hydroxybutyrate and DPN^+ and the amounts of DPNH formed were measured. Phosphate buffer was used at pH 7 and tris buffer at the more alkaline pH values. Controls containing all reagents except β -hydroxybutyrate or acetoacetate showed no significant changes in extinction.

The results of 14 individual measurements showed good agreement (Table 1). The mean value for K was 1.42 ± 0.19 (S.D.) $\times 10^{-9}$. From this value and the E'_0 for the DPN^+ -DPNH system of -0.320 v at pH 7.0 and 25° (Burton, 1957), E'_0 for the β -hydroxybutyrate system is calculated to be -0.266 v. This figure is much less negative than that calculated by Burton (1957) and slightly less negative than those arrived at (for 38°) by Green *et al.* and Hoff-Jørgensen. For a full discussion of any error in Burton's figure for E'_0 of the DPN system see Clark (1960); the figure of -0.320 v may be a little too high. The above K value is very similar to that recently obtained at 32° by Doudoroff, Merrick & Contopoulou (1961), using a preparation of β -hydroxybutyric dehydrogenase from *Rhodospirillum rubrum* (1.3×10^{-9}).

SUMMARY

1. The equilibrium constant of the reaction between diphosphopyridine nucleotide and β -hydroxybutyrate

$$K = \frac{[\text{acetoacetate}] [\text{DPNH}] [\text{H}^+]}{[\beta\text{-hydroxybutyrate}] [\text{DPN}^+]}$$

was determined by using D(-)- β -hydroxybutyric dehydrogenase from *Rhodopseudomonas spheroides* and found to be 1.42×10^{-9} at 25° . It is calculated from this value and the redox potential of the diphosphopyridine nucleotide system that the redox potential of the β -hydroxybutyrate-acetoacetate system is -0.266 v (pH 7.0, 25°).

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Effects of Iron Deficiency and of Zinc Deficiency on the Composition of *Mycobacterium smegmatis*

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Certain cultures of *Mycobacterium smegmatis* in Proskauer & Beck medium grew poorly and showed abnormalities in composition (Winder & Denny, 1956). It was found that this condition resulted from a deficiency of both iron and zinc in some batches of this medium, which relies on chance contamination of its constituents to provide these metals. Bacteria from these doubly deficient cultures had a low ribonucleic acid and deoxyribonucleic acid concentration from the time of growth inhibition, and possibly preceding it (Winder & Denny, 1959).

It has been known for some time that mycobacteria require iron (Sauton, 1912) and zinc (Drea, 1956) for growth. Both metals are probably essential for all organisms and each has been shown to be a component of a number of enzymes (Nicholas, 1957; Granick, 1958; Hoch & Vallee, 1958; Patwardhan, 1960; Kupiecki & Coon, 1960). Almost all of these enzymes are concerned with oxidation-reduction processes.

This paper is concerned with a comparison between the changes in amounts of some important components in *M. smegmatis* which take place when

growth inhibition is brought about by deficiency of these metals, and when it is brought about by exhaustion of other components of the medium.

MATERIALS AND METHODS

Materials. Chemicals for the growth medium were of analytical-reagent grade, except for L-asparagine, which was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., and magnesium citrate 'true', which was obtained from British Drug Houses Ltd., Poole, Dorset. Iron was used as $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and zinc as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. Ether was shaken with an acid solution of FeSO_4 and distilled to remove peroxides.

Organism used. The strain of *Mycobacterium smegmatis* used is of unknown origin. It has been maintained in these Laboratories for a number of years and is very similar to N.C.T.C. 8152.

Microscopic examination. Heat-fixed smears were stained by the Ziehl-Neelsen stain, 20% (v/v) sulphuric acid being used for decoloration. The average size of the bacteria in a smear was estimated by measurements made on photographs (magnification $\times 1400$) obtained with a Zeiss Photomicroscope.

Treatment of medium and glassware. The medium used was Proskauer & Beck medium prepared as recommended by the American Trudeau Society (1950). The content of trace metals was greatly decreased by autoclaving with 1% (w/v) of activated alumina (May and Baker Ltd.) at 10 lb./in.² for 10 min., following the recommendations of Donald, Passey & Swaby (1952). When cool, the medium was filtered through Whatman no. 542 filter paper; the first 50 ml. of each batch was discarded and the rest was pooled and dispensed into the culture flasks. Conical flasks (250 ml.) containing 100 ml. of medium, or Roux bottles (1 l.) containing 250 ml. of medium, were used as the culture vessels.

The culture glassware was cleaned overnight in strong chromic acid, rinsed four times with tap water, soaked for 2 hr. in tap water and rinsed four times with tap water and twice with glass-distilled water. It was plugged with non-absorbent cotton and sterilized by dry heat at 160° for 1 hr.

Metals were added to the media at these concentrations: normal media, 2 $\mu\text{g.}$ of Fe^{3+} and 0.4 $\mu\text{g.}$ of Zn^{2+} /ml.; iron-deficient media, 0.06 $\mu\text{g.}$ of Fe^{3+} and 0.4 $\mu\text{g.}$ of Zn^{2+} /ml.; zinc-deficient media, 2.0 $\mu\text{g.}$ of Fe^{3+} and 0.012 $\mu\text{g.}$ of Zn^{2+} /ml. Addition of other trace elements was unnecessary (Winder & Denny, 1959).

