Respiration and Nutritional Requirements of Certain Members of the Genus *Mycobacterium*

BY N. L. EDSON AND G. J. E. HUNTER (Travis Research Fellows), *Travis Laboratory, Medical School, University of Otago, New Zealand*

(Received 23 August 1943)

Much investigation [reviewed by Calmette, 1923; Long, 1923; Knight, 1936] has shown that many acid-fast bacilli can be cultivated in synthetic media. The utilization of specific carbon and nitrogen sources has also been studied [Kendall, Day & Walker, 1914; Long, 1921-22; see also Knight, 1936; Merrill, 1931].

With the exception of Uyei [1927], who employed the tubercle bacillus, authors have not defined precisely the size of the inoculum, and this is now known to be a critical factor in experiments on bacterial nutrition. Accordingly, we have reinvestigated the growth requirements of certain *Mycobacteria* in order to determine the effects of varying the size of inoculum. The use of defined inocula and other rigid conditions has made it possible to demonstrate the stimulant action of certain accessory nutrients in an unequivocal manner.

EXPERIMENTAL

Organisms. The following species were utilized: *Myc. phlei* a (Veterinary Research Station, Glenfield, N.S.W.), *Myc. phlei* b (McMaster Veterinary Laboratory, Sydney), *Myc. phlei* (no. 525), *Myc. smegmatis* (no. 523), *Myc. stercoris* (no. 3820), *Myc. sp. Karlinski* (no. 2071), *Myc. sp. leprous Kedrowsky* (no. 509), *Myc. ranae* (no. 2891), *Myc. butyricum* (no. 337), *Myc. tuberculosis hominis*, avirulent strain T₁ (Commonwealth Serum Laboratories, Melbourne; originally from the Royal Institute of Public Health, London), *Myc. tuberculosis hominis*, virulent strain H 37 (Commonwealth Serum Laboratories, Melbourne; originally from the U.S.A.). The species designated by catalogue numbers were obtained from the National Collection of Type Cultures, Lister Institute, London.

In this laboratory, stock cultures were maintained on Dorset's egg medium and subcultured every fifth week. Prior to experimental use the organisms were grown for several generations on the surface of meat infusion broth containing 5% of glycerol. Broth cultures, except those of tubercle bacilli, were 7 days old when used. All organisms were cultivated at 38°. The cultural characteristics of the three specimens of *Myc. phlei* were identical.

METHODS

Washed bacterial suspensions. In both respiratory and nutritional experiments the bacteria were removed from the surface of the broth, washed three times with sterile

distilled water (by centrifugation), and a known weight resuspended in a measured volume of water. The notorious difficulty of obtaining homogeneous suspensions of acid-fast bacilli was overcome in two ways as follows:

(a) *Preparation of inocula for nutritional tests.* Small quantities of bacteria (up to 60 mg. wet wt.) were successfully dispersed by light hand-grinding with 0.5 ml. of water in a test-tube and plunger apparatus made from two closely fitting tubes, the contiguous surfaces of which had been carefully ground. The suspension was washed from the grinder and diluted with sterile distilled water.

(b) *Preparation of suspensions for manometric work.* Larger quantities (up to 2 g. wet wt.) were dispersed by gentle shaking in a small stainless steel ball-mill similar to that described by Langner & Forrester [1939]. The mill, containing bacteria, 10 ml. of sterile distilled water and three steel balls (diam. $\frac{3}{8}$ in.), was clamped to a Warburg shaker and shaken outside the thermostat for 20 min. at the rate of 40 oscillations/min. The suspension was diluted to an appropriate volume. Microscopic examination of stained smears revealed no disruption of bacterial substance or loss of acid-fast properties.

Bacterial suspensions prepared by these methods are satisfactory for the work described, and possess a degree of stability approximating to that of a yeast suspension of corresponding density. Suspensions of *Myc. smegmatis* and *Myc. stercoris* tend to flocculate more rapidly than the others.

Measurement of respiration. Oxygen consumption was measured by the manometric method of Warburg, the usual conical vessels being employed. The gas space contained oxygen and the temperature of the thermostat was 38°. The main compartment of the Warburg cup contained 1 ml. of 0.2 M sodium phosphate buffer, pH 7.4, to which were added 2 ml. of bacterial suspension. Substrate solutions were added from the side-bulb after 30 min. equilibration. Experiments were conducted for times ranging from 1 to 4 hr.

Oxygen uptake (Q_{O_2}) is expressed as μ l. O₂ consumed/mg. dry wt. bacteria/hr. The dry weight was determined by drying 6 ml. of suspension to constant weight at 107°. 2 ml. of suspension generally contained 12-15 mg. dry weight of bacteria.

Nutritional experiments. In this work we have adhered as closely as possible to the principles stressed by Knight & Fildes [1933] and Fildes & Richardson [1935]. In adapting these principles to experiments on the slowly growing *Mycobacteria* the chief considerations were the use of a fluid synthetic medium containing substances of the highest purity, and the addition of a washed inoculum which was

large enough to produce growth in a reasonable time, but not so heavy as to carry over significant amounts of medium from the mother culture or to provide significant quantities of growth stimulants arising from cellular disintegration.

In order to ascertain the requisite inoculum and to test the ability of each organism to grow with different sources of carbon, exploratory experiments were conducted with $6 \times \frac{3}{4}$ in. test-tubes containing 5 ml. of medium. The inoculum used initially was 1 mg. wet weight of bacilli in one drop (0.05 ml.) of suspension; the result was a guide to the inoculum permissible in decisive experiments, all of which were performed in 20 ml. of medium contained in 100 ml. pyrex Erlenmeyer flasks. The depth of fluid was 6–8 mm.

