3 ml.) added slowly with stirring. No immediate precipitation occurred, but after standing overnight at room temperature a gelatinous precipitate settled out. This was filtered off and dried (0.48 g.). Most of the colour was adsorbed in this precipitate, and on addition of more water (5 ml.) the remainder of the mycobactin was precipitated as a colourless jelly, which gave on drying a white powder (1.42 g.).

Activity of side fractions. Fractions *a*, *d*, *f*, *j* and *l* had no growth-promoting activity, whilst the amount of mycobactin in fractions *b*, *h* and *i* was too small to make their purification worth while; fractions *o*, *m* and *q* were kept for recycling.

Alternative extraction procedures. The method described above involves considerable labour in the early stages. In some experiments water was removed from the cells by drying in a ventilated oven at 75°. The product was then milled and extracted repeatedly with hot acetone. After removal of inactive material which settled out on cooling the solution, the solvent was removed and the residue was ground with light petroleum in a mortar. Successive extractions with this solvent yielded a dry brick-red powder containing most of the mycobactin and a very dark syrup which was soluble in water and could thus be separated from the required product. It was possible to proceed from this stage to the direct isolation of a crystalline aluminium complex.

The crude product obtained as described above (2.5 g.) was dissolved in CHCl_3 (50 ml.) and applied to a 3 cm. column containing 100 g. neutral Al_2O_3 . The column was washed successively with 2, 5 and 10% (v/v) ethanol in CHCl_3 . The washings removed coloured impurities. The column retained a series of brownish bands and a broad zone fluorescing blue in ultraviolet light. The latter zone was removed and eluted by refluxing with ethanol (3 × 150 ml.). The brownish ethanolic extract was concentrated to small bulk and allowed to stand, when crystals of crude Al mycobactin separated (0.40 g.). The crystals were purified by repeated crystallization from ethanol or acetone, finally giving a product, m.p. 214–215°. The crystals obtained in this way were always reddish brown, the amount of colour varying from one preparation to another. A typical analysis gave C, 62.7; H, 8.3; N, 7.85; Al, 2.5%. Table 1 shows weight recoveries and biological activities of intermediate concentrates in a complete preparation of crystalline Al mycobactin from dried bacteria.

Metal-free mycobactin could also be prepared from the crude product described above. For this purpose it was first purified by passing a CHCl_3 solution through short columns of MgSO_4 which absorbed some dark-coloured tars. After removal of the solvent, the residue was treated successively with ether and benzene, to take out impurities soluble in these solvents. This gave a light-brown powder suitable for conversion into the Cu complex and treatment by methods already described.

In most of the large-scale preparations that have been carried out, Cu was removed by treatment with H_2S , but

this method was somewhat unsatisfactory owing to the tendency to formation of colloidal CuS and to some contamination of the product with sulphur compounds. The use of ion-exchange resin was subsequently found to be advantageous, although there was some loss of product due to adsorption on the resin. Dry Zeo-Karb 225 in the acid form was ground in a Christie and Norris mill to pass a 200-mesh sieve and washed free from fine particles by repeated settling in ethanol with removal of the supernatant. The resin (90 g. dry wt.) was allowed to settle in a 2.5 cm. tube and the ethanol was displaced by 40% (v/v) CHCl_3 in ethanol. Cu mycobactin (20 g.) in the same solvent (1.5 l.) was allowed to flow through the column (time required, approx. 24 hr.) and the column washed with the solvent (500 ml.). The issuing solution was free from copper and evaporation gave mycobactin (15 g.). The resin could be recovered by passing through it 2N-HCl in ethanol.

Analytical methods

Melting points are corrected. Spectra were measured on a Beckman spectrophotometer (cell length 2 cm. unless otherwise stated).

Electrometric titrations in methanol. The electrode assembly consisted of a glass electrode conditioned to the solvent, and a reference electrode of Hg-HgCl-2M-methanolic-LiCl connected with the titration cell by a capillary bridge containing LiCl solution. Titration was carried out in a cell of 3 ml. capacity with magnetic stirring, the alkali solution being added from a micrometer syringe. The solvent used was methanol containing 20% (v/v) CHCl_3 to increase the solubility of mycobactin. Readings were made on the pH scale of the instrument, but owing to the nature of the solvent used the results are arbitrary and have no significance in terms of pH. The magnitude of the readings was found to vary somewhat from one experiment to another, though the form of the titration curve remained the same. In plotting the results the readings have been adjusted so that the maximum reading is the same in each case.

Titration of weakly basic groups. Approx. 100 mg. of mycobactin or a derivative dissolved in 5 ml. glacial acetic acid was titrated with 0.1M-HClO₄ in glacial acetic acid using crystal violet as indicator (Nadeau & Branchen, 1935). The solution was standardized against 5 ml. 0.04M-*p*-toluidine in glacial acetic acid under exactly similar conditions.