Growth and harvesting. The media were inoculated with about 6 $\mu\text{g.}$ (wet wt.) of *M. smegmatis*/ml.; the bacteria had been taken from the surface of a 3–4-day-old culture and lightly ground in Proskauer & Beck medium. After the required incubation period at 37° without shaking, the pellicle was transferred to a small sintered-glass funnel and washed rapidly with water. It was then transferred to 16 mm. $\times$ 125 mm. test tubes (about 30 mg. of 'insoluble' nitrogen/tube) and extracted as described.

Extraction procedures. The bacteria were treated successively at 0° with 10 ml. of acetone for 1 hr., 10 ml. of acetone for 2 hr. (if necessary the bacteria were stored under acetone at this stage at –15° for several days) and 10 ml. of acetone for 1 hr. After each extraction the

mixture was centrifuged at 0°. The supernatants were pooled for porphyrin determination.

The residues were similarly extracted successively at 0° with 3 ml. of 10% (w/v) trichloroacetic acid for 3 hr., 1 ml. of 5% (w/v) trichloroacetic acid for 1 hr., 1 ml. of 5% trichloroacetic acid for 15 min. and 1 ml. of 5% trichloroacetic acid for 15 min. The supernatants were pooled for the determination of the acid-soluble compounds.

The residues were extracted successively with 10 ml. of ethanol for 15 min., 10 ml. of ethanol for 18 hr. and three times successively with 10 ml. of ethanol-ether (3:1) for 4 min. at 60°. After each extraction the mixture was centrifuged. For the last three extractions warm water was added to the centrifuge buckets. The supernatants were pooled and used for phospholipid determinations.

To the residues was added 5 ml. of *N*-KOH; the mixture was stirred frequently for 2 hr., the volume was made to 10 ml. with *N*-KOH, the suspension was well mixed and a sample (2 ml.) was taken for determination of 'insoluble' nitrogen. The remainder was incubated at 25° for 18 hr., neutralized with 60% (w/w) HClO_4 and trichloroacetic acid was added to give a 5% (w/v) solution; the mixture was left at 0° for 18 hr. and centrifuged. The residues were washed with two successive 5 ml. volumes of 5% trichloroacetic acid. The supernatants and washings were pooled, made to 20 ml. with 5% trichloroacetic acid and samples were taken for 'insoluble' polyphosphate determination.

The residues were extracted with 10 ml. of 5% trichloroacetic acid at 90° for 15 min.; the suspension was left at 0° for some time and centrifuged. The residues were stirred with 5 ml. of 5% trichloroacetic acid and the suspension was centrifuged again. The supernatants were pooled, made to 20 ml. with 5% trichloroacetic acid and samples were taken for determination of DNA and 'insoluble' inorganic polyphosphate. The residue, consisting of waxes, proteins, polysaccharides, etc., was discarded.

The extraction and centrifuging was done at 18–20° unless otherwise stated. The residues were stirred several times during the extractions, with very thin glass rods. Centrifuging was done at 2000*g* for 3–5 min. Supernatants were removed carefully with fine Pasteur pipettes.

On occasion the KOH extraction of RNA was replaced by treatment with 5% trichloroacetic acid at 20° for several days (the exact length of time being determined by following the $E_{260}/E_{268.5}$ ratios of the extracts), which extracted RNA and converted DNA into apurinic acid (Winder & Denny, 1956). RNA and DNA were determined in these extracts as described by Winder & Denny (1956). The results confirmed those given by the modified Schmidt-Thannhauser method described above.

When total lipid, dry weight and acid-soluble nitrogen were to be determined the washed bacteria were placed in tared 16 mm. $\times$ 125 mm. test tubes, freeze-dried over P_2O_5 and weighed. They were extracted twice with 10 ml. of ethanol for 1 hr., twice with 10 ml. of ethanol for 10 min. at 70°, twice with 10 ml. of ethanol-ether (3:1) for 10 min. at 60° and twice with 7 ml. of benzene for 10 min. at 75°. These extracts were transferred to tared test tubes, dried under a stream of warm air and then over P_2O_5 , and weighed. The cells were further extracted in the usual manner with cold 5% trichloroacetic acid and total nitrogen was determined in the residues. The acid extracts were pooled and total nitrogen was determined in them.

Porphyrin determination. Coproporphyrin in the acetone

extracts was determined by the method of Todd (1949). All the red fluorescent material in these extracts behaved as coproporphyrin in this procedure.

Porphobilinogen was sought by the methods of Mauzerall & Granick (1956).

Determination of acid-soluble compounds. The pooled trichloroacetic acid extracts were immediately shaken thoroughly with two successive portions (15 ml.) of ether to remove trichloroacetic acid, neutralized and made to 6 ml. Orthophosphate was determined in a sample by the method of Fiske & Subbarow (LePage, 1949). Another sample was treated with $N-H_2SO_4$ at 100° for 10 min., the orthophosphate was determined and the acid-labile phosphate was obtained by difference. Another sample was ashed (LePage, 1949), the total phosphate was determined and the acid-stable phosphate was found by difference.

ATP was estimated enzymically with 3-phosphoglycerate and crystalline phosphoglycerate kinase, crystalline triose phosphate dehydrogenase with DPNH being used as the indicator reaction. (Details of this unpublished method of Bücher & Schuart and a kit for the determination were obtained from C. F. Boehringer und Soehne GmbH., Mannheim, Germany.)

The sum of the oxidized pyridine nucleotides (DPN plus TPN) was determined by the cyanide-addition reaction and the results were confirmed on occasion by the butan-2-one fluorescence method. Reduced diphosphopyridine nucleotide was determined with alcohol dehydrogenase on a 0.1 M- Na_2CO_3 extract from the bacteria. The methods for all these determinations were those described by Ciotti & Kaplan (1957), except that in the cyanide method extra blanks, consisting of the analytical solutions treated with 0.05 M-glycine buffer, pH 11.2, instead of cyanide, were included.

