The Cultivation of a Single Strain of *Actinomyces israelii* in a Simplified and Chemically Defined Medium

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SUMMARY

Growth curves were determined for a single strain of *Actinomyces israelii* grown in systematic modifications of the partially defined medium of Howell & Pine (1956). These determinations resulted in a progressive simplification of the Howell & Pine medium and in the elucidation of a completely defined medium containing 335 $\mu\text{g.}$ total-N/ml. instead of 948 $\mu\text{g.}$ total-N/ml. in the original medium; the yield of organism was increased concomitantly from 55 $\mu\text{g.}$ total-N/ml. of medium to 80 $\mu\text{g.}$ total-N/ml. of medium. This simplified medium contained no ammonium sulphate, purine or pyrimidine bases but twenty amino acids (of which L-cysteine and L-tryptophan were found to be essential) and sixteen growth factors.

INTRODUCTION

Actinomyces israelii is a Gram-positive slow-growing anaerobic to micro-aerophilic organism forming small (0.5–2.5 mm. diameter) white coherent ‘bread-crumb’ or ‘cauliflower’ colonies which grow at the bottom of vessels of liquid media, leaving a clear supernatant medium at all stages of growth. Microscopic observation of pure cultures shows a non-sporing rebranching filamentous organism, the filaments seldom exceeding 1 μ in diameter (Erikson, 1940, 1949; Erikson & Porteous, 1955). The organism has a reputation for being difficult to isolate in pure culture and to maintain in laboratory culture for prolonged periods (see Erikson, 1940, 1953; Holm, 1948, 1951; Thomson & Lovestedt, 1951). Rosebury, Epps & Clark (1944) recommended cultivation in alternate complex undefined media. Other references to the use of complex media are given by Erikson & Porteous (1953) who made the first successful attempt to maintain *A. israelii* in simpler media. They succeeded in obtaining good growth of 6 strains in serial subculture in a medium composed of equal volumes of broth + 1% (w/v) casein hydrolysate + 0.5% (w/v) glucose; 5 of these strains were trained to grow in continued subculture, though in diminished yield, in a medium composed of 99 volumes of 1% (w/v) casein hydrolysate + 1 volume of heart broth + 0.5% (w/v) glucose. These authors could not replace the broth by chemically defined nutrients but showed that *A. israelii* could be maintained in these media for periods up to 2 years. They concluded that the ‘short life, . . . and at best lessened activity’ of *A. israelii* reported by earlier workers who used more complex media might have been due to the presence in those media of growth inhibitory substances. Howell & Pine (1956) reported a medium which

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was chemically defined except for the starch and possibly the oleic acid that it contained, and which supported the growth of 13 strains of anaerobic *Actinomyces* in serial subculture; 5 of the strains were typical of *A. israelii* as defined above. The present work is in continuation of that by Erikson & Porteous (1953) and is based on the significant advance made by Howell & Pine (1956). A preliminary account of the work has been published (Christie & Porteous, 1959).

METHODS

Organism. The organism used was one of a collection of strains of *Actinomyces israelii* maintained by Dr Dagny Erikson and given to one of us (J.W.P.) in 1953. Cultures were freeze-dried at that time and maintained for various periods since 1956 in medium S or medium A of Erikson & Porteous (1953).

Freeze-drying. Cultures were routinely freeze-dried at intervals of 3–6 months by using an LB5 rotary dryer (Edwards High Vacuum, Ltd., Crawley, Sussex) and a suspension medium of equal volumes of ox-heart broth, horse serum (Burroughs Wellcome and Co., London) and 15% (w/v) glucose solution.

Reagents. All media and analytical solutions were made up in distilled water unless stated to the contrary. The following compounds were used in preparing media: KH_2PO_4 , $(\text{NH}_4)_2\text{SO}_4$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, Na acetate. $2\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, glucose (analytical reagent quality); $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ (reagent quality); casein hydrolysate (vitamin-free for biological assay; batch 1650; Allen and Hanburys, Ltd.); *p*-aminobenzoic acid, oleic acid, haemin (British Drug Houses, Ltd.); folic acid, pimelic acid, pyridoxal.HCl, pyridoxamine.HCl, thiamine.HCl, thioctic acid, purine and pyrimidine bases (L. Light and Co. Ltd.); biotin, calcium pantothenate, inositol, nicotinic acid, nicotinamide (Roche Products Ltd., Welwyn Garden City, Hertfordshire); glutathione (Distillers Company Ltd.); coenzyme A (70% assay; Sigma Chemical Company; agents G. T. Gurr Ltd., London); vitamin B₁₂ (crystalline; Glaxo Laboratories); the amino acids were the best quality available, and, except for L-cysteine.HCl, were not further purified.

