Growth of a *Leptothrix* Strain in a Chemically Defined Medium

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(Received 6 March 1964)

SUMMARY

A *Leptothrix* strain was found to be a motile aerobe which did not ferment sugars but utilized some of them for growth. It required cystine and a source of molybdenum or vanadium, and preferred ornithine or arginine as its N-source. Liquid media of defined chemical composition have been developed for its cultivation, and growth characteristics in these media determined.

INTRODUCTION

Little is known about the metabolism of the organisms of the genus *Leptothrix* (Bergey, 1957). The object of the present work was to develop a chemically defined medium for the growth of one of these organisms in preparation for a study of its metabolism.

METHODS

The strain chosen was obtained through the courtesy of Professor B. W. Lacey (Westminster Hospital School of Medicine), who isolated it in 1947 and classified it as a *Leptothrix*; it probably originated as an airborne contaminant (private communication). Since it grew moderately well at 25°, without the long delay caused by higher temperatures, on the surface of nutrient agar slopes buffered to pH 7.0, this temperature was used throughout the investigation. All media were prepared from the best available quality of chemicals in demineralized water of minimum resistance 2×10^6 ohm/cm. Exploratory experiments in liquid cultures were made in 6 in. $\times$ $\frac{5}{8}$ in. Pyrex tubes and the amount of growth estimated by an 'EEL' nephelometer calibrated to give results in equiv. mg. dry-wt. organism/ml. More exact results were obtained when necessary by measurements of the absorption of light (583 m μ) by killed samples from 50 ml. cultures in 7 in. $\times$ 1 in. Pyrex tubes, in a 'Unicam 500' spectrophotometer calibrated to give results in equiv. mg. dry-wt. organism/ml. Filtered and sterile air (Morrison, Griffiths & Harris, 1955) passed through a glass tube drawn out at the end of a fine capillary was used to aerate liquid media.

RESULTS

Characteristics of the organism

On the surface of nutrient agar (Oxoid containing KH_2PO_4 , 5.4 g./l., adjusted to pH 7.0 with NaOH) the initial smooth round colonies gave way later to a spreading growth typical of a motile organism; motility was observed in a hanging drop. In liquid peptone media (various) the organism grew only on the surface with the

formation of a pellicle. When the liquid peptone media were aerated there was a small amount of growth distributed evenly throughout the culture which was not increased by repeated subcultivation. The peptone cultures contained long slender threads and short rod-like forms, the former predominating in older cultures and the latter in younger ones. The Gram-staining technique gave variable results. Polychrome methylene blue stained the sheaths of filaments, the organisms within the sheaths and the individual shorter rods; the stained organisms exhibited a characteristic granular appearance and had parallel sides with characteristic oval or spherical terminal bodies which stained more strongly and there seemed to be protoplasmic 'bridges' between organisms in a sheath. The presence of granules or spores was not shown by Neisser's or Moeller's methods; the organism was not acid-fast according to the Ziehl-Neelsen method (Fairbrother, 1953).

When subjected to the usual biochemical tests by the media and methods recommended by the Oxoid Division of Oxo Ltd., and/or by Difco Laboratories, the organism (within 6 weeks) did not produce gas from any of the test sugars. It produced acid only from glucose, sucrose, lactose and dulcitol. It did not produce indole from tryptophan, hydrogen sulphide from sulphur-containing amino acids, acetylmethylcarbinol from glucose, nor did it grow on, or produce alkali from, media containing ammonium citrate as the sole carbon and energy source. It possessed a urease and a catalase. The production of acid from glucose, sucrose, lactose and dulcitol was confirmed in the conventional Warburg apparatus, which showed also a simultaneous uptake of oxygen.

The organism did not grow autotrophically on the solid or liquid media based on manganese salts used for *Leptothrix ochracea* (Lieske, 1919), in the iron bicarbonate liquid medium used for *Spirophyllum ferrugineum* (Lieske, 1911), nor did it grow heterotrophically in the medium formulated for the filamentous sheathed iron bacterium *Sphaerotilus natans* (Stokes, 1954); the sources of carbon tested were sucrose, glucose, lactose, acetate, succinate, malate, fumarate, citrate, pyruvate. It produced thin spreading colonies which developed brown edges on Mn-agar (Mulder, 1964).