Soluble inorganic polyphosphate was determined by the methods of Juni, Kamen, Reiner & Spiegelman (1948).

For determination of the total purine and pyrimidine bases, 3.5 ml. of the neutralized extract was freeze-dried, 0.1 ml. of 60% (w/w) perchloric acid was added, and it was heated at about 105° for 1.5 hr. Water (0.2 ml.) was added, the suspension was centrifuged and 0.01 ml. was pipetted on to Whatman no. 1 paper which had been washed successively with 0.05 M-sodium tetraborate, 10% (v/v) acetic acid containing 0.1% of ethylenediaminetetra-acetic acid, and water. The bases were then chromatographed with propan-2-ol-conc. HCl-water (Wyatt, 1951) eluted and the amounts determined by the differential-extinction technique (Bendich, 1957).

Lipid phosphorus. A portion of the lipid extract was evaporated to dryness, ashed with 10 N- H_2SO_4 with repeated additions of H_2O_2 , and phosphate was determined.

Ribonucleic acid. Samples of the extracts, containing 5% of trichloroacetic acid, were heated for 15 min. at 90° , suitably diluted with water, read at 268.5 m μ and the RNA phosphorus was calculated, a figure of 9800 being used for the atomic extinction coefficient with respect to phosphorus [i.e. $30.98 E/cl$, where E is extinction, c concentration of phosphorus (g./l.) and l the thickness of the absorbing layer (cm.)]; Logan, Mannell & Rossiter, 1952].

Deoxyribonucleic acid. Samples of the extracts were suitably diluted with water, read at 268.5 m μ and the DNA phosphorus was calculated by using an atomic extinction coefficient for phosphorus of 9800 (Logan *et al.* 1952).

'Insoluble' inorganic polyphosphate. The samples were made normal with respect to H_2SO_4 , heated at 100° for 10 min. and the orthophosphate was determined. Allowance was made for liberation of phosphate from the nucleic acids by subtraction of 23% of the RNA phosphorus and 14% of the DNA phosphorus in the samples.

Base composition of ribonucleic acid. A KOH extract of the cells, prepared as described above, was neutralized with 60% (w/w) perchloric acid, left for some hours at 0° and clarified by centrifuging. The supernatant was diluted and chromatographed on Dowex 1 (chloride form) columns, following the recommendations of Cohn (1957).

Nitrogen determination. The 2 ml. samples for 'insoluble' nitrogen determination, and the pooled extracts for acid-soluble nitrogen, were transferred to 16 mm. $\times$ 125 mm. test tubes, followed by 1 ml. of H_2SO_4 , 0.5 ml. of saturated KH_2SO_4 and 0.25 ml. of 1% (w/v) selenious acid. The tubes were heated on a sand bath until the liquid was colourless (about 24 hr.). After dilution, ammonia was determined in Conway units with boric acid as absorbent (Conway, 1957).

All the results in this paper are quoted in terms of the insoluble nitrogen.

Instruments. A Beckman DU spectrophotometer was used for all photometric work, a Radiometer 21 pH meter (Radiometer, Copenhagen) for pH determination and a Hanovia Chromatolite (Hanovia Ltd., Slough, Bucks.) for examination of chromatograms.

RESULTS

Under the conditions of these experiments all the cultures had formed a thin pellicle on the surface of the medium by the second day, and thereafter growth consisted in the thickening of this pellicle. In the normal cultures (i.e. those provided with adequate iron and zinc) rapid growth continued to about the third day and had almost ceased by the fourth day, owing to almost simultaneous exhaustion of the source of carbon (glycerol) and of nitrogen (asparagine) (Fig. 1). When the concen-

Fig. 1. Growth of *M. smegmatis* (as measured by increase in insoluble nitrogen) on: ●, normal medium (2 μ g. of iron and 0.4 μ g. of zinc/ml.); □, iron-deficient medium (0.06 μ g. of iron and 0.4 μ g. of zinc/ml.); ○, zinc-deficient medium (2 μ g. of iron and 0.012 μ g. of zinc/ml.). Figs. 2-6 are based on results from this set of cultures.

tration of either glycerol or asparagine in the medium was reduced, growth slowed earlier and the final yield decreased.

The iron-deficient cultures grew better initially than did the normal cultures, presumably because of a slight inhibitory effect of Fe^{3+} ions at $2\text{ }\mu\text{g./ml.}$ After 2.3 days the traces of iron or zinc in the deficient media had been exhausted to a sufficient extent to lead to a substantially diminished growth rate (Fig. 1). The cultures continued to grow slowly, however, presumably because of either a slow leaching of metals from the glassware or the existence of systems adequate for slow growth that were less metal-dependent.

The lag period and initial growth rate of *M. smegmatis* in Proskauer & Beck medium were unfortunately rather variable, so that at a given age, particularly in the early stages of growth, both the yield and the physiological condition of the cultures varied somewhat. The variation was reduced, but not eliminated, by using inocula of the same age and by treating media and glassware always in the same manner. Within each experiment, the contents of several replicate flasks were pooled to give each sample where possible. The results illustrated in Figs. 1-6 were all obtained from one experiment.