Measurement of pH values. A Model 30 pH meter (Electronic Instruments Ltd., Richmond, Surrey) was used except for measurements on 0.1 ml. samples of inoculated media which were made with a capillator indicator set (British Drug Houses Ltd., Poole, Dorset).

Sterilization. All glassware was autoclaved at 120° for 20 min. Maintenance media were heat sterilized and dispensed as described by Erikson & Porteous (1953); chemically defined media, or component parts thereof, were sterilized by filtration through a P11 porcelain filter (Baird and Tatlock Ltd., London) or through a cellulose acetate filter membrane (A. Gallenkamp and Co. Ltd., London).

Culture vessels. Unless otherwise stated, rimless Pyrex tubes (12 × 75 mm.) containing 2 ml. liquid medium were used. Small volumes of all media were dispensed as described by Erikson & Porteous (1953).

Anaerobiosis. Stock cultures were maintained under hydrogen in an anaerobic jar (Erikson & Porteous, 1953). Experimental media were sealed in individual culture vessels with a layer of sterile liquid paraffin.

Inoculation technique. All inoculations were made with Pasteur pipettes. In all quantitative growth experiments, preparation of inoculum and quantitative inoculation were carried out as described by Christie & Porteous (1960). In the

routine maintenance of stock cultures, the larger colonies in tubes of liquid medium were broken up, so far as possible, with the tip of a Pasteur pipette and four to eight of the resulting small clumps transferred to each 2 ml. volume of fresh medium. Cultures were incubated at 37°.

Tests for culture purity. All cultures were tested periodically as follows. (i) Small fragments of colonies were stained with 0.1 % (w/v) methylene blue and examined microscopically under a cover-slip with an oil-immersion objective. Cultures were rejected when they showed any signs of contamination or when they failed to accord with the description given in the introductory paragraph of this paper. (ii) Replicate tubes of medium were inoculated and incubated aerobically; cultures were rejected when growth occurred in a second aerobic subculture (Erikson & Porteous, 1955). (iii) Duplicate nutrient agar plates were inoculated by spreading fragments of colonies over the agar surfaces; both plates were incubated at 37°, one aerobically, the other under hydrogen. Cultures were rejected when the aerobic plate showed any growth which survived a second aerobic subculture or when the anaerobic plate failed to produce typical *Actinomyces israelii* colonies only, i.e. abruptly raised, nodular colonies (Erikson & Porteous, 1953, 1955). (iv) Biphaseic agar-gel media were inoculated as described by Erikson & Porteous (1955); cultures were retained only when the colonies which developed in these media were discrete, compact, nodular, and free from any 'tailing'. Medium S of Erikson & Porteous (1953) was used in all the above culture tests.

Analytical techniques. (i) Growth curves were determined by inoculating replicate tubes of liquid medium with *Actinomyces israelii* in the manner already described. At appropriate times after inoculation and incubation, two pairs of tubes were withdrawn; colonies were quantitatively removed from a pair of tubes by Pasteur pipette into an 18 × 150 mm. Pyrex test tube, the colonies allowed to sediment in the tube and then washed twice by sedimentation in a total of 25 ml. distilled water. Colonies from the other pair of culture tubes were treated in the same way and the duplicate washed samples analysed for total-N content; all growth yields quoted are the average of two such determinations expressed as $\mu\text{g.}$ total-N content of the colonies harvested from 1 ml. medium, after correction for the total-N content of the corresponding inoculum. The remaining medium from each pair of culture tubes was filtered through Whatman No. 1 filter paper to remove any liquid paraffin and the duplicate filtrates taken for analysis. All results quoted are the average of the duplicate determinations expressed as the quantity of the compound determined per ml. medium.

(ii) Total-N determinations on harvested colonies and on media were done in duplicate as described by Christie & Porteous (1960), using the digestion apparatus described by Porteous (1960).

(iii) α -Amino-N was determined by the method of Pope & Stevens (1939) on 1–5 ml. samples of medium.

(iv) Ammonia-N was determined by Conway's method (1950), water blanks and ammonium sulphate standards being included with each set of determinations.