A series of parallel experiments, in which two lots of bacteria were washed from 3 to 9 times, one with distilled water and the other with 0.9% NaCl, gave identical results, showing that three washings were adequate, and that water was no more deleterious to the bacteria than saline.

In all experiments the ammonium ion was either the sole or the chief source of nitrogen. Organic materials were synthetic products, or analytical reagents where these were available; inorganic materials were of analytical grade. The following solutions were prepared:

(a) Basal salt mixture contained 1.0 g. of $(\text{NH}_4)_2\text{SO}_4$; 4.5 g. of KH_2PO_4 ; 2.0 g. of NaCl; 0.25 g. of NaHCO_3 ; 26.0 ml. of NaOH and distilled water to make the total volume 600 ml.

The pH was 7.5. The solution was distributed in test-tubes (3 ml.) or in Erlenmeyer flasks (12 ml.) and autoclaved at 15 lb. for 15 min.

(b) 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (autoclaved).

(c) 10% solutions of various carbon-containing substances (carbon-source) sterilized by filtration (Seitz EK) or by autoclaving, according to heat stability. Acids were used as neutral sodium salts.

(d) Solutions of substances under test, which were added in small volume and in low concentrations, were filtered or autoclaved according to their stability. Whenever stability

was in doubt, both autoclaved and filtered specimens were tested. Acids were neutralized with NaOH, bases with HCl.

Solutions of classes (b), (c) and (d) were added aseptically to the distributed salt mixture, and, after all additions had been made, sterile distilled water was added to bring the final volume to 5 ml. in test-tubes, and to 20 ml. in flasks. Thus 600 ml. of original salt mixture were diluted to 1000 ml. The final concentration of magnesium sulphate was 0.04 g./litre, and that of carbon source 1–2%.

Media were inoculated by cautious addition of 0.05 ml. of bacterial suspension to the surface, which was subsequently disturbed as little as possible. Test-tubes were slanted on trays. All vessels were plugged with cotton-wool, remaining unsealed unless experiments were prolonged beyond 7 days. Cultures were incubated at 38° and observed daily.

At the termination of an experiment the growth was transferred to tightly packed asbestos on a Gooch crucible, washed three times with distilled water, and dried to constant weight at 107°. Although this procedure involves a small loss of bacteria, the weights are sufficiently accurate for the purpose of the experiments described.

RESULTS

Respiration measurements

The oxygen consumption of several species in the presence and absence of substrates is shown in Table 1. When no substrate is added, there is an oxygen uptake which remains constant for about 2 hr. and diminishes slowly thereafter. The value of Q_{O_2} varies with the species, and bears some relationship to the rate of growth in nutrient broth. In the presence of most substrates oxygen consumption increases above the 'resting' value. There is one notable exception: citrate causes no significant change in respiration under the conditions used. Moreover, there was no oxidation of citrate when the suspension medium was phosphate saline [Krebs, 1933] or the basal salt mixture described above. Bacterial suspensions which have been heated in a boiling water-bath for 10 min. have no oxygen uptake either in presence or absence of added substrate.

Table 1. *Respiration of Mycobacteria with various substrates*

Substrate (0.02 M)	Uptake of oxygen (Q_{O_2}) with							
	<i>Myco.</i> <i>phlei</i>	<i>Myco.</i> <i>smegmatis</i>	<i>Myco.</i> <i>stercoris</i>	<i>Myco.</i> sp. <i>Karlinski</i>	<i>Myco.</i> <i>rauae</i>	<i>Myco.</i> sp. <i>leprosus</i> <i>Kedrowsky</i>	<i>Myco.</i> <i>butyricum</i>	<i>Myco.</i> <i>tuberculosis</i> <i>hominis</i> T 1
None	- 7.1	- 16.2	- 12.1	- 18.9	- 11.4	- 7.7	- 9.2	- 2.0
Glucose	- 27.9	- 22.6	- 15.3	- 21.9	- 32.3	- 7.9	- 12.9	- 3.8
Fructose	- 9.9	- 26.6	- 15.5	- 26.3	- 20.1	- 8.5	- 15.5	—
Glycerol	- 8.8	- 27.7	- 23.0	- 35.2	- 20.5	- 10.4	- 27.1	- 3.2
Sodium <i>dl</i> -lactate	- 25.7	- 42.6	- 27.5	- 36.2	- 16.1	- 9.1	- 11.0	- 3.7
Sodium pyruvate	- 11.3	- 31.9	- 24.2	- 34.9	- 35.9	- 9.8	- 15.4	- 2.6
Sodium acetate	- 9.3	- 40.1	- 25.2	- 46.9	- 30.6	- 13.3	- 22.3	- 2.5
Sodium succinate	- 11.2	- 26.6	- 22.3	- 24.7	- 18.1	- 8.0	- 9.3	—
Sodium citrate	- 7.1	- 15.2	- 11.9	- 19.9	- 12.6	- 7.5	- 9.1	- 2.3
<i>dl</i> -Alanine	- 8.7	- 17.4	- 14.1	- 22.6	- 20.8	- 8.1	- 10.3	—

(Medium: 0.066 M phosphate buffer, pH 7.4, at 38°. Gas: oxygen.)

It will be seen that the members of the acid-fast group exhibit considerable differences in their ability to oxidize the same substrate. For example, the respiration rate of *Myco. phlei* is increased three- to fourfold by glucose and lactate, whereas pyruvate, acetate, succinate and glycerol are oxidized slowly; on the other hand, the oxygen consumption of *Myco. ranae* is rapid in presence of pyruvate, acetate and glucose, but much slower in presence of lactate and succinate. *Myco. butyricum* shows preference for glycerol and acetate, whilst *Myco. sp. Karlinski* presents the highest rate with acetate, and distinctly lower—though still considerable—rates with glycerol, lactate and pyruvate.