Ribonucleic acid

In the normal cultures the RNA/nitrogen ratio fell steeply after about 2.7 days (Fig. 2), i.e. at approximately the same time as the growth rate

diminished. The ratio was fairly constant at about half the earlier value after about 4 days. When the medium contained one-quarter of the normal concentration of glycerol or asparagine, growth slowed earlier, and the RNA/nitrogen ratio likewise fell earlier than in the normal medium. The ratio fell lower in the low asparagine cultures than in the normal cultures (Fig. 2), indicating that a deficiency of nitrogen has a stronger effect on the RNA content than on the protein content of the cells.

The slowing of growth that took place in the metal-deficient cultures at about 2.3 days was also accompanied by a fall in RNA/nitrogen ratio. In most experiments, as in that illustrated in Figs. 1-6, the fall in RNA/nitrogen ratio appeared to precede the inhibition of growth, but this has not been decided conclusively owing to the slight variation in initial growth rate from flask to flask. The ratio fell earlier in the zinc-deficient cultures than in the iron-deficient cultures, perhaps because of the deficiency developing slightly earlier in the former cultures. In older cultures, the RNA/nitrogen ratio remained higher in both of the metal-deficient cultures than in the normal cultures.

Deficiency of both iron and zinc did not affect the base ratios in the RNA (Table 1). These ratios were similar to those for RNA for other mycobacteria (Khouvine, Barbier & Wyssman, 1953; Khouvine & Wyssman, 1954).

Deoxyribonucleic acid

In normal cultures the DNA/nitrogen ratio diminished slightly between 2.3 and 2.7 days, but as the growth rate fell off the ratio rose steeply and continued to rise until growth ceased, by which time the value had about doubled (Fig. 3) When growth was slowed earlier through reduction of either the glycerol or asparagine concentration in

Fig. 2. RNA content in *M. smegmatis* grown on: ●, normal medium ($2\text{ }\mu\text{g.}$ of iron and $0.4\text{ }\mu\text{g.}$ of zinc/ml.); □, iron-deficient medium ($0.06\text{ }\mu\text{g.}$ of iron and $0.4\text{ }\mu\text{g.}$ of zinc/ml.); ○, zinc-deficient medium ($2\text{ }\mu\text{g.}$ of iron and $0.012\text{ }\mu\text{g.}$ of zinc/ml.); ▲, medium containing 25% of normal glycerol; ■, medium containing 25% of normal asparagine.

Table 1. Base composition of ribonucleic acid from a normal culture of *Mycobacterium smegmatis* and from a culture deficient in both iron and zinc

M. smegmatis was grown for 3 days at 37° on medium containing $2\text{ }\mu\text{g.}$ of iron and $0.4\text{ }\mu\text{g.}$ of zinc/ml. in the normal culture, and $0.6\text{ }\mu\text{g.}$ of iron and $0.012\text{ }\mu\text{g.}$ of zinc/ml. in the deficient culture. RNA was extracted and hydrolysed by a modified Schmidt-Thannhauser procedure and the nucleotides were separated on a Dowex 1 (chloride form) column.

Base	Normal culture (% of total bases)	Metal-deficient culture (% of total bases)
Adenine	22.7	22.5
Guanine	38.1	37.5
Uracil	16.25	16.4
Cytosine	23.0	23.3

the medium, the rise in DNA/nitrogen ratio took place earlier. Hence limitation of growth through limitation of carbon or nitrogen, or of both, led to a rise in DNA/nitrogen ratio.

When growth was limited through a deficiency of either iron or zinc, however, the DNA/nitrogen ratio did not rise appreciably (Fig. 3). In both these cultures at about the time of growth inhibition the ratio was slightly, but significantly, below that in the normal cultures. In the iron-deficient cultures the ratio fell further than in the zinc-deficient cultures.

The increased DNA/nitrogen ratio in the older normal cultures did not seem to be due to extracellular DNA, as with some micro-organisms (Cattin & Cunningham, 1958), since the pellicles were not 'slimy'. Hence it probably represented an increase in the amount of nuclear material per unit of cytoplasm.

Microscopic observations were in keeping with this interpretation. The bacteria in normal cultures showed changes similar to those described for a number of mycobacteria by Brieger, Cosslett & Glauert (1954) and by others. During the lag phase the short rods and coccoid forms in the inoculum elongated, and from then to about the third day the bacteria consisted of short- to medium-length rods. Subsequently the average length of the rods

in the normal cultures decreased until the bacteria consisted of very short rods and coccoid forms. When the medium contained a reduced concentration of either glycerol or asparagine the decrease in cell size took place earlier. Qualitatively, these alterations in cell size are what would be expected from the changes in the DNA/nitrogen ratio, but they are quantitatively greater, suggesting that the longer rods are multicellular.

For the first 2 days the bacteria in the metal-deficient cultures were microscopically similar to those in the normal cultures. Thereafter the rods in the iron-deficient cultures increased in length instead of decreasing, until many assumed the character of long, filamentous structures. This elongation was greater than would be expected from changes in the DNA/nitrogen ratio over the same period, unless it is assumed that the number of cells in these rods was also increasing. In the zinc-deficient cultures the rods did not lengthen as much.

Fig. 3. DNA content in *M. smegmatis* grown on: ●, normal medium (2 μ g. of iron and 0.4 μ g. of zinc/ml.); □, iron-deficient medium (0.06 μ g. of iron and 0.4 μ g. of zinc/ml.); ○, zinc-deficient medium (2 μ g. of iron and 0.012 μ g. of zinc/ml.); ▲, medium containing 25% of normal glycerol; ■, medium containing 25% of normal asparagine.