RESULTS

Media; terminology. All experimental media were derived from that published by Howell & Pine (1956) by progressive elimination of certain components. The composition of the Howell & Pine medium, referred to hereafter as medium HP, is shown in Tables 1 and 2; progressive modifications of this medium are designated HP1, HP2, etc. Any component omitted from a member of this series of media was omitted from all subsequent members of the series. Inoculation into a new

Table 1. *Composition and preparation of medium HP (Howell & Pine, 1956)*

	per litre	
	(g.)	(mg.)
1. Major salts		
KH_2PO_4	15.0	—
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	—	200.0
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	—	20.0
$\text{CH}_3\text{COONa} \cdot 2\text{H}_2\text{O}$	—	300.0
2. Minor salts		
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	—	4.0
$\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$	—	0.15
$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	—	0.15
3. Glucose	10.0	—
4. $(\text{NH}_4)_2\text{SO}_4$	1.0	—
5. Cysteine.HCl	1.0	—
6. Glutathione	—	500.0
7. L-Asparagine	—	100.0
8. L-Tryptophan	—	40.0
9. Purines and pyrimidines		
Adenine sulphate	—	20.0
Guanine hydrochloride	—	20.0
Uracil	—	20.0
Xanthine	—	20.0
Thymine	—	20.0
10. (a) Casein hydrolysate (vitamin free); or (b) amino acids as listed in Table 4, column A	4.0	—
11. Vitamins and growth factors as listed in Table 2	—	—
12. Purified potato starch	—	500.0

A solution of the above components, less the starch, was adjusted with 2N-KOH to the required pH value (7.2 in the present work) before dilution to a known volume with water and sterilization by filtration. Heat sterilized starch solution was then added to give the desired starch concentration and to dilute other components to the required concentration.

Table 2. *Growth factor components of medium HP (Table 1)*

11. Growth factors	(mg./l.)		($\mu\text{g./l.}$)
<i>p</i> -Aminobenzoic acid	2	DL-thioctic acid	100
Thiamine.HCl	2	Biotin	100
Riboflavin	2	Pimelic acid	100
Nicotinic acid	1	Haemin	200
Nicotinamide	1	Folic acid	500
Pyridoxal.HCl	1	Vitamin B ₁₂	1
Pyridoxamine.HCl	1	Citrovorum factor	100
Ca pantothenate	2		
Inositol	2		
Oleic acid	1		
Coenzyme A (70 % assay)	1		

member of the series of experimental media was always made from a mother culture in the simplest available medium of the series.

Selection of test organism. All eleven organisms available were isolated from human actinomycotic lesions. All strains were microscopically, macroscopically and physiologically typical of *Actinomyces israelii* when grown in medium S of Erikson & Porteous (1953). Two strains failed to grow in medium HP1 (medium HP, less citrovorum factor and less coenzyme A; Tables 1, 2). Three other strains survived only a few serial subcultures in this medium. The remaining 6 strains survived serial subculture in medium HP1. The techniques available for preparing uniform inocula of *A. israelii* (Christie & Porteous, 1960) and for measuring the growth of the inoculum (see: Methods, Analytical techniques) restricted the number of strains which could be investigated quantitatively and exhaustively. Strain Wills was selected as apparently representative of the 6 strains available which would grow in medium HP1; all results presented below refer to strain Wills of *A. israelii*.

Optimum initial pH value of the medium. Good growth occurred when the initial pH value of medium HP1 lay between 7.0 and 7.6; outside these limits the yield of organism decreased rapidly until at an initial pH of 6.6 or 7.8 the inoculum failed to grow. In further experiments all media were adjusted initially to pH 7.2.

Change in the pH value of the medium during growth of Actinomyces israelii

Preliminary experiments showed (Fig. 1) that (a) the pH value of medium HP1 decreased rapidly from pH 7.1 to 5.5 during the period when the increase in cell nitrogen was rapid with respect to time (analogous to the logarithmic growth phase of simple bacteria); (b) after growth ceased, the pH value of the medium continued to decrease to a limiting value of pH 5.0. These observations were found to be valid in several subsequent modifications of the medium; colonies harvested during the period of most rapid growth (pH of medium = 7.1 to 6.1) continued to grow at the same rate when subcultured to a medium of the same initial composition and pH 7.2; colonies harvested during the stationary phase (pH of medium 5.5 or less) showed a lag of 30–60 hr. when subcultured to fresh medium of the same initial composition and pH 7.2. pH measurements were therefore routinely used to determine the approximate growth phase of cultures, particularly of those intended as sources of inocula for quantitative growth experiments designed to measure the response of *Actinomyces israelii* to major components of medium HP.