Simplification of the composition of semi-solid media

During the early work excellent growths were obtained on the surface of 'S.I.M. medium' (Difco) which contains (% w/v): 0.8 Difco Beef Extract, 0.02 Peptonized Iron, 3.0 peptone, 0.0025 sodium thiosulphate and 1.5 agar. The addition of 0.5 % (w/v) Difco yeast extract to this medium yielded luxuriant growth. The separate ingredients of this reinforced medium were tested individually and in all mixtures (the sample of peptonized iron was obtained through the courtesy of Difco Laboratories). Thiosulphate had no effect on growth, the peptonized iron alone yielded poor growth; but beef extract + peptone + peptonized iron + yeast extract were needed together to obtain luxuriant growth. Cultivation of the organism on slopes containing progressively less of the beef extract, until this was eliminated, yielded a substrain (I) which grew abundantly on peptone + peptonized iron + yeast extract. A similar procedure eliminated the peptonized iron to produce substrain II which grew abundantly on peptone + yeast extract. The yeast extract was similarly eliminated to yield substrain III which grew well on semi-solid peptone agar; the amount of growth declined when the concentration of peptone was less than 1 % (w/v); nine different peptones were equally effective.

Growth in liquid media

Substrain II grew in aerated liquid media containing (g./l.): 5.4 KH_2PO_4 ; 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 30.0 peptone; 5.0 yeast extract; adjusted to pH 7.0. Substrain III grew in a corresponding liquid medium from which the yeast extract had been omitted. Both strains were cultured in liquid media in which casein hydrolysate (prepared in the laboratory by Dr D. E. Griffiths and containing only amino acids) progressively replaced the peptone. Substrain II yielded substrain IV_A which grew readily in the aerated buffered solution of casein hydrolysate + yeast extract. Substrain III did not grow when the concentration of casein hydrolysate exceeded the equivalent of one-third of the peptone; only when yeast extract was re-introduced was it possible to obtain substrain IV_B which grew readily on the aerated buffered solution of casein hydrolysate + yeast extract. Substrains IV_A and IV_B were indistinguishable. Substrain IV grew abundantly in aerated media with casein hydrolysate + yeast extract, moderately in media with peptone or yeast extract alone, and not at all in media with only casein hydrolysate. Liver extract could replace the yeast extract but gave dark-coloured media. Thus substrain IV required a factor or factors provided by yeast extract, liver extract or peptone, but growth on one of these alone was limited by a shortage of amino acid present in the casein hydrolysate. The substrain which was used in the subsequent work can be preserved by cultivation on media containing (g./l.): 5.4 KH_2PO_4 ; 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 10.0 casein hydrolysate; 5.0 yeast extract; 1.5 agar; adjusted to pH 7.0 with NaOH.

Table 1. *Amount of growth of Leptothrix at 25° and composition of the medium*

Concentration of casein hydrolysate (%, w/v)	Concentration of yeast extract (%, w/v)		
	0.1	0.2	0.5
	Growth equiv. (mg. dry-wt. organism/ml.)		
No glucose			
0.00	0.14–0.16	0.26–0.29	0.55–0.60
0.50	0.38–0.42	0.54–0.58	0.67–0.74
1.00	0.62–0.68	0.73–0.80	0.89–0.99
1.2 % (w/v) glucose			
0.00	0.23–0.26	0.55–0.60	0.63–0.70
0.50	0.40–0.44	0.71–0.79	0.83–0.92
1.00	0.70–0.78	0.92–1.01	1.06–1.16

Medium also contained (g./l.): 5.4 KH_2PO_4 ; 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; adjusted to pH 7.0.

The amount of growth in aerated liquid media with yeast extract alone was almost linearly dependent on the concentration of the yeast extract and reached the equivalent of 0.55–0.60 mg. dry-wt. organism/ml. at 0.5 % (w/v) yeast extract. The addition of casein hydrolysate, or of glucose, increased the response of growth to concentration of yeast extract. The two effects were additive (see Table 1). Other additions of carbon and energy sources had a similar effect to that of glucose in media containing 0.5 % (w/v) casein hydrolysate: 0.5 % (w/v) acetate or glutamate was as effective as 1.2 % (w/v) glucose, 0.5 % (w/v) aspartate was 70 % as effective, and

various sugars at 1.2 % (w/v) and dicarboxylic acids at 0.5 % (w/v) are from 15 to 50 % as effective.