Fig. 4. Insoluble polyphosphate content in *M. smegmatis* grown on: ●, normal medium (2 μ g. of iron and 0.4 μ g. of zinc/ml.); □, iron-deficient medium (0.06 μ g. of iron and 0.4 μ g. of zinc/ml.); ○, zinc-deficient medium (2 μ g. of iron and 0.012 μ g. of zinc/ml.); ▲, medium containing 25% of normal glycerol; ■, medium containing 25% of normal asparagine.

Insoluble inorganic polyphosphate

In normal cultures the insoluble inorganic polyphosphate/nitrogen ratio began to rise shortly after the second day and rose steeply after 2·7 days, when growth became distinctly slower (Fig. 4). The rise was due to approaching exhaustion of nitrogen in the medium, as was shown by the fact that the ratio increased earlier and rose higher in media containing a reduced concentration of asparagine (Fig. 4). The inorganic polyphosphate/nitrogen ratio did not rise very high in normal medium owing to the fact that this medium is balanced so that the carbon source approaches exhaustion soon after the nitrogen source. When the concentration of glycerol in the medium was reduced, so that carbon deficiency appeared before nitrogen deficiency, no increase in inorganic polyphosphate/nitrogen took place (Fig. 4).

In zinc-deficient cultures the insoluble-polyphosphate/nitrogen ratio started to increase probably even before growth inhibition appeared, and it rose continuously to a high value (Fig. 4). In iron-deficiency a rise in polyphosphate took place early, but the rise rapidly levelled off and the final ratio was little above that in the normal cultures.

Acid-soluble compounds

Stable acid-soluble phosphate/nitrogen values in bacteria from the normal cultures reached a peak at about the third day and fell thereafter (Table 2). The value was much lower in glycerol-deficient

cultures and much higher in nitrogen-deficient cultures from the third to the fourth day. In both metal-deficient cultures the value had fallen by the third day (Table 2).

The labile acid-soluble phosphate/nitrogen ratio altered little between the second and the fourth days in normal cultures (Table 2). Again glycerol shortage reduced the value and nitrogen shortage increased it. The value in both the metal-deficient cultures fell between the third and the fourth days (Table 2).

In normal cultures the ATP/nitrogen ratio fell fairly steadily with increasing age after the second day (Fig. 5). In iron-deficient cultures the ratio rose above normal in the early deficiency state and then fell continuously, though it usually remained higher than in normal cultures of the same age (Fig. 5). In zinc-deficient cultures the ATP/nitrogen ratio was constant from 2·3 to 3 days, rose to a peak at about the fourth day and then declined (Fig. 5).

The excess of acid-labile phosphate over that accounted for as ATP was mostly soluble inorganic polyphosphate in the samples in which this was estimated. The content of this substance was not followed in detail, however.

In all cultures the amount of reduced pyridine nucleotides was very low compared with the amount of oxidized pyridine nucleotides.

In normal cultures the ratio of oxidized pyridine nucleotides/nitrogen remained practically constant over the period studied (Fig. 6). The values obtained

Table 2. *Lipid, nitrogen and acid-soluble phosphorus in Mycobacterium smegmatis*

Growth medium	Age (days)	Acid-soluble phosphorus		Lipid		Nitrogen	
		Acid-labile (mg. of P/g. of insoluble N)	Acid-stable (mg. of P/g. of insoluble N)	Phospho- lipid P (mg. of P/g. of insoluble N)	Total lipid (g./g. of insoluble N)	Acid- soluble (mg. of N/g. dry wt.)	Insoluble (mg. of N/g. dry wt.)
Normal (2 µg. of iron and 0·4 µg. of zinc/ml.)	2	6·6	19·2	8·0	1·96	5·62	78·0
	3	7·0	21·6	7·4	1·55	6·16	82·8
	4	7·1	16·0	7·3	1·20	5·50	83·3
	5	—	—	—	0·91	4·13	88·8
Nitrogen-deficient (25% of normal asparagine, normal metals)	3	8·6	22·7	10·8	—	—	—
	4	8·8	32·0	19·3	—	—	—
Glycerol-deficient (25% of normal glycerol, normal metals)	3	5·9	10·4	7·0	—	—	—
	4	4·5	9·0	6·4	—	—	—
Iron-deficient (0·06 µg. of iron and 0·4 µg. of zinc/ml.)	2	6·5	20·0	8·0	1·85	6·04	82·3
	3	7·2	18·4	9·1	1·99	3·43	79·9
	4	6·0	13·1	10·1	1·68	—	82·4
	5	—	—	—	1·56	3·39	87·9
Zinc-deficient (2 µg. of iron and 0·012 µg. of zinc/ml.)	2	6·5	20·3	8·0	1·99	5·94	79·0
	3	7·2	19·6	9·4	2·07	4·83	74·0
	4	5·5	16·8	11·2	2·27	4·85	68·5
	5	—	—	—	1·81	5·31	70·7

were about three times those recorded for *Mycobacterium phlei* by Weber & Swartz (1960). In the iron-deficient cultures, on the other hand, oxidized nucleotide/nitrogen dropped early, even before measurable growth inhibition had taken place, and remained low. In zinc-deficient cultures the ratio fell later than in the iron-deficient cultures, but the final value was similar.

In view of the increase in diphosphopyridine-

Fig. 5. ATP content in *M. smegmatis* grown on: ●, normal medium (2 µg. of iron and 0.4 µg. of zinc/ml.); □, iron-deficient medium (0.06 µg. of iron and 0.4 µg. of zinc/ml.); ○, zinc-deficient medium (2 µg. of iron and 0.012 µg. of zinc/ml.).