Growth in synthetic media

Utilizable carbon sources

If the metabolism of the *Mycobacteria* is predominantly aerobic—as generally believed—there should be some relationship between the rate of oxidation of a given substrate and rate of growth in a synthetic medium containing that substrate as the sole source of carbon. In the experiments described in Table 2, the concentration of carbon

Selection of an inoculum suitable for critical experiments

Bacterial suspensions were prepared, by serial dilution, to contain amounts of wet bacilli varying from 10^{-1} to 10^{-8} mg./drop (0.05 ml.). Although bacilli, both clumped and isolated, could be detected microscopically, turbidity was no longer visible in a suspension of 10^{-4} mg./drop. Several tubes of glycerol broth were inoculated with each dilution, 1 drop to 5 ml. of medium. Subsequent growth indicated that suspensions containing less than 10^{-5} mg./0.05 ml. have a random distribution of bacilli, which renders them unsuitable for nutritional experiments [cf. Berg, 1941]. Suspension of 10^{-1} to 10^{-5} mg./drop gave growth in all tubes. With 10^{-1} to 10^{-3} mg./drop, growth appeared on the third day, but with 10^{-4} mg./drop not until the fifth day.

Hence an inoculum of 10^{-2} mg. was selected for most of the work, because it ensured approximately equal seedings and growth in a reasonable time. This inoculum contained about 500,000 bacilli. *Myco. ranae* is exceptional in that it shows less tendency to clump, and so it may be used in higher dilutions.

Growth with small inocula

The results of seeding 20 ml. of synthetic medium, and of glycerinated broth, with relatively small quantities of bacteria are shown in Table 3. When

Table 2. Growth of *Mycobacteria* in synthetic media containing various sources of carbon

Organism	Growth with source of carbon as 1% of						
	Glucose	Fructose	Glycerol	dL-Lactate	Pyruvate	Acetate	Succinate
<i>Myco. phlei</i>	+++	±	±	++++	+++	++	+
<i>Myco. smegmatis</i>	+++	++++	++++	+++	++++	+	+
<i>Myco. stercoris</i>	++	+++	++++	++++	++++	+	±
<i>Myco. sp. Karlinski</i>	+++	+++	++++	++++	++++	++++	++
<i>Myco. ranae</i>	++	++++	+	±	++++	++++	++
<i>Myco. sp. Kedrowsky</i>	±	++	++	±	++++	++	+
<i>Myco. butyricum</i>	+	±	+	±	+	±	±

With citrate (0.5%) as source of carbon, no growth occurred with any organism.

Number of positive signs indicates rate of growth. ± = trace of growth.

(Nitrogen source, $(\text{NH}_4)_2\text{SO}_4$. Time, 7 days. Inoculum, 1 mg. seeded in 5 ml. of medium.)

source was 1% and the inoculum was relatively heavy (1 mg. wet wt. bacilli/tube). Gross differences in rate of growth were assessed by daily inspection. Although the first visible evidence of multiplication was often the appearance of 'bottom granules', the chief mass of growth was confined to the surface.

The results demonstrate that certain species of *Mycobacteria* are able to grow when heavily seeded in synthetic media containing carbonaceous material which they can oxidize: and that, in general, the rate of growth shows some degree of correspondence to the increased oxygen uptake in phosphate buffer (Tables 1, 2). With citrate there was no increased oxygen consumption, and there was no growth in synthetic medium.

no growth occurred in 7 days, the plug was sealed with paraffin wax and incubation continued.

The ability of an inoculum of 0.01 mg. to multiply to considerable proportions in 7 days is illustrated by the figures for glycerol broth. In synthetic media, however, there is no growth if the carbon source is glucose, fructose, glycerol, acetate, succinate or citrate. Lactate and pyruvate are exceptional. *Myco. phlei*, *Myco. smegmatis* and *Myco. Karlinski* grew in both lactate and pyruvate, whilst *Myco. stercoris* produced slight growth in lactate.

Table 3 also shows that an inoculum of 0.01 mg. remains viable after 7 days, and is able to attain weighable proportions with certain sources of carbon. *Myco. smegmatis* reached a growth of 7.5 mg. in succinate after 5 weeks; *Myco. Karlinski*, 15 mg.

Table 3. *Growth of Mycobacteria in synthetic media in presence and absence of citrate*