Attempts were made to replace the factor(s) provided by yeast extract, but without success. These tests included single peptides, partially hydrolysed casein, acetylated amino acids, NAD, NADP, purines, pyrimidines and the vitamins known to be present in yeast extract. The material required is unusual and fractionation of the yeast extract was needed.

Fractionation of yeast extract

Colouring matter was adsorbed from aqueous solutions of yeast extract by dry alumina (activated by heating to 150° for 2 hr), exhaustively dried silica and activated charcoal. The charcoal adsorbed also amino acids and the material required particularly efficiently from cold solutions at pH 3.5–4.5, but subsequent preferential elution of the active material was not obtained. The cation exchange resins Amberlite IR120 and IRC50 in the hydrogen form retained the active material with some amino acids; the more strongly acidic IR 120 (H) being the more efficient. The impure active material was recovered from resin IR 120 by elution with *m*-ammonia solution. The amount of amino acid present in this eluate was decreased either by a long period of dialysis or by acetylation of the amino acids (Cheronis, 1954) and a second retention of the active material on IR 120 (H), but acetylation caused some loss of activity and both procedures were too cumbersome for routine use.

Unlike non-polar solvents the partially water-miscible alcohols extracted all the active material together with some colouring matter and some amino acids from aqueous solutions of yeast extract. The extraction was most efficient from slightly acidic cold solutions. The pronounced tendency for emulsion formation and the need for large volumes of alcohol were overcome by slow extraction in the apparatus devised by Kutscher & Steudel (Umbreit, Burris & Stauffer, 1951); modified by the insertion of an extra condenser to ensure that the extracting alcohol was cold, and rates of flow adjusted so that emulsion was not returned to the boiler. The active material in 100 g. yeast extract was removed by continuous extraction in this way for 16 days. The extract in *n*-butanol stored overnight at 0° deposited a light brown powder which contained three-quarters of the active material but the remainder could be recovered only as a syrup which was difficult to fractionate further. The light brown powder dissolved readily in water. When ice-cold acetone was added slowly to an ice-cold 10 % (w/v) aqueous solution adjusted to pH 2 with nitric acid, a buff-coloured precipitate containing nucleotides (Le Page & Mueller, 1949) formed which was removed by a no. 3 sintered-glass filter. The washed and dried precipitate, which accounts for 20–24 % by weight of the material extracted by *n*-butanol, had no growth-promoting properties. The supernatant liquor adjusted to pH 7 and evaporated *in vacuo* yielded a yellowish active powder. A concentrated aqueous solution of this powder yielded a further inactive precipitate when diluted slowly with absolute ethanol until the solution contained 95 % (v/v) ethanol. Evaporation of the supernatant liquor *in vacuo* yielded an active pale orange powder, which accounted for about 6 % of the original weight of yeast extract. Chromatographic analysis showed the presence of norvaline, norleucine, alanine, serine, together with traces of other amino acids. This fraction was not an adequate source of nitrogen-

containing nutrients for the organism. The fraction was soluble in *n*-butanol and in ethanol and dry activated alumina removed the active material from solutions in these solvents, but none of the active material was recovered when the extraction was from *n*-butanol and only traces were recovered when it was from ethanol. This refined butanol-extracted material was a satisfactory source of the material needed to permit an investigation of the amino acid requirements of the *Leptothrix* used. This investigation, reported in detail below, showed that L-arginine and DL-cystine are necessary nutrients, and a new basal medium containing (g./l.): 12 glucose; 1 L-arginine hydrochloride; 0.1 DL-cystine; 5.4 KH_2PO_4 ; 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; adjusted to pH 7.0, was used for testing further fractions of the yeast extract for the active material.