Fig. 6. Content of pyridine nucleotides in *M. smegmatis* grown on: ●, normal medium (2 µg. of iron and 0.4 µg. of zinc/ml.); □, iron-deficient medium (0.06 µg. of iron and 0.4 µg. of zinc/ml.); ○, zinc-deficient medium (2 µg. of iron and 0.012 µg. of zinc/ml.).

nucleotidase activity in zinc-deficient, though not in iron-deficient, cultures of *Neurospora* (Nason, Kaplan & Colowick, 1951), it appeared possible that this enzyme might be responsible for destruction of DPN in our zinc-deficient cultures, or in the extracts, in view of the fact that this enzyme is only partially precipitated by trichloroacetic acid and its activity can be restored after removal of the acid (Kaplan, 1955). However, we could find no evidence for the presence of this enzyme in any cultures of *M. smegmatis* (Winder, O'Hara & Ratledge, 1961). Certainly, such activities as could have escaped detection could not have been responsible for the destruction of DPN after extraction, though it is conceivable that destruction could have taken place in the living cell.

Amounts of total acid-soluble adenine, guanine, uracil and cytosine/unit nitrogen were also determined (Table 3). These amounts fell slightly in the normal cultures at the third day, i.e. at the time of growth inhibition. In the metal-deficient cultures at the time of their growth inhibition (2.3–2.6 days) amounts of adenine and guanine were little if at all below normal, whereas uracil and cytosine appeared to be slightly above normal. Thymine contents were too low for accurate determination by the methods used, though there was a suggestion that values were low in the iron-deficient cultures. The contents of all these bases in metal-deficient cultures increased from the fourth to the fifth days, suggesting autolytic breakdown of nucleic acids at this stage.

It will be seen from Table 3 and Figs. 5 and 6 that most of the acid-soluble adenine is accounted for as ATP and pyridine nucleotides.

Lipids

The total lipid/nitrogen ratios are given in Table 2. This ratio fell by about half in normal cultures between the second and the fifth days. The decrease that took place in the deficient cultures was considerably smaller. Phospholipid/nitrogen ratio also fell slightly in normal cultures, a fall which was accentuated when glycerol was deficient. When nitrogen was deficient the ratio increased instead. Phospholipid/nitrogen rose in both the metal-deficient cultures.

Nitrogen

Values for acid-soluble and insoluble nitrogen on a dry-weight basis are given in Table 2. Insoluble nitrogen/dry wt. rose slightly between the second and the fifth days in normal and in iron-deficient cultures. In zinc-deficient cultures there was a slight fall. Acid-soluble nitrogen/dry wt. fell after the third day in normal cultures. In zinc-deficient cultures this value fell after the second day, but

Table 3. *Acid-soluble purines and pyrimidines in Mycobacterium smegmatis*

All values (μ moles/g. of insoluble N) were determined after hydrolysis by 60% (w/w) perchloric acid at 105° for 1.5 hr. (see Materials and Methods section).

Growth medium	Age (days)	Adenine	Guanine	Uracil	Cytosine
Normal (2 μ g. of iron and 0.4 μ g. of zinc/ml.)	2.25	128	20.7	—	—
	2.65	122	—	42.0	11.9
	3	107	14.0	31.8	7.4
	4	90	9.3	35.7	10.8
	5	82	10.0	34.2	6.8
Iron-deficient (0.06 μ g. of iron and 0.4 μ g. of zinc/ml.)	2.25	122	21.9	44.7	—
	2.65	111	13.1	49.8	13.9
	3	83	12.0	50.6	8.4
	4	62	16.1	26.7	10.3
	5	95	20.1	41.3	13.3
Zinc-deficient (2 μ g. of iron and 0.012 μ g. of zinc/ml.)	2.25	123	19.0	46.4	—
	2.65	119	13.6	44.6	13.7
	3	112	11.6	48.2	15.6
	4	101	15.0	35.3	14.3
	5	109	18.2	58.5	26.5

increased again at the fifth day, possibly because of autolysis. In iron-deficient cultures a larger fall took place after the second day.

Coproporphyrin

Coproporphyrin was the only free porphyrin found, in keeping with the observations of Todd (1949) and Bariéty, Gajdos, Gajdos-Torok & Sifferlen (1957). The content varied considerably from culture to culture. In normal cultures the content reached a maximum (100–150 μ g./g. of nitrogen) and fell thereafter. When the nitrogen in the medium was reduced the porphyrin content increased several-fold. In iron deficiency the content was lower than normal up to the fourth day, after which it rose steeply. In zinc-deficient cultures the content was above normal up to the third day and at the fifth day, but was usually subnormal between these.

No porphobilinogen was found in any of these cultures.

DISCUSSION

The metal-deficient cultures can be compared directly with cultures of the same age only before the third day. After that, the metal-deficient cultures have a comparative surplus of carbon and nitrogen sources and of the non-limiting metal.

The insoluble polyphosphate content reflects the balance between supply of and demand for 'high-energy' phosphate, since this polyphosphate can function in the reversible synthesis of ADP and ATP (Yoshida & Yamataka, 1953; Hoffmann-Ostenhof, Kenedy, Keck, Gabriel & Schönfellinger, 1954; Kornberg, Kornberg & Simms, 1956; Winder & Denny, 1957). The exhaustion of nitrogen supplies in normal and nitrogen-deficient cultures

presumably led to decreased demands on high-energy phosphate for anabolic processes, with its consequent accumulation as insoluble polyphosphate. Earlier exhaustion of the energy source in carbon-deficient cultures, and later exhaustion in normal cultures, stopped the accumulation. The accumulation of polyphosphate in iron-deficient and particularly in zinc-deficient cultures showed that growth in these was not inhibited by failure of respiration or of oxidative phosphorylation. This is interesting, since most of the enzymes known to contain these metals are respiratory enzymes. Iron deficiency led subsequently to inhibition of energy-supplying reactions, indicated by the fact that the polyphosphate accumulation was comparatively small.