Quantitative acetylation. The substance (0.05–0.08 mequiv.) was weighed into a 10 mm. tube. Acetic anhydride in dry pyridine (1M, 0.1 ml.) was added from a micrometer syringe and the tube at once sealed. The tube was immersed 1 hr. in a boiling-water bath, then cooled, opened, and tube and contents transferred to a 25 ml. conical flask. Water (2 ml.) was added and the residual acetic acid titrated with 0.03N-NaOH using phenolphthalein as indicator. Blank determinations were carried out under identical conditions. In the case of substances having an acid group, additional controls were included in which the substance was dissolved in pyridine and titrated with alkali as before; the acid equivalent was then used to correct the acetic acid titration.

General properties and derivatives

Mycobactin. The product obtained from the extractions described above was a white powder, not visibly crystalline. Mycobactin had m.p. 165–166.5°, $[\alpha]_D^{25}$ –19° in absolute CHCl_3 (c, 4.9). Absorption maxima in methanol: 250 m μ .

(ϵ = 14000), 311 m μ . (ϵ = 3700); maxima in CHCl_3 at 253 and 314 m μ . Equivalent weight by titration with HClO₄ in glacial acetic acid: 870; 868; 873. (Found: C, 65.15, 64.7, 64.65; H, 8.55, 8.5, 8.55; N, 8.35, 8.4, 8.3. Tests for S, halogens, P and -OCH₃ negative, ash absent. $\text{C}_{47}\text{H}_{75}\text{O}_{10}\text{N}_5$ requires C, 64.9; H, 8.7; N, 8.05%.)

Salts of mycobactin. For conversion into the hydrochloride, mycobactin (1 g.) was dissolved in absolute CHCl_3 (25 ml.) and dry ether (50 ml.) added with stirring. No immediate precipitation occurred. The solution was quickly cooled in an ice bath and dry HCl passed through the solution. The gelatinous precipitate of the hydrochloride was centrifuged, washed three times with dry ether and dried in a vacuum desiccator. The hydrochloride was not obtained wholly pure. The product was a white, amorphous powder of indefinite melting point (80–100°), showing a blue-white fluorescence in ultraviolet light. Absorption maxima: 270 m μ . (ϵ = 13400); 338 m μ . (ϵ = 3200). (Found: N, 7.9; Cl, 4.2. $\text{C}_{47}\text{H}_{75}\text{O}_{10}\text{N}_5 \cdot \text{HCl}$ requires N, 7.7; Cl, 3.9%.)

For preparation of the picrate, mycobactin (1 g.) was dissolved in absolute CHCl_3 (30 ml.) and treated with picric acid (0.4 g.) in CHCl_3 (20 ml.). Ether (140 ml.) was added and the yellow precipitate washed with ether, filtered and dried, giving 0.90 g., m.p. 146–147°. It was further purified by reprecipitating twice from CHCl_3 by addition of ether. The product, still slightly impure, was a yellow powder without visible crystalline structure, m.p. 150–152°. (Found: C, 58.4; H, 7.4; N, 9.7. $\text{C}_{47}\text{H}_{75}\text{O}_{10}\text{N}_5 \cdot \text{C}_6\text{H}_3\text{O}_7\text{N}_3$ requires C, 57.9; H, 7.2; N, 10.2%.)

Aluminium mycobactin. The conversion of mycobactin into its Al complex may be brought about by adsorption on to neutral Al_2O_3 and subsequent elution with ethanol as described on p. 604. This procedure, however, always gives a brown product, possibly due to the uptake of traces of Fe from the adsorbent. Colourless crystals may be prepared by the use of Al isopropoxide, though the yields are low. Losses occur through the formation of an insoluble by-product, possibly polymeric. Mycobactin (1 g.) was dissolved in CHCl_3 (20 ml.). One equivalent of Al isopropoxide (0.235 g.) dissolved in CHCl_3 (5 ml.) was added, and the mixture allowed to stand overnight at 20°. The solution was evaporated to small bulk (1–2 ml.) and an excess of ether (50 ml.) added. The solution was allowed to stand 6 hr. and then filtered from some flocculent precipitate (0.20 g.). The filtrate was evaporated and the residue dissolved in warm ethanol (3 ml.), filtered, and allowed to crystallize. Yield, 0.35 g. (m.p. 214–215.5°). **Aluminium mycobactin** had m.p. 216.5–217° (after three crystallizations). $[\alpha]_D^{25} + 376^\circ$ in absolute CHCl_3 (c, 4.0). Absorption maxima in methanol: 265 m μ . (ϵ = 18 200); 342 m μ . (ϵ = 5300); maxima in CHCl_3 at 267 and 347 m μ . (Found: C, 63.45, 62.9, 63.3, 63.1; H, 7.9, 8.3, 8.25, 7.9; N, 7.95, 7.9, 7.85, 7.85; Al, 3.1, 3.0. $\text{C}_{47}\text{H}_{75}\text{O}_{10}\text{N}_5\text{Al}$ requires C, 63.1; H, 8.1; N, 7.8; Al, 3.0%.)