Organism	Dura- tion (days)	Inoculum (mg.)	Growth (mg. dry wt.) with source of carbon as 1% of												Control glycerol broth 46.0
			Glucose		Fructose		Glycerol		dl-Lactate		Pyruvate		Acetate		Succinate
			Con- trol	Citrate	Con- trol	Citrate	Con- trol	Citrate	Con- trol	Citrate	Con- trol	Citrate	Con- trol	Citrate	
<i>Myc. phlei</i>	7	0.01	Nil	10.1	Nil	Trace	Nil	Nil	15.2	24.2	15.0	20.1	Nil	9.0	Trace
		0.001	—	—	—	—	—	—	14.1	22.0	—	—	—	—	—
		0.0001	—	—	—	—	—	—	2.0	20.7	—	—	—	—	—
	21	0.01	10.2	—	—	—	Trace	—	—	—	—	—	—	—	—
<i>Myc. smegmatis</i>	42	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—
	56	0.01	—	—	—	—	—	—	—	—	—	—	—	—	—
		1.0	—	—	—	—	8.4	—	—	—	—	—	—	—	—
	7	0.01	Nil	14.0	Nil	23.4	Nil	17.0	42.2	44.8	13.3	27.2	Nil	9.8	16.3
<i>Myc. stercoris</i>	35	0.01	—	—	Nil	—	—	—	—	—	—	—	Nil	—	—
	63	0.01	—	—	Nil	—	—	—	—	—	—	—	Nil	—	—
	7	0.01	Nil	13.7	Nil	20.8	Nil	6.2	Trace	28.9	Nil	21.2	Nil	15.3	14.7
	21	0.01	—	—	Nil	—	Nil	—	27.8	—	Nil	—	Nil	—	—
<i>Myc. sp. Karlini</i>	63	0.01	—	—	Nil	—	Nil	—	—	—	Nil	—	Nil	—	—
	7	0.01	Nil	8.7	Nil	9.1	Nil	6.0	3.1	18.9	3.7	16.2	Nil	2.0	4.4
		0.001	Nil	7.4	—	—	—	—	11.3	27.7	Trace	13.2	—	—	—
		0.0001	Nil	4.5	—	—	—	—	—	—	Nil	24.7	—	—	—
<i>Myc. sp. Kedrowsky</i>	42	0.01	—	—	Nil	—	Nil	—	—	—	—	—	15.0	—	—
	63	0.01	—	—	Nil	—	Nil	—	—	—	—	—	—	—	—
	7	0.01	Nil	Trace	Nil	7.0	Nil	2.5	Nil	—	3.9	5.3	Nil	3.5	—
	35	0.01	Nil	—	Nil	—	Nil	—	Nil	—	—	—	Nil	—	—
<i>Myc. butyricum</i>	7	0.01	Nil	Nil	Nil	Nil	Nil	Trace	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	21	0.01	Nil	Nil	Nil	Nil	Nil	—	Nil	Nil	Nil	Nil	Nil	Nil	Nil
<i>Myc. ranae</i>	7	0.0001	Trace	5.1	Nil	Nil	Trace	0.1	Nil	10.1	1.8	9.7	Trace	2.3	Nil
	14	0.0001	—	—	Trace	8.2	—	—	—	—	—	—	—	—	Nil

(Nitrogen source, $(\text{NH}_4)_2\text{SO}_4$. Volume of medium, 20 ml. Conc. of sodium citrate, 0.01 %.)

after 6 weeks in acetate. *Myco. stercoreis*, which showed only a trace of growth in lactate after 1 week, increased to 27.8 mg. in 3 weeks, and *Myco. Kedrowsky* presented a weighable growth in pyruvate after 5 weeks, although no trace was visible at the end of the first week. *Myco. phlei* subsisting on glucose gave somewhat irregular results: in some experiments growth began after 10 days and increased to 10 mg. by the end of the third week, whereas in others no growth was obtained in 9 weeks. None of the organisms studied showed any growth in synthetic medium containing citrate as the sole source of carbon, even when the flasks were incubated for 20 weeks.

Growth usually began as small 'bottom granules' and later extended to cover the surface with a thin film, the thickness of which varied with the organism. In lactate, growth was always confined to the surface, where it began as a thin, veil-like pellicle. *Myco. phlei* presented its normal pigmentation only in the presence of glycerol [see Ingraham & Steenbock, 1935]; with other sources of carbon the pigmentation was faint. *Myco. ranae*, which is emulsified with ease, grew so rapidly at 38° that the inoculum was reduced to 0.0001 mg.

The experiments recorded in Table 3 show that the amount of growth in synthetic media is a function of both time and size of inoculum.

The stimulant effect on growth of the addition of various substances to the medium

The effect of citrate. The effect of adding a small quantity of citrate can be seen in Table 3. Uniform results, quantitatively of the same order, were obtained with five different samples of commercial citric acid, and also with a specimen of synthetic citric acid prepared by the method of Dünschmann [1891]. Controls containing citrate as the sole carbonaceous material in concentrations ranging from 0.0001 to 2.0% have never shown the slightest trace of growth.

Table 3 shows that when the size of the inoculum and the time of the experiment are fixed arbitrarily, a small amount of citrate (0.01%) can be a factor which determines growth in a synthetic medium; and that when some growth occurs in the absence of citrate, it always reaches greater proportions in the presence of citrate. The yields were unaffected by doubling the usual concentrations of the sources of carbon and nitrogen. The effect occurs with all organisms except tubercle bacilli (see later) and *Myco. butyricum*, which begins to grow in the presence of glycerol and citrate, but ceases after a short time.

The citrate effect was examined more extensively, *Myco. phlei* being used as a convenient organism. Table 4 illustrates the influence of concentration.

Table 4. *Growth of Myco. phlei in a synthetic medium containing citrate*

Incubation (days)	Inoculum (mg. wet wt.)	Conc. of citrate (%)	Bacterial growth (mg. dry wt.)
7	0.01	Nil	Nil
		0.1	7.7
		0.01	8.1
		0.001	Nil
		0.0001	Nil
30	0.01	Nil	Nil
		0.001	12.0
7	0.01	Nil	Nil
		0.01	8.1
	0.001	Nil	Nil
		0.01	6.1
7	0.0001	Nil	Nil
		0.01	3.4
	0.1	Nil	6.4
		1.0	8.6
		0.1	16.7
		0.01	13.3
		0.001	9.2

Note. In a series of 10 observations (inoculum 0.01 mg., citrate concentration 0.01%), the mean growth in 7 days was 9.6 mg. (highest value 12.7, lowest 7.7).

(Carbon source, 1% glucose; nitrogen source, $(\text{NH}_4)_2\text{SO}_4$; volume of medium, 20 ml.)

The optimal concentration for a short experiment was of the order 0.1–0.01%; 1% of citrate appears to inhibit the effect. It is noteworthy that an inoculum as small as 10^{-4} mg. can be stimulated to grow in 7 days by the presence of 0.01% of citrate.

The experiments of Table 5 show that the citrate effect continues undiminished through serial subcultures. The organisms were inoculated as a washed suspension prepared in the usual manner. Three flasks were set up at each transfer: a control without citrate, and two containing 0.01% of citrate. One of the resulting cultures was weighed and the other used for the next transfer.