Molecules of *M* value less than 3500–4000 can enter the particles of the dextran Sephadex G 25 (Pharmacia, Uppsala, Sweden) swollen with water. When an aqueous solution is allowed to percolate into the gel, molecules larger than *M* 3500–4000 remain in the void volume of the gel and are removed immediately by washing. The molecules which have penetrated the gel particles are eluted in fractions according to the ease with which they pass in and out of the particles. The concentration of solution that can be used is limited solely by viscosity. In separate experiments 40 g. Difco yeast extract in 200 ml. of (a) demineralized water, (b) demineralized

Table 2. *Fractionation of yeast extract by elution from Sephadex G-25*

Solvent	Volume of eluate before the arrival of active material including void volume (ml.)	Active eluate	
		Volume (ml.)	% of original yeast-extract
Water	570	380	3.8–4.2
Dilute aqueous NH_3	570	380	14–16
Dilute aqueous HCl	495	250	17
5 % (v/v) aqueous ethanol	495	250	21

water adjusted to pH 9.0 with ammonia, (c) demineralized water adjusted to pH 3.0 with acetic acid, (d) 5 % (v/v) ethanol in demineralized water, were allowed to enter a column $1\frac{1}{2}$ in. in diameter and 19 in. long containing 50 g. (dry) Sephadex G 25 swollen with the solvent. The gels were eluted slowly with solvents of the same composition, the void volumes (294 ml.) being collected as an initial fraction, whereas the subsequent eluates were collected in 5 ml. fractions automatically. The fractions containing active material were located by testing whether 0.1 ml. added to 10 ml. of basal medium permitted growth of the organism. The active fractions were combined, evaporated to dryness and the weights of solid material determined (Table 2). The distribution of the active material in the fractions when water alone was used showed an early and late peak of concentration suggesting that the material occurred in more than one form.

Although water was the most efficient solvent, in practice the gel became pocketed with bubbles of CO_2 and its surface grew moulds. Consequently in routine productions dilute aqueous ammonia was used since this left the column ready for use again after the elution. The active material present in 40 g. yeast extract was recovered in 5.6–6.4 g. of dry powder in 49 hr but it was mixed with amino acids

which were nutrients of the *Leptothrix*. This powder is referred to subsequently as material I.

The active material and much of the colouring matter in material I was adsorbed on DEAE Sephadex A50 fine-grade dextran when 2 g. of material I dissolved in 10 ml. of water percolated through a column of 5 g. (dry wt.) of this dextran swollen with water. This dextran is an anion exchanger in the chloride form with a capacity of 3–4 m-equiv./g. (dry wt.). Elution with water removed the active material in a 20 ml. fraction which yielded 0.46 g. of almost colourless solid when evaporated *in vacuo* (material II).

Alternatively, material I was acetylated in 6 g. portions (Cheronis, 1954). The active material was retained by a column of 40–50 ml. of Amberlite CG120 (H) 16–50 mesh (twice the amount needed to retain the sodium ions present) from the acetylated product dissolved in 40 ml. water, and recovered by elution with aqueous ammonia (M). After a repetition of the process, the product (0.7 g. (dry wt.)) contained active material but did not give a positive test with ninhydrin. This material dissolved in 5 ml. water was allowed to percolate into a column of 5 g. DEAE Sephadex A25 dextran (medium grade corresponding to A50) swollen with water. The remaining colouring matter was retained strongly at the top of the column and on elution with water, after the void volume had been displaced, the active material was recovered in 25–30 ml. solution. Vacuum distillation and exhaustive drying over anhydrous CaCl_2 yielded 0.03 g. of a faintly yellow solid which was strongly hygroscopic and dissolved readily in water but not in absolute ethanol. It was obtained as a fine powder by precipitation from aqueous solution by ethanol, recovery being complete when the liquid contained 90 % (v/v) ethanol. One mg. of this material (material III) permitted growth in 30 l. of basal medium to a population equiv. 0.5 mg. dry-wt. organism/ml.