Single crystal oscillation X-ray photographs were taken by Miss Bailey and Dr A. F. Wells, who report the following: 'The crystals are monoclinic and layer line measurements give (a) = 15.81 ± 0.1 Å; (b) = 12.31 ± 0.1 Å; (c) = 13.54 ± 0.1 Å. Angle β = 101.5° ± 1°. Density by flotation in NaI solution, 1.175. Mol. wt. per unit cell = 1828 (± 4%). The dimensions given are the mean of four determinations, but the accuracy is somewhat low owing to the form of the product which crystallizes in thin, fragile plates.'

For conversion of Al mycobactin into the metal-free compound, the crystalline product (1 g.) was dissolved in ethanol (20 ml.), and cupric acetate (0.2 g. monohydrate) in

ethanol (10 ml.) added; the Cu complex was slowly precipitated on standing. After 6 hr. the complex was filtered, washed with ethanol and dissolved in CHCl_3 (20 ml.). The CHCl_3 solution was washed twice with 1% (v/v) acetic acid and twice with water, and then evaporated to half bulk to remove dissolved water. Methanol (25 ml.) was added and H_2S passed through the solution. Excess of the gas was driven off by a stream of air and the CuS filtered off. The filtrate was evaporated and the residue dissolved in CHCl_3 (20 ml.) and passed through a short column of MgSO_4 (1 g.) to remove colloidal CuS . The solution was filtered, evaporated to 10 ml., treated with dry ether (30 ml.) and allowed to stand 4 hr. The precipitate was filtered off, washed with ether and dried giving a white, amorphous powder (0.82 g.), m.p. 165–166°, having a spectrum identical with that of mycobactin.

Copper mycobactin. Mycobactin (1 g.) was dissolved in ethanol (40 ml.) and cupric acetate (0.3 g. monohydrate) in ethanol (20 ml.) added. The solution was allowed to stand 6 hr. and the copper complex filtered off. It was dissolved in CHCl_3 (40 ml.) and the solution washed twice with 1% acetic acid and twice with water. It was then evaporated to 10 ml., filtered and ether (30 ml.) added. The flocculent precipitate was filtered and dried giving a grey-green powder (0.95 g.). *Copper mycobactin* had no m.p., it charred above 200°. Absorption maxima in absolute CHCl_3 262 $\mu\mu$. ($\epsilon = 18\,900$); 354 $\mu\mu$. ($\epsilon = 4500$). (Found: C, 60.4; H, 7.6; 7.45; Cu, 6.7, 7.2 $\text{C}_{47}\text{H}_{78}\text{O}_{10}\text{N}_6\text{Cu}$ requires C, 60.6; H, 7.9; N, 7.5; Cu, 6.8%.)

Diacetyl mycobactin. Mycobactin (1 g.) was dissolved in redistilled pyridine (5 ml.) and acetic anhydride (0.25 ml.) added. The solution was allowed to stand 1 hr. at 20° and then diluted with water (20 ml.). The product was extracted with CHCl_3 , the solution washed successively with dilute HCl , NaHCO_3 solution and water and then evaporated to small volume (3–4 ml.). Dry ether (15 ml.) was added and the mixture allowed to stand 4 hr. The gelatinous precipitate was filtered off, washed with ether and dried giving a white, amorphous powder (0.90 g.). The m.p. was unchanged after reprecipitation. *Diacetylmycobactin* had m.p. 134–135.5°; $[\alpha]_D^{25} - 6.7 \pm 0.5^\circ$ in absolute CHCl_3 (c, 4.0). Absorption maxima: 250 $\mu\mu$. ($\epsilon = 12\,800$); 312 $\mu\mu$. ($\epsilon = 3900$). Equivalent wt. by titration with HClO_4 in acetic acid, 957. (Found: C, 64.05, 63.95, 64.3; H, 8.35, 8.2, 8.15; N, 7.65, 7.7, 7.7. $\text{C}_{51}\text{H}_{79}\text{O}_{12}\text{N}_6$ requires C, 64.2; H, 8.3; N, 7.3%.)

DISCUSSION

The isolation of mycobactin reported here forms a preliminary to degradative work leading to determination of its structure which will be discussed in later papers. The properties at present reported show that mycobactin is a compound entirely different from any other known growth factor.