Table 5. *Serial transfer of Myco. phlei in a synthetic medium*

	Growth (mg. dry wt.) with	
	Control (no citrate)	0.01% citrate
Original culture	Nil	8.3
1st subculture	Nil	8.7
2nd "	Nil	8.7
3rd "	Nil	8.0
4th "	Nil	8.5
5th "	Nil	9.5, 9.5 (duplicates)

(Carbon source, 1% glucose; nitrogen source, $(\text{NH}_4)_2\text{SO}_4$. Time of incubation, 7 days; inoculum, 0.01 mg. wet wt.)

The following substances related to or derived from citric acid were entirely devoid of stimulant action, when used in equivalent concentrations ($3.9 \times 10^{-4} M$): Tricarballic acid, aconitic acid (*trans*), trimethyl citrate, citramide, acetyl-citric acid, *dl*-citramalic acid, itaconic acid, citraconic acid, citrazinic acid, citrazinamide, and *iso*-nicotinic acid. The last three were tried because it was

thought possible that ammonia might condense with citric acid to give a pyridine derivative as an intermediary metabolite.

The citric acid (initially 1.49 mg. in 20 ml.) remaining in the medium after 7 days was determined by the method of Pucher, Sherman & Vickery [1936]: about one-half of the citric acid had disappeared.

Table 6. *The stimulant effect of amino-acids on the growth of Mycobacteria in a synthetic medium*

Substance added (0.01 %)	Growth (mg. dry wt.) with					
	<i>Myco.</i> <i>phlei</i>	<i>Myco.</i> <i>smegmatis</i>	<i>Myco.</i> <i>stercoris</i>	<i>Myco.</i> <i>Kedrowsky</i>	<i>Myco.</i> <i>Karlinski</i>	<i>Myco.</i> <i>rauae</i>
(a) Source of carbon, 1 % glucose						
Syn. <i>dl</i> -Alanine	11.8	13.2	8.0	0.5	8.0	Trace
Syn. <i>dl</i> -Valine	10.4	9.1	7.7	—	6.4	Trace
Syn. <i>dl</i> -Leucine	13.6	6.6	5.4	0.7	5.6	Trace
Syn. <i>dl</i> -Norleucine	8.1	6.2	15.7	0.5	5.6	Trace
Syn. <i>dl</i> -Glutamic acid	10.6	31.6	13.9	0.8	7.3	Trace
<i>l</i> (-)-Tryptophan	11.0	8.5	1.6	0.2	10.3	Trace
<i>l</i> (-)-Proline	10.2	2.6	1.2	0.5	9.5	Trace
Syn. β -Alanine	Nil	Nil	Nil	Nil	Nil	Nil
Syn. <i>dl</i> - β -Amino- <i>n</i> -butyric acid	Nil	Nil	Nil	Nil	Nil	Nil
Syn. <i>dl</i> - δ -Amino- <i>n</i> -valeric acid	Nil	Nil	Nil	Nil	Nil	Nil
None	Nil	Nil	Nil	Nil	Nil	Nil
(b) Source of carbon, 1 % glycerol						
Syn. <i>dl</i> -Alanine	0.5	17.2	—	—	—	—
Syn. <i>dl</i> -Glutamic acid	1.0	13.5	6.3	—	—	—
Syn. <i>dl</i> -Leucine	0.1	—	—	—	—	—
<i>l</i> (-)-Proline	—	15.4	—	—	—	—
None	Nil	Nil	Nil	—	—	—

Note. (1) Controls in which the source of carbon was omitted showed no growth. (2) The prefix 'syn.' means 'synthetic'. (Main nitrogen source, $(\text{NH}_4)_2\text{SO}_4$. Time of incubation, 7 days. Inoculum, 0.01 mg. to 20 ml. of medium in all cases except *Myco. ranae*, with which the inoculum was reduced to 0.00001 mg.)

Table 7. *The stimulant effect of amino-acids on the growth of Myco. phlei*

Addition to medium		Growth (mg. dry wt.) with source of carbon as 1 % of		Addition to medium		Growth (mg. dry wt.) with source of carbon as 1 % of	
		Glucose	Suc- cinate			Glucose	Suc- cinate
Substance	Conc. (%)			Substance	Conc. (%)		
None	—	Nil	Nil	Syn. <i>dl</i> -Lysine	0.01	12.0	—
Syn. <i>dl</i> -Alanine	0.02	11.8	1.7	Syn. <i>dl</i> -Aspartic acid	0.01	11.6	1.9
<i>l</i> (+)-Alanine	0.01	11.5	—	<i>l</i> (-)-Asparagine	0.01	11.7	—
Syn. β -Alanine	0.01	Nil	Nil	Syn. <i>dl</i> -Glutamic acid	0.01	10.6	2.4
Syn. <i>dl</i> -Serine	0.01	11.2	—	<i>l</i> (+)-Glutamine	0.01	7.1	—
<i>l</i> (-)-Cystine	0.01	2.8	—	Syn. <i>dl</i> - α -Amino- <i>n</i> -caprylic acid	0.01	10.5	Trace
Syn. <i>dl</i> -Phenylalanine	0.02	10.3	—	<i>l</i> (+)-Arginine	0.01	7.3	—
<i>l</i> (-)-Phenylalanine	0.01	9.2	—	<i>l</i> (+)-Ornithine	0.01	3.5	—
<i>d</i> (+)-Phenylalanine	0.01	9.2	—	<i>l</i> (-)-Histidine	0.01	9.8	—
<i>l</i> (-)-Tyrosine	0.01	8.9	—	<i>l</i> (-)-Proline	0.01	10.2	Trace
Syn. <i>dl</i> - α -Amino- <i>n</i> -butyric acid	0.01	7.4	—	<i>l</i> (-)-Hydroxy-proline	0.01	9.1	—
Syn. <i>dl</i> -Threonine	0.01	Trace	—	Glycine	0.01	9.7	—
Syn. <i>dl</i> -Methionine	0.01	9.1	—	Glycyl-glycine	0.01	9.2	—
Syn. <i>dl</i> -Leucine	0.01	13.6	Trace	Glycyl- <i>l</i> (-)-leucine	0.01	3.6	—
Syn. <i>dl</i> -Isoleucine	0.01	7.0	—	<i>l</i> (-)-Leucyl-glycine	0.01	15.0	—
				Glutathione	0.01	8.6	—
				Casein	0.01	7.5	—
				Peptone (Witte)	0.01	10.3	—