The decrease in RNA and increase in DNA relative to insoluble nitrogen on growth inhibition due to exhaustion of carbon or nitrogen, or of both, in the medium indicated a greater inhibition of RNA than of protein synthesis, and a greater inhibition of protein than of DNA synthesis. This pattern is usual in transition of bacterial cultures from log phase to stationary phase (Herbert, 1961). In fact, the inhibition of RNA synthesis may lead to the inhibition of protein synthesis and of growth. Hence the decrease in RNA relative to insoluble nitrogen that took place in iron- and zinc-deficient cultures at about the time of growth inhibition was not unusual, and as in normal cultures it may be the cause of growth inhibition. However, the absence of an increase in DNA relative to nitrogen, and with iron deficiency a slight decrease, suggest that shortage of either of these metals has a specific inhibitory effect on DNA synthesis.

There is a general occurrence of iron and zinc in RNA from different sources (Wacker & Vallee, 1959).

The decrease in ATP relative to insoluble nitrogen in normal and in iron-deficient cultures, at a time when amounts of insoluble polyphosphate on the same basis were increasing, indicated a decreased synthesis of adenosine rather than shortage of phosphate or energy. A similar inhibition of adenosine synthesis did not seem to develop in the zinc-deficient cultures.

The normal cultures showed that a fall in pyridine nucleotides relative to insoluble nitrogen was not a necessary accompaniment of growth inhibition. Hence the fall in the content of these in iron-deficient cultures even before growth inhibition, and the rather later fall in zinc-deficient cultures, suggests a specific effect of deficiency of these metals on pyridine nucleotide synthesis. The higher than normal content of ATP when pyridine nucleotide content had fallen below normal suggested that adenine nucleotide deficiency cannot be invoked to explain the failure of pyridine nucleotide synthesis. Hence an inhibition of the synthesis of nicotinic acid or of its incorporation into the pyridine nucleotides seems probable.

A possible action of zinc in nicotinic acid synthesis is suggested by the fact that nicotinic acid is derived from tryptophan in animals and in *Neurospora*, and tryptophan synthesis has been shown to be defective in zinc-deficient higher plants and *Neurospora* (Tsui, 1948; Nason, 1950; Nason *et al.* 1951). However, the relationship between tryptophan and nicotinic acid has been found not to apply to some bacteria (Yanofsky, 1954) and therefore may not apply to *M. smegmatis*. Other possibly relevant observations are that iron is a component of tryptophan pyrrolase (Tanaka & Knox, 1959) and that Fe^{2+} ions are required by the pyridine ribosidase of certain bacteria (Van Eys, 1960).

DPN synthesis is particularly associated with nuclei (Hogeboom & Schneider, 1952) and hence the constant DPN/nitrogen ratio in normal cultures may represent a fortuitous balance between the increasing content of nuclear material (indicated by the increased DNA/nitrogen) and a fall in DPN synthesis per unit of such material. The lack of increase in nuclear material in the metal-deficient cultures would then account for the fall in DPN.

The decreased protein content of the zinc-deficient cultures is in keeping with other findings (Nason *et al.* 1951; Possingham, 1956) suggesting a special inhibitory effect of zinc deficiency on protein synthesis. An increase in acid-soluble nitrogen, such as has been observed in zinc-deficient plants (Possingham, 1956), was found only in the late stages of zinc deficiency in our cultures.

Patterson (1960) found that zinc stimulated free porphyrin formation in *Mycobacterium avium*,

apparently in disagreement with our findings. However, the discrepancy might be explained by the higher concentration of zinc (4 $\mu\text{g./ml.}$) used by him.

Filamentous forms of bacteria arise in response to a considerable range of stimuli (Hinshelwood, 1946; Nickerson, 1948; Loveless, Spoerl & Weisman, 1954). They seem to result in some cases from inhibition of DNA synthesis without proportional inhibition of cell growth (Jeener & Jeener, 1952; Caldwell & Hinshelwood, 1950; Webb & Nickerson, 1956; Katchmann, Fetty & Busch, 1959) and in other cases by inhibition of cell division or cell separation (Caldwell & Hinshelwood, 1950; Webb, 1953; Spoerl & Looney, 1958). Filament formation in iron deficiency in *M. smegmatis* may fall into the first category since it is accompanied by a reduced DNA/unit nitrogen, but since elongation of the bacilli is greater than would be expected from the change in DNA concentration, iron deficiency may also have an effect on cell division or cell separation. The smaller degree of elongation in zinc deficiency would also appear to be due to partial failure of cell division or separation. There are higher requirements for zinc and probably for iron for cell separation than for growth in *Coccidioides immitis* (Goldschmidt & Taylor, 1958).

SUMMARY

1. *Mycobacterium smegmatis* required about 2 $\mu\text{g.}$ of iron/ml. and 0.4 $\mu\text{g.}$ of zinc/ml. for full growth on Proskauer & Beck medium. If the concentration of either metal was decreased, growth slowed sooner than normally and the final yield was reduced.