Mycobactin occurs in relatively high concentrations (1% of dry weight) in *Myco. phlei* grown on suitable media, and its biological activity accounts for most if not all of the growth-promoting action towards *Myco. johnei* possessed by simple extracts of *Myco. phlei*. Mycobactin or some related compound is probably present also in *Myco. tuberculosis* and other mycobacteria which are known to promote

the growth of *Myco. johnei*. Proof that mycobactin itself is responsible for these effects will have to await the isolation of the growth factors from these organisms.

The question arises whether mycobactin is a compound found only in the mycobacteria or whether it has a wider distribution. The rather dubious growth stimulation of *Myco. johnei* by extracts of a variety of plant and animal tissues might indicate the presence of minute amounts of mycobactin, but could well be due to quite unrelated substances. There may be small amounts of mycobactin in the intestine which are not readily detected by the method of assay but the fact that *Myco. johnei* grows in the intestinal mucosa and mesenteric glands of the ox does not prove this to be true. It is probable that *Myco. johnei* can synthesize mycobactin when growing *in vivo* just as adapted strains may do when growing on synthetic medium.

Most of the known growth factors are of widespread occurrence, being found both in animals and in micro-organisms. Each compound of this kind probably has a characteristic biochemical function which is similar in different organisms even when they are of the most diverse types. This universality of function represents the biochemical unity underlying many living processes. Superimposed on these fundamental reactions, there are presumably certain metabolic reactions specific to particular organisms or groups of organisms leading to the production of characteristic compounds not found in other organisms. Thus, some peculiarity in the metabolism of the mycobacteria results in the production of fatty products having structures unusual among naturally occurring compounds. Little is known of the mechanism of such metabolic peculiarities. If mycobactin proves to be a growth factor specific to the mycobacteria in occurrence and action, it will be of unusual interest as an essential link in a type of metabolism confined to this particular genus. It may therefore have a special importance in relation to chemotherapy. Many attempts have been made to prepare synthetic drugs having chemical structures resembling those of known bacterial growth factors in the hope of bringing about bacteriostasis by the blocking of essential enzyme systems. Generally speaking these attempts have been unsuccessful in producing drugs of practical importance. The reasons for the failure appear to be due largely to the fact that most known growth factors for micro-organisms are also important substances in human metabolism. The result is that bacteriostatic drugs of this kind are often too toxic to be of chemotherapeutic value. An example of this effect is found in pyrithiamine, a structural analogue of thiamine (Woolley & White, 1943a, b). Another difficulty which may arise is illustrated by pantoyl taurine, an analogue of

pantothenic acid (McIlwain & Hawking, 1943). The failure of this substance as a drug may be attributed to the presence of pantothenic acid in the body of the host in a concentration sufficient to nullify the action of the drug. There is reason to hope that these difficulties would not arise with drugs designed to simulate some feature of the mycobactin structure. Such drugs might prove of importance in the chemotherapy of tuberculosis or leprosy.

SUMMARY

1. Methods for large-scale production of *Mycobacterium phlei* are described: the organism contained large amounts of growth factor for *Myco. johnei* when grown on media made from beef, but not on other media.

2. A method for the assay of the growth factor is described.

3. The factor has been isolated as a crystalline aluminium complex and also in an amorphous metal-free form. The name 'mycobactin' is suggested for the compound.

4. Mycobactin is a hitherto undescribed compound. Evidence is given in support of the empirical formula $C_{47}H_{76}O_{10}N_8$. Details are given of its general properties and of a number of derivatives.

5. The concentration of mycobactin in living *Myco. phlei* grown on beef infusion medium is estimated to be about 3 mg./g. Optimum growth of *Myco. johnei* is promoted by the presence of 40–80 µg. mycobactin/ml. of medium.

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Techniques in Tissue Metabolism

2. APPLICATION OF ELECTRICAL IMPULSES TO SEPARATED TISSUES IN AQUEOUS MEDIA

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Fluctuating electrical-potential gradients exist in the greater part of the organs of the animal body. Characteristics of the gradients produced in nervous and muscular elements are used to indicate the functional state of organs *in vivo*; applied impulses greatly change the activity of such organs and the metabolic activities of their constituent tissues. The application of electrical impulses *in vitro* to separated tissues under conditions which allow active metabolism has been described in previous papers from these laboratories (McIlwain, 1951a; McIlwain, Anguiano & Cheshire,

1951; McIlwain & Gore, 1951; Kratzing, 1951). Large changes in the metabolic behaviour of certain tissues have been caused, while others were unaffected.

The present paper describes: (1) apparatus chosen and devised to furnish and measure the electrical impulses applied in such experiments; (2) arrangements of electrodes for leading the impulses to the tissue; and (3) measurement of the effects of the impulses on (a) the electrical fields set up by the impulses around the tissue, and (b) the metabolic response of the tissue.