Note. Controls in which the carbon source was omitted gave no growth.

(Main source of nitrogen, $(\text{NH}_4)_2\text{SO}_4$. Time of incubation, 7 days. Inoculum, 0.01 mg. to 20 ml. of medium.)

The effect of amino-acids, peptides and proteins. An effect similar to that of citrate is produced by small concentrations of amino-acids, dipeptides and proteins. The results are given in Tables 6 and 7.

Although amino-acids are not essential nutrients, growth is promoted by α -amino-acids, which are constituents of protein, and in which the α -amino group is free or bound in peptide linkage. The active agent appears to be either the whole amino-acid molecule, or the part of it represented by the grouping $=C(NH_2)-COOH$, since this is the only structure common to the 23 amino-acids which induced growth. There is no significant difference between the activity of those stereo-isomers that could be tested; the effect varies quantitatively, and is least with *dl*-threonine. Proline and hydroxyproline act like the α -amino-acids. Since these are protein constituents and probably convertible into α -amino-acids during metabolism, their stimulant action is not regarded as exceptional. No special importance is attached to the effect of crude proteins (peptone and casein); or to that of glutathione, since spontaneous decomposition into cysteinylglycine and pyrrolidone carboxylic acid may occur during 7 days' incubation at 38°.

When the free amino group is blocked by acetylation or benzylation, conjugated with cholic acid, or otherwise substituted, the stimulant action is lost. β -Amino-acids, and others in which the amino group is displaced still farther from the carboxyl group, are also inactive.

Myc. phlei was tested more thoroughly than the other organisms. The following additional substances (concentration 0.01 %) failed to stimulate the growth of *Myc. phlei* subsisting on glucose: (1) sarcosine, 2,5-di-keto-piperazine, *N*-benzoylglycine, glycocholic acid, *N*-acetyl-alanine, *N*-benzoyl-alanine, *N*-phenyl-glycine, α -amino-phenylacetic acid, *dl*- ϵ -amino-caproic acid, *dl*- α -amino-*iso*-butyric acid, *dl*- α -amino- α -methyl-*n*-butyric acid; (2) urea, guanidine, creatine, taurine, glucosamine, and succinimide.

The effect of histamine. When a number of amines, amides and heterocyclic bases was tested, the only one that proved to be active was histamine. The magnitude of the effect is shown in Table 8.

The following were negative: (1) tyramine, tryptamine, putrescine, betaine, choline, ethanolamine, ethylamine, propylamine, *n*-butylamine, and *sec*-octylamine; (2) acetamide, propionamide, *n*-butyramide, *n*-caproamide, and malonamide; (3) pyrrole, pyridine, pyrrolidine and piperidine. The concentration in each case was 0.01 %.

The effect of other organic substances. Many other organic substances were tested, but all were devoid of stimulant properties in so far as they produced no growth in 7 days. The organism used was *Myc. phlei*, and 0.01 mg. was seeded in 20 ml. of synthetic

Table 8. *Stimulation of the growth of Myco. phlei by histamine*

Addition to medium		Bacterial growth (mg. dry wt.)
Substance	Conc. (%)	
None	—	Nil
Histamine hydrochloride, synthetic (Eastman Kodak Co. Ltd.)	0.01	9.2
Histamine hydrochloride (British Drug Houses, Ltd.)	0.01	9.0
Histamine phosphate (British Drug Houses, Ltd.)	0.01	9.1
Sodium citrate	0.01	10.4

(Synthetic medium, volume 20 ml. Carbon source, 1 % glucose. Nitrogen source, $(NH_4)_2SO_4$. Inoculum, 0.01 mg. Incubation, 7 days.)

medium containing 1 % of glucose. Although it is unnecessary to list the whole series as negative, the fact that the following were ineffectual is recorded:

(1) Normal saturated fatty acids from C_1 to C_{18} , crotonic and oleic acids, β -hydroxy-*n*-butyric acid, *iso*-butyric and α -hydroxy-*iso*-butyric acids, normal chain dicarboxylic acids from C_2 to C_8 , and hydroxy-malonic acid (concentrations, 0.01 %).

(2) Fructose monophosphate, arabinose, *d*-ribose, inositol, sorbitol, glycerol, ethylene glycol, ethanol, methanol, dihydroxy-acetone, gluconic, glyceric, lactic, pyruvic, *l*-malic, fumaric, *d*-tartaric and α -keto-glutaric acids (concentrations, 0.01 %).

(3) Uracil, adenine, adenosine, guanine, hypoxanthine, allantoin, alloxan, and hydantoin (concentrations, 0.01 %).

(4) Aneurin (100 μ g.), riboflavin (50 μ g.), nicotinic acid (100 μ g.), phthiocol (20 μ g.), pyridoxin (50 μ g.), pantothenic acid (100 μ g.), pyocyanine (500 μ g.), cytochrome *c* (100 μ g.), haemin (1 mg.), 2-methyl-1:4-naphthoquinone (saturated), ascorbic acid, indole acetic acid, β -indole propionic acid, α -naphthalene acetic acid, *p*-aminobenzoic acid and nicotinamide (all 2 mg.). The quantities in brackets are the amounts present in 20 ml. of medium.