2. Ribonucleic acid (per g. of insoluble nitrogen) fell at about the time of growth inhibition because of deficiencies of iron, zinc, carbon or nitrogen, or of both carbon and nitrogen. Ribonucleic acid from metal-deficient cultures had a normal base composition.

3. Deoxyribonucleic acid (per g. of nitrogen) increased when either carbon or nitrogen became deficient. In zinc deficiency there was no increase, and in iron deficiency there was a slight fall.

4. The bacteria decreased in size when carbon or nitrogen became deficient. The length of the bacteria increased several times during iron deficiency and somewhat less in zinc deficiency.

5. In zinc deficiency insoluble inorganic polyphosphate (per g. of nitrogen) increased several-fold, suggesting that energy- and phosphate-using reactions were inhibited rather than respiration. In normal and in iron-deficient cultures the polyphosphate increase stopped after a short time, suggesting that energy-supplying reactions also were inhibited.

6. In both metal deficiencies acid-labile acid-soluble phosphate (per g. of nitrogen) fell about a day after growth inhibition, and acid-stable acid-soluble phosphate fell rather sooner.

7. Adenosine triphosphate (per g. of nitrogen) fell in both normal and iron-deficient cultures at about the time of growth inhibition, suggesting a relative slowing of adenylic acid synthesis. In zinc-deficient cultures it remained high.

8. The amount of pyridine nucleotides (per g. of nitrogen) remained high in normal cultures. It fell early in zinc-deficient and particularly in iron-deficient cultures.

9. Total lipids (per g. of nitrogen) fell less in both metal-deficient cultures than in normal cultures, and phospholipids rose in metal deficiency.

10. Insoluble nitrogen (per g. dry wt.) rose slightly in normal and iron-deficient cultures, and fell in zinc deficiency. Acid-soluble nitrogen (per g. dry wt.) fell more rapidly than normal in both deficient cultures.

11. In normal cultures coproporphyrin (per g. of nitrogen) rose to a peak and then fell with increasing age. In nitrogen deficiency amounts were greatly increased. In early zinc deficiency amounts were higher, and in early iron deficiency lower, than normal. At later stages amounts in both these cultures rose.

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Iodine Metabolism in the Thyroid Gland of the Rat

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The steps that lead from the concentration of iodide by the thyroid gland to the formation of the thyroid hormones thyroxine and 3,5,3'-tri-iodothyronine are not yet fully understood. It is generally held that 3-monoiodotyrosine is the first organic iodo-compound formed. In the normal gland, much of this is rapidly converted into di-iodotyrosine.

The suggestion of Harington & Barger (1927) that thyroxine was formed in the thyroid by a coupling reaction between two molecules of di-iodotyrosine has not been seriously disputed. However, the biosynthetic mechanism whereby tri-iodothyronine is formed is still a matter of debate. Gross & Pitt-Rivers (1953) suggested that it might arise by a partial deiodination of thyroxine, but they produced no evidence in support of this hypothesis. Roche, Michel, Michel & Lissitzky (1952) had failed to demonstrate any deiodination of thyroxine by thyroid slices *in vitro*, although they found that in the same conditions iodide was readily obtained from both the iodotyrosines. They concluded that the biosynthesis of tri-iodothyronine probably involved coupling between one molecule each of mono- and di-iodotyrosine.

The likelihood that monoiodotyrosine is the precursor of di-iodotyrosine in thyroglobulin is strongly supported by the work of Taurog, Tong & Chaikoff (1958), who showed that at early time intervals after a single injection of ^{131}I in rats the monoiodotyrosine ^{131}I content of the thyroid hydrolysates rose rapidly and thereafter fell to a final concentration of about 15% of the total ^{131}I of the thyroid gland. The radioactivity in di-iodotyrosine reached a maximum after that in monoiodotyrosine had passed its peak, and remained at a concentration of over 40% of the total ^{131}I for many hours; 75 hr. after the injection there was only a slight fall in the

radioactive di-iodotyrosine concentration although the radioactive thyroxine content of about 14% of the total ^{131}I had reached a maximum value 25–30 hr. after the injection. These authors did not investigate tri-iodothyronine in their thyroid hydrolysates.

The high monoiodotyrosine ^{131}I to di-iodotyrosine ^{131}I ratios in the thyroid found by Taurog *et al.* (1958) soon after administration of ^{131}I were not observed by Bois & Larsson (1958); they were unable to detect any changes in the ratio of radioactive monoiodotyrosine to di-iodotyrosine in hydrolysates of rat thyroid 30 min. to 7 days after injection of ^{131}I . The incorporation of ^{131}I into thyroxine and tri-iodothyronine increased with time. Further, the ^{131}I content of both the iodothyronines rose when the animals were fed on an iodine-deficient diet, but the increase was much more pronounced for tri-iodothyronine than for thyroxine. Since the glands of the iodine-deficient animals contained more radioactive monoiodotyrosine than those of animals on the basic diet, it was concluded that tri-iodothyronine arose from coupling of the two iodotyrosines.

Plaskett (1961*a, b*) investigated the distribution of ^{131}I in the two rings of thyroxine and tri-iodothyronine obtained from hydrolysates of thyroids of rabbits injected with ^{131}I ; 24 hr. after the injection, the specific radioactivity of iodine was equal in both rings of both these amino acids. He suggested that his findings were compatible with thyroxine being the biological precursor of tri-iodothyronine.

Feuer & Vekardi (1957) and Feuer (1959) found that up to 6 hr. after injection of ^{131}I into rats the radioactivity in tri-iodothyronine exceeded that in thyroxine, and fell as that of the thyroxine rose. They suggested that tri-iodothyronine was the pre-