Analysis of the isolated material III

When the isolated material III was heated to dryness with concentrated nitric acid a white crystalline powder was obtained which amounted to 48.88 % of the original weight. Analysis of an aqueous solution of this powder by standard procedures (Morrison, 1961) isolated appreciable quantities of molybdenum, which according to the *Biochemist's Handbook* (1961a) accounts for $5\text{--}9 \times 10^{-6}$ % of the dry weight of baker's yeast and $9\text{--}13 \times 10^{-5}$ % of brewer's yeast. Microtitration with lead nitrate, with diphenylcarbazine as indicator (Evans, 1939), established that the ash contained 16.22 % (w/w) Mo. corresponding to 7.93 % (w/w) Mo in material III. The content of Mo in material I was 2.31 % (w/w).

The amino acid requirements of the Leptothrix

The organism was grown aerobically in a basal medium (containing (g./l.): 5 KH_2PO_4 ; 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 12 glucose; 5 material I; adjusted to pH 7.0), basal medium + 0.1 % (w/v) nitrogenous material supplements (casein hydrolysate and individual amino acids). The sizes of the populations produced are compared in Table 3. Only DL-cystine had an effect comparable to that of casein hydrolysate, and only DL-cysteine was inhibitory. With the latter a few cultures showed a slight deposit after 800 min. (the usual period of lag) and then a slow growth after 26–33 hr to a population equiv. 0.31–0.36 mg. dry-wt. organism/ml. When the tests

were repeated at 0.5% (w/v) of supplement, L-arginine gave a maximum growth equiv. 0.25 mg. dry-wt. organism/ml. The effects of lower concentrations of DL-cystine are shown in Table 4. DL-Cystine at 0.0005–0.001% (w/v) was required for maximal growth and when its concentration was below 0.0005% (w/v) the rate of growth decreased.

Table 3. *Effect of casein hydrolysate and single amino-acids on the growth of Leptothrix at 25°*

Medium	Maximum growth equiv. mg. dry-wt. organism/ml.
Basal	0.15
Basal + casein hydrolysate	0.56
Basal + DL-cystine	0.38
Basal + L-ornithine	0.23
Basal + L-arginine or tyrosine	0.21
Basal + DL-cysteine	Trace
Basal + any one of others present in the hydrolysate	0.14–0.18

Basal medium (g./l.): 12 glucose, 5 purified butanol extract of yeast extract, 5.4 KH_2PO_4 , 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; buffered to pH 7.0. Nitrogenous material supplements at 0.1% (w/v).

Table 4. *Effect of cystine on the growth of Leptothrix at 25°*

Initial concentration of DL-cystine (%, w/v)	Maximum growth equiv. mg. dry-wt. organism/ml.	Mean generation time (min.)
0	0.15	420
0.00001	0.16	420
0.0001	0.23	400
0.0005	0.36	220
0.001	0.38	200
0.002–0.020	0.38	200

Basal medium as for Table 3.

The other amino acids at 0.1% (w/v) were retested in media to which 0.01% (w/v) DL-cystine had been added (Table 5). Citrulline and urea were tested also since with arginine and ornithine (which increased the amount of growth markedly) they are members of the ornithine cycle (Krebs, 1934). Whenever the addition was of an amino acid containing two nitrogen atoms per molecule the culture showed two successive phases of logarithmic growth at different rates, and all cultures showed a lag of about 13 hr. Mixtures of the amino acids which extended growth were tested; rates of growth as well as amounts of growth being measured (Table 6). Of the seven media which gave large populations, L-ornithine was present in four, L-arginine, L-proline and L-glutamate in three, and L-hydroxyproline in one (when with L-ornithine). L-Ornithine was present in the four media which gave the most rapid growths in phase one, but L-arginine was present in the four media which gave the most rapid growths in phase two. The fastest growths in both phases were in media containing L-ornithine and L-arginine. Different samples of L-ornithine gave slightly different results; chromatographic analysis detected traces of L-proline in some of the samples. Consequently L-arginine was preferred as a N-source.

Table 5. *Effect of amino acids on the growth of Leptothrix at 25° in media containing cystine*

Medium	Amount of growth equiv. mg. dry-wt. organism/ml.
Basal	0.38
Basal plus L-arginine	0.63–0.75
Basal plus L-ornithine	0.60–0.75
Basal plus L-proline	0.57–0.69
Basal plus L-glutamate	0.42–0.50
Basal plus L-hydroxyproline	0.39–0.44
Basal plus any one of others present in the hydrolysate	0.38–0.41
Basal plus citrulline	0.29
Basal plus urea	0.38

Basal medium as for Table 3 plus 0.01 % (w/v) cystine.