The effect of calcium and iron. It has been shown that certain *Mycobacteria* will grow in a synthetic medium containing an oxidizable source of carbon if the seeding is sufficiently large and the incubation time sufficiently long; and that the lag period can be shortened, and growth enhanced, by addition of citrate, α -amino-acids and histamine, in low concentrations. The basis of the synthetic medium is a neutral solution containing NH_4^+ , K^+ , Na^+ , Mg^{++} , SO_4^- , PO_4^- , Cl^- , HCO_3^- and CO_3^- . Since traces of calcium and iron are present as impurities in the reagents, the question arose as to whether the amounts of these, and possibly of other elements, were optimal for growth.

Hay ash, rich in Ca^{++} and Fe^{+++} , was prepared and used according to Fildes & Richardson [1935], the result being a considerable stimulation of growth. The separate effects of Ca and Fe were

also investigated: the addition of either in low concentration was followed by growth, the character of which depended on the element. Results with *Myco. phlei* as test organism are given in Table 9. Ca was added as chloride, Fe as ferrous sulphate or ferric chloride, the solutions being added aseptically to the final medium. Ca was also added as CaCO_3 , before autoclaving, in quantity just sufficient to saturate the basal salt mixture.

In the presence of Fe, initially Fe^{++} or Fe^{+++} , *Myco. phlei* formed a pigmented granular bottom growth, later extending to the surface as a thin yellow film; but in the presence of Ca the bottom growth developed as a slimy, whitish deposit and the surface pellicle was white. The effective concentrations are given in Table 9, which also permits a comparison with the effects of citrate and hay ash, and shows that when Mg^{++} is not added to the basal salt mixture, the stimulant effect of citrate can still be evoked. Increasing the concentration of Mg^{++} to the point of precipitating MgNH_4PO_4 does not stimulate growth.

Table 9. *Effect of inorganic substances on the growth of Myco. phlei*

Addition to medium		
Material	Amount added to 20 ml. of medium	Bacterial growth (mg. dry wt.)
None	—	Nil
Sodium citrate	2 mg.	12.6
Hay ash	Equiv. to 1 mg. ash	7.8
Fe^{+++}	75 μg .	7.5
	50 μg .	1.8
	20 μg .	0.1
	10 μg .	Nil
	50 μg .	1.5
Fe^{++}	50 μg .	1.5
Ca^{++}	100 μg .	7.6
	50 μg .	3.0
	20 μg .	Nil
CaCO_3	Saturated	7.0
Mg^{++}	50 μg .	Nil
Sodium citrate (Mg^{++} not added to basal mixture)	2 mg.	9.7

(Carbon source, 1% glucose. Nitrogen source, $(\text{NH}_4)_2\text{SO}_4$. Inoculum, 0.01 mg. Time of incubation, 7 days.)

A control test using the ash of analytical sodium citrate gave negative results. Other ions were tested with negative results: Ni^{++} , Co^{++} , Mn^{++} , Cu^{++} , Cd^{++} , Zn^{++} , Sn^{++} , Al^{+++} , Ba^{++} , La^{+++} , Ce^{++++} , Th^{++++} , Tl^+ , Br^- , I^- , all 50 μg /flask. Sodium silicate (2 mg.) also gave negative results.

Earlier, anomalous results were encountered in testing some possible growth stimulants, which were dissolved in the basal salt mixture and filtered through a Seitz pad. Growth occurred, although it was unexpected in view of other general findings.

When the experiments were repeated by adding the unsterilized materials to autoclaved salt mixture, there was no growth. Attention was thus directed to the Seitz filtrate, which was slightly turbid and gave a test with ammonium oxalate indicating the presence of about 100 μg . of Ca^{++} /20 ml.; the Prussian blue test for Fe^{+++} [Feigl, 1937] revealed about 1–10 μg . of Fe^{+++} /20 ml., but the α - α' -dipyridyl test for Fe^{++} [Feigl, 1937] was negative. Subsequently it was discovered that both water and saline could extract detectable quantities of Ca^{++} and Fe^{+++} from filter pads in a single filtration. These findings are described elsewhere [Hunter, 1943], and it will suffice to state that they do not invalidate any observations recorded in this communication. The aseptic addition of a small volume of filtered solution (up to at least 2 ml.) to a synthetic medium, the final volume of which is 20 ml., does not convey sufficient Ca or Fe to affect the growth of *Mycobacteria* under the conditions we have employed.

Preliminary observations on the growth of tubercle bacilli and Myco. butyricum, no. 337

In studying the growth of tubercle bacilli (strains T₁ and H 37) in synthetic media we have found that small inocula do not propagate during an incubation period of 6 weeks. It was necessary to use either 5 mg. of washed suspension or a washed pellicle large enough to cover a 2 mm. loop. Mother cultures were 3 weeks old. Growth was examined in media containing 1% of one of glycerol, glucose or *D*-lactate, with and without addition of hay ash, citrate and amino-acids, separately and together. The surface of glycerol-broth medium in the control tubes was covered within 3 weeks, but growth was poor in the synthetic media, amounting to no more than a thickening of the inoculum or a slight patchy extension. Growth could be stimulated to normal dimensions by addition of small quantities of Marmite or muscle *Kochsaft*.

It has been seen that a small inoculum of *Myco. butyricum* grows poorly in the synthetic medium. Larger inocula in the form of washed surface pellicles also failed to exhibit extension in a glycerol medium to which hay ash, citrate, and amino-acid mixtures had been added. Again muscle *Kochsaft* and Marmite stimulated rapid growth approaching that of broth controls.