Progressive simplification of the Howell & Pine medium

In all experiments reported hereafter, glucose and the major and minor salts were retained in experimental media at the concentrations given in Table 1 (items 1, 2 and 3); coenzyme A and citrovorum factor were omitted from all media and the remainder of the growth factors (Table 1, item 11) retained at the concentrations shown in Table 2. Table 3 shows the contribution of the various major components (see Tables 1, 2) to the total-N content of medium HP1.

The requirements for ammonium sulphate, L-cysteine and glutathione in the growth medium for *Actinomyces israelii* Wills strain were investigated in turn; the requirement for purine and pyrimidine bases was then investigated before modifying the amino acid concentration of the resulting medium. In these investigations

growth curves were determined in the manner described. With an exception (noted below) all inocula were taken from a mother culture in the rapid growth phase. All growth curves for nutritionally adequate media were of the general form shown in Fig. 1, i.e. a lag of 20–30 hr. followed by a period of rapid growth, with the culture entering the stationary phase between 70 and 90 hr. after inoculation.

Fig. 1. Changes in the pH value of medium HP1 (Table 6) during growth of the Wills strain of *Actinomyces israelii* at 37° under liquid paraffin seals. ■—■, pH; ●—●, growth.

The successive modifications to medium HP1 and the maximum growth obtained in the modified media are shown in Table 6. Five concentrations of ammonium sulphate were tested in modifications of medium HP1. Since the presence or absence of ammonium sulphate did not affect the growth rates or yields of organism this salt was omitted from medium HP1 to give medium HP2 which was then modified to contain L-cysteine at six concentrations within the range 0–1000 µg./ml. No growth was obtained when L-cysteine was omitted; at initial concentrations between 20 and 100 µg. cysteine/ml. there was a roughly linear growth response and maximal growth was obtained with an initial L-cysteine concentration of 200 µg./ml.

Table 3. *Calculated total-N content of the major nitrogen-containing components of medium HP (Table 1)*

Component	Calculated total-nitrogen content (µg.N/ml. medium)
Casein hydrolysate	520*
(NH ₄) ₂ SO ₄	212
L-cysteine.HCl	90
Glutathione	68
Purine and pyrimidine bases	32
L-Asparagine	21
L-Tryptophan	5

Total: 948

* This value determined experimentally on a separate stock solution of casein hydrolysate.

Medium HP2 modified to contain cysteine at this concentration was termed medium HP3, with a glutathione content of 500 $\mu\text{g./ml.}$ In initial experiments medium HP3 was modified to contain 0, 100, 250 and 500 $\mu\text{g.}$ glutathione/ml. The corresponding growth curves are shown in Fig. 2. In further experiments only the first and last of the above modifications of medium HP3 were used; normal growth curves were obtained which are also shown in Fig. 2. Both sets of results showed that the final cell yield was independent of the initial glutathione concentration. In the initial experiments there was a pronounced lag which was most marked in the absence of glutathione from the medium; the inocula in this experiment were taken from a culture in the stationary phase (pH of medium at harvest = 5.0), but the longest lag was greater than could be accounted for solely by the use of stationary phase inocula. In the second experiment there was no apparent lag whether glutathione was present or absent; the inocula in this instance were taken from a culture in its most rapid phase of growth (pH of medium at harvest = 6.6). Although there did not seem to be a specific requirement for glutathione it was retained at a concentration of 100 $\mu\text{g./ml.}$ for further experiments and medium HP3 modified in this way became medium HP4.

Fig. 2. Growth of the Wills strain of *Actinomyces israelii* at 37° under liquid paraffin seals in 2 ml. volumes of medium HP3 (Table 6) modified to contain various initial concentrations of glutathione ($\mu\text{g./ml.}$ medium): $\circ-\circ$, 500 (medium HP3); $\blacktriangle-\blacktriangle$, 250; $\blacktriangledown-\blacktriangledown$, 100 (medium HP4, Table 6); $\bullet-\bullet$, 0. Growth of an inoculum taken from a stationary phase mother culture is indicated by a full line; growth of an inoculum taken from a rapidly growing mother culture is indicated by a broken line.

Omission of the purine and pyrimidine bases from medium HP4 did not affect the yield of organism and gave medium HP5 which became the basal medium for investigation of the amino acid requirements.