Table 6. *Growth of Leptothrix at 25° in media containing at 0.1 % (w/v) L-arginine, L-ornithine, L-hydroxyproline, L-proline and L-glutamate either singly or two at a time*

Amino acids present	Maximum growth equiv. mg. dry-wt. organism/ml.	Mean generation times (min.)	
		Phase 1	Phase 2
None	0.37–0.41	200	—
L-Arginine	0.75	200	260
L-Arginine plus L-ornithine	> 0.80	110	230
L-Arginine plus L-proline	> 0.80	170	350
L-Arginine plus L-hydroxyproline	0.76	180	430
L-Arginine plus L-glutamate	> 0.80	150	280
L-Ornithine	0.75	130	420
L-Ornithine plus L-proline	> 0.80	150	390
L-Ornithine plus L-hydroxyproline	> 0.80	150	520
L-Ornithine plus L-glutamate	> 0.80	130	500
L-Proline	0.69	210	770
L-Proline plus L-hydroxyproline	0.69	210	860
L-Proline plus L-glutamate	> 0.80	210	660
L-Glutamate	0.50	190	—

Basal medium as for Table 3: +0.01 % (w/v) DL-cystine.

Carbohydrate requirements

The abilities of a number of substances at 0.5% (w/v) to replace glucose in the medium were re-examined. Acetate, DL-malate, fumarate or α -ketoglutarate gave the same rate of growth as glucose (mean generation time, m.g.t., 200–210 min. for the first phase of logarithmic growth), whereas oxaloacetate gave a slower one (m.g.t. 320 min.) compared with the rate in absence of an added C-source (m.g.t. 230 min.). Glucose or acetate produced slightly greater amounts of growth than the other compounds, but all, with the exception of citrate, which was inhibitory, yielded denser populations than were obtained in absence of an added C-source.

Chemically defined media

The sodium salts chromate, manganate, molybdate, selenate, selenite, tellurate, tellurite, tungstate, and the chlorides chromous, chromic, manganous, were tested as

alternatives to the yeast extract, or materials I, II or III, needed to complete the basal medium ((g./l.) 12 glucose; 1 L-arginine hydrochloride; 0.1 DL-cystine; 5.4 KH_2PO_4 ; 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; adjusted to pH 7.0). Only sodium molybdate and sodium vanadate allowed growth to take place. The effective ranges of concentration of these materials were very different; and whereas with molybdate the medium was colourless (unless the molybdate was in excess, when the production of phosphomolybdate caused an intense yellowing), with vanadate the cultures were pale yellow-green initially, then transitionally purple and finally dingy brown as growth ceased. Because of these colourings the amounts of growth in the presence of higher

Fig. 1

Fig. 2

Fig. 1. Growth of *Leptothrix* at 25° and availability of molybdenum or vanadium. Medium contained (g./l.): 30.0 glucose; 1 L-arginine hydrochloride; 0.1 DL-cystine; 5.4 KH_2PO_4 ; 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; adjusted to pH 7.0. Molybdenum supplied as: molybdate, ∇ ; material I, Δ ; material III, $\square$. Rates of growth as mean generation times: all sources of molybdenum between the broken lines; vanadate, $\blacktriangle$.

Fig. 2. Growth of *Leptothrix* at 25° and the concentrations of nutrients in the medium. Medium contained (g./l.): 0.1 DL-cystine; 5.4 KH_2PO_4 ; 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; adjusted to pH 7.0; plus glucose (mM): $\circ$, 30; $\bullet$, 66. Percentage of total growth by the second phase in media containing 66 mM-glucose, ∇ .

concentrations of molybdate, and all concentrations of vanadate, were not measured. The relations between rate of growth and concentration of (1) material I, (2) material III, (3) sodium molybdate, (4) sodium vanadate, and the relations between amount of growth and the first three of these supplements are shown in Fig. 1.