These observations suggest that the tubercle bacillus and *Myco. butyricum* (no. 337) have a more exacting nutrition than the other organisms studied.

DISCUSSION

When certain species of *Mycobacteria* are lightly seeded in a synthetic medium under the conditions described in this paper, it is possible to demonstrate

the effectiveness of growth stimulants which operate in concentrations of the order $1 \times 10^{-3} M$ or lower. The active substances belong to four apparently independent classes: (1) citric acid, (2) α -amino-acids, (3) histamine, (4) ions of calcium and iron. They are termed 'growth stimulants' because they do not conform to the definition of essential growth factors [Fildes & Richardson, 1935].

Many investigators have found that citric acid is an advantageous constituent of media specially designed for cultivation of tubercle bacilli. Under the conditions of our experiments low concentrations of citric acid do not stimulate the growth of tubercle bacilli, but this substance effectively reduces the lag period and promotes the growth of acid-fast saprophytes. Experiments with *Myc. phlei* show that citric acid is not oxidized as a source of energy; nor does it catalyze the oxidation of carbohydrate after the manner of the citric acid cycle [Krebs & Johnson, 1937], since intermediates of the cycle—such as α -keto-glutaric, succinic, fumaric and *l*-malic acids—do not stimulate growth.

Although citric acid is not oxidized, it disappears from the medium. It is possible that the organisms normally synthesize small amounts of citric acid from a variety of sources of carbon during the lag period. If this were so, the stimulant effect of citric acid would be explained.

The stimulant properties of amino-acids have been observed with other bacterial families, e.g. *Esch. coli* [Sahyun, Beard, Schultz, Snow & Cross, 1936] and propionic acid bacteria [Wood, Anderson & Werkman, 1938]. The possibility that citric acid is concerned in the synthesis of amino-acids was

considered, but no evidence to support the suggestion was obtained.

Sauton [1912] and Long & Seibert [1926] found that Fe enhances the growth of tubercle bacilli. Our experiments show that the growth of acid-fast saprophytes can be stimulated by very low concentrations of Ca and Fe; but in the absence of added Ca^{++} , Mg^{++} or Fe^{+++} growth still occurs if citrate be present, showing that if any, or all, of these ions be essential, sufficient is provided by the impurities of analytical reagents.

It must be emphasized that the *Mycobacteria* used in this work have been cultivated on laboratory media for years and have had ample opportunity to adapt themselves to artificial conditions. Although the stock cultures have been kept on a rich medium, it is possible that freshly isolated organisms would have more exacting nutritional demands.

SUMMARY

1. The respiration of certain species of *Mycobacteria* has been determined in presence and absence of substrates. Species vary widely with respect to the respiration of 'resting' cells and to oxygen consumption in the presence of different substrates.

2. Growth of small inocula in a synthetic medium has been studied. It was found that under defined conditions the growth of some species is stimulated by citric acid, α -amino-acids, histamine, calcium and iron.

We wish to thank Prof. C. E. Hercus and the W. H. Travis Trustees for their continued interest in this work; and we are indebted to Dr H. A. Krebs for correcting the proofs.

REFERENCES

- Berg, W. N. [1941]. *Amer. Rev. Tuberc.* **43**, 685.
 Calmette, A. [1923]. *Tubercle Bacillus Infection and Tuberculosis in Man and Animals*, Chap. II. Baltimore: Williams and Wilkie Co.
 Dünschmann, M. [1891]. *Liebigs Ann.* **261**, 162.
 Feigl, F. [1937]. *Qualitative Analysis by Spot Tests*. New York: Nordemann Publishing Co.
 Fildes, P. & Richardson, G. M. [1935]. *Brit. J. exp. Path.* **16**, 326.
 Hunter, G. J. E. [1943]. *Biochem. J.* **37**, 577.
 Ingraham, M. A. & Steenbock, H. [1935]. *Biochem. J.* **29**, 2553.
 Kendall, A. I., Day, A. A. & Walker, A. W. [1914]. *J. infect. Dis.* **15**, 417.
 Knight, B. C. J. G. [1936]. *Spec. Rep. Ser. Med. Res. Coun. Lond.*, no. 210, p. 83 *et seq.*
 — & Fildes, P. [1933]. *Brit. J. exp. Path.* **14**, 112.
 Krebs, H. A. [1933]. *Hoppe-Seyl. Z.* **217**, 191.
 Krebs, H. A. & Johnson, W. A. [1937]. *Enzymologia*, **4**, 148.
 Langner, P. H. & Forrester, J. S. [1939]. *J. Immunol.* **37**, 133.
 Long, E. R. [1921–2]. *Amer. Rev. Tuberc.* **5**, 857.
 — [1923]. *The Chemistry of Tuberculosis*, Section I, Chap. II. Wells, G., deWitt, L. M. and Long, E. R. Baltimore: Williams and Wilkie Co.
 — & Seibert, F. B. [1926]. *Amer. Rev. Tuberc.* **13**, 393.
 Merrill, H. M. [1931]. *J. Bact.* **21**, 361.
 Pucher, G. W., Sherman, C. C. & Vickery, H. B. [1936]. *J. biol. Chem.* **113**, 235.
 Sahyun, M., Beard, P., Schultz, E. W., Snow, J. & Cross, E. [1936]. *J. infect. Dis.* **58**, 28.
 Sauton, B. [1912]. *C.R. Acad. Sci., Paris*, **155**, 1860.
 Uyei, N. [1927]. *J. infect. Dis.* **40**, 425, 433.
 Wood, H. G., Anderson, A. A. & Werkman, C. H. [1938]. *J. Bact.* **36**, 201.