Howell & Pine (1956) supplemented their casein hydrolysate medium (Tables 1, 2) with L-asparagine and with L-tryptophan. Omission of L-asparagine from medium HP4 decreased the yield of organism by 15%. When L-tryptophan was omitted from medium HP4 no growth occurred. Both these amino acids were therefore retained at the original concentration in all subsequent media.

Howell & Pine (1956) replaced the casein hydrolysate in their medium by an

amino acid mixture (item 10*b*, Table 1) which was supposed to simulate the concentrations of amino acids supplied by the casein hydrolysate. In the experiments of Howell & Pine replacement of their casein hydrolysate by this amino acid mixture had no significant effect on the rate of growth or on the final yields of organism with the three organisms they tested, one of which was identified as *Actinomyces israelii*, one as probably *A. israelii* and one as *A. naeslundii*. Replacement of the casein hydrolysate of medium HP5 by the amino acid mixture used by Howell & Pine gave medium HP6/A in which the yield of organism was only 65% of that in the casein hydrolysate medium HP5 (Table 6). It appeared that the amino acid mixture did not simulate the casein hydrolysate used in the present work. In a comparative experiment medium HP5 was therefore modified by substituting two amino acid mixtures ('A' and 'B', Table 4) for the casein hydrolysate to give media HP6/A and HP6/B, differing in the proportions of the amino acids present but essentially identical in their total-N content. Concurrent experiments with medium HP5 had shown that starch (Table 1) was not required for the growth of *A. israelii* Wills strain. Starch was therefore omitted from media HP6/A and HP6/B. The yield of organism obtained in medium HP6/A was only 74% of that obtained in medium HP6/B, where the yield was 87% of that in the casein hydrolysate medium HP5 (Table 6). Medium HP6 containing amino acid mixture 'B' (Table 4) was therefore adopted for further experiments.

Further experiments showed that medium HP7 (medium HP6/B modified to contain glutamic acid at a concentration of 100 µg./ml. and each of the other amino acids of mixture 'B' (Table 4) at a concentration of 50 µg./ml.) would just support

Table 4. *The composition of two amino acid mixtures (A and B) used in the preparation of the completely defined media HP6/A and HP6/B (Table 6), together with the total-N contribution of the amino acids to the media. Amino acid mixture (A) was that used by Howell & Pine (1956)*

Amino acid	Concentration in the medium (µg./ml.)		Total-N contribution to the medium (µg. N/ml.)	
	(A)	(B)	(A)	(B)
Glycine	20	200	3.7	37.3
L-Alanine	220	200	34.6	31.4
DL-Serine	308	200	41.0	26.7
L-Threonine	152	200	17.9	23.5
L-Leucine	388	200	41.5	21.4
L-Isoleucine	252	200	26.9	21.4
DL-Valine	260	200	31.1	24.0
L-Glutamic acid	932	1000	88.8	95.2
L-Aspartic acid	244	200	25.7	21.0
L-Lysine	304	200	58.3	38.4
L-Arginine	172	200	55.3	64.0
L-Histidine	84	200	22.8	54.2
L-Proline	320	200	39.0	24.4
L-Hydroxyproline	12	200	1.3	21.4
L-Phenylalanine	200	200	17.0	17.0
L-Tyrosine	268	200	20.7	15.5
L-Tryptophan*	48	—	6.5	—
DL-Methionine	136	200	12.8	18.8
Total:	4320	4200	544.9	555.6

* Already present, together with L-cysteine and L-asparagine in the basal medium (Table 1).

growth of *Actinomyces israelii* strain Wills. Earlier attempts to obtain a nitrogen balance sheet for the growth of the organism in medium HP1 (Fig. 1) failed because of the low yields of organism relative to the nitrogen content of the medium (Table 6). The attempt was repeated for growth in medium HP7 with the results shown in Table 5. The ammonia-N content of the medium (arising from the ammonia used to dissolve haemin in preparing the medium) was essentially unaltered at the end of the growth period. The yield of organism balanced the decrease in the α -amino-N content of the medium and this decrease accounted for 97% of the decrease in the total-N content of the medium.

Table 5. Nitrogen balance after growth of the Wills strain of *Actinomyces israelii* for 6 days at 37° under liquid paraffin seal in medium HP7 (Table 6)

	Calculated 0 days	Determined		Increase or decrease
		0 days $\mu\text{g. N/ml. medium}$	6 days $\mu\text{g. N/ml. medium}$	
Organism (total-N)	—	0	29	+ 29