The rates of growth of cultures containing sodium molybdate were not highly reproducible, but the cultures that started to grow with a mean generation time of 80 to about 130 min. clearly had a second phase of logarithmic growth with a mean generation time of 160–200 min. Below approximately $5\mu\text{M}$ the concentration of molybdate controlled the initial rate of growth. In the amount and rate of growth the

organism showed no preference for any of the forms of molybdenum tested in the basal medium. Molybdate was very much more effective than vanadate, which had a critical concentration above which it progressively decreased the rate of growth. In subsequent work 1 g./l. sodium molybdate was the supplement used to complete the medium.

Amount of growth and the concentration of nutrients

In the chemically defined medium DL-cystine was a negligible source of nitrogen, carbon and energy as compared with the L-arginine and glucose. The sole N-source, L-arginine, was also a possible source of carbon and energy and thus the availability of L-arginine may have affected the utilization of glucose and vice versa. The dependence of amounts of growth on the initial concentration of glucose in the presence of different initial concentrations of L-arginine hydrochloride, and on the initial concentration of L-arginine in the presence of different concentrations of glucose, are shown in Figs. 2 and 3. Without glucose there was negligible growth but in its presence L-arginine caused a considerable increase in the ratio of cell material produced to glucose consumed. This is explicable if the arginine is used as a source of C but not as a source of energy. Glucose has a much smaller sparing action on the utilization of L-arginine. Unlike the cases of coliform organisms growing in glucose + ammonium salt + buffer media (Dagley, Dawes & Morrison, 1951) the amounts of growth of the *Leptothrix* were not linearly dependent on the initial concentrations of nutrients. Particularly with respect to L-arginine the production of *Leptothrix* was more efficient when the amount of nutrient severely limited the amounts of growth. These cultures showed two logarithmic phases of growth and the proportion of the total amount of growth which occurred in the first phase was greater when the total growth was limited severely by a low initial concentration of L-arginine. This is shown also in Fig. 2 for media containing 66 mM-glucose.

Effect of serial subcultivation in the defined medium on the behaviour of the Leptothrix strain

After serial subcultivation in the defined medium cultures of the organism showed only one phase of growth, but there was some dependence of the rate of growth on the concentrations of glucose and L-arginine (Table 7). The amounts of growth were not detectably different from those shown in Figs. 2 and 3. Thus serial subcultivation had increased the rate of the second phase of growth and the two phases merged.

Table 7. *Effect of glucose and L-arginine concentrations on growth of Leptothrix at 25°*

Conc. glucose (mM)	66	66	30	30
Conc. arginine (mM)	47.5	4.75	47.5	4.75
Mean generation time (min.)	84	96	102	102

Basal medium (g./l.): 0.1 DL-cystine; 1 Na₂MoO₄·2H₂O; 5.4 KH₂PO₄; 0.4 MgSO₄·7H₂O; adjusted to pH 7.0.

The initial pH value may affect the amount of growth (Fig. 4). Growth took place in the media initially at pH values greater than 5.1 and less than 8. When the medium contained initially only 30 mM-glucose and 4.75 mM-L-arginine the amount of growth was not dependent on the initial pH value when this was between 5.6 and 7.7, but in media containing 66 mM-glucose and 47.5 mM-L-arginine the amount of growth

was dependent on the initial pH value being greatest at pH 7. Thus in the less nutritious medium initially between pH 5.6 and 7.7 the concentrations of nutrients were limiting whereas in the richer medium growth ceased for a reason which was related to the initial pH value.

Effect of temperature on growth of the Leptothrix

The organism was subcultivated in the defined medium six times at each of a number of progressively higher temperatures. After each change of temperature the lag phase was prolonged in the first two subcultivations but then decreased progressively; after acclimatization to 37° the lag phase was decreased to about 2 hr. The rates of growth were still erratic but showed an overall increase with increase of temperature, the shortest mean generation times recorded being (min.): 83 at 25°; 64 at 30°; 48 at 34°; 37 at 37°. After six subcultivations at 37° the organism had the same nutritional requirements as at 25°.

Fig. 3

Fig. 4

Fig. 3. Growth of *Leptothrix* at 25° and the concentrations of nutrients in the medium. Medium contained (g./l.): 0.1 DL-cystine; 5.4 KH_2PO_4 ; 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; adjusted to pH 7.0; plus L-arginine (mM): ○, 4.75; ●, 47.5.

Fig. 4. Growth of *Leptothrix* at 25° and the initial pH of the medium. Medium contained (g./l.): 0.1 DL-cystine; 5.4 KH_2PO_4 ; 0.4 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; pH adjusted with NaOH; plus: ●, 66 mM-glucose + 47.5 mM-L-arginine; ○, 30 mM-glucose + 4.75 mM-L-arginine.

The role of molybdenum and cystine

Measurements of the consumption of oxygen by suspensions of the *Leptothrix* (equiv. 1.5 mg. dry-wt. organism/ml. and washed twice by isotonic buffer) in glucose + buffer solutions showed that molybdate and cystine both enhanced the rate of oxidation and that these effects were additive. In the absence of added molybdate the oxidation was of short duration, but in its presence continued to the limit measurable by the Warburg apparatus. It has not yet been possible to demonstrate an inability to consume oxygen in the absence of a source of molybdenum but this may be because of the extremely small amount of molybdenum required.

Similarly measurements (Friedemann & Haugen, 1943) of the aerobic production of pyruvate from glucose established that: in the absence of a source of molybdenum no pyruvate was produced in 12 hr; in the presence of sodium molybdate, or of

material III, pyruvate production became significant after 5 hr and thereafter accelerated rapidly to a maximum steady rate; in the presence of yeast extract, material I or material II, the maximum rate was established after 2 hr. Thus the source of molybdenum was required for oxidative processes and the active form is not molybdate itself though the *Leptothrix* can utilize molybdate to supply its needs.

DISCUSSION

The strain of *Leptothrix* examined was exacting in two respects: (i) it required for growth a Mo-containing compound and cystine; (ii) it was a strict aerobe, incapable of fermenting sugars. It has been established that the need for molybdenum was connected with oxidative processes since there was stimulation of oxygen uptake in glucose + buffer solutions when molybdenum was provided, and vanadate, which could replace molybdate, showed progressive valency changes as the cultures grew. In particular the need for Mo was concerned with the oxidative production of pyruvate from glucose. A requirement for Mo is known for the reductive systems of *Neurospora*, soya bean and probably *Escherichia coli*, where it is also thought to act as an electron carrier: the relevant literature has been reviewed by Nason (*Biochemists' Handbook*, 1961*b*). Other biological uses of Mo are known: it affects the nitrogen metabolism (Mulder, 1948) and the growth (Mulder, 1954) of some higher plants and micro-organisms; molybdate is metabolized by *Azotobacter* (Keeler, 1958) and by certain farm animals (Stewart, Farmer & Mitchell, 1946). The concentration of molybdenum ($m\mu M$) required by *Azotobacter* (Burk, 1934) are not as small as with the present *Leptothrix* and vanadium can replace molybdenum (Horner, Burk, Allison & Sherman, 1942) in the case of *Azotobacter* more easily. The strain of *Leptothrix*, growing in a chemically defined medium, provides an opportunity to study mechanisms which may be of widespread importance.

In the defined medium the *Leptothrix* showed no preference for the materials isolated from yeast extract over molybdate when growth rates were the criterion. However, this was probably because the rate of growth was not controlled by a step in the part of the mechanism which required Mo: the measurements of pyruvate production showed a clear preference for the form of Mo occurring in the yeast extract, and that the acetylation procedure used in the isolation of material III had been detrimental. The need for Mo may be exhibited generally by organisms of the *Sphaerotilus-Leptothrix* group since all the media used by Mulder (1964) in his survey of them contained either yeast extract (Difco) or a basal culture solution containing (mg./l.) $0.05 \text{ Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$.

As the *Leptothrix*'s requirement for Mo was almost specific this strain might provide the basis of a micro-biological assay for extremely low concentrations of this element.

The authors wish to acknowledge the technical help of Mrs Eiry Jones and supporting grants to C.J.E. from Monsanto Ltd. and The Pharmaceutical Society of Great Britain.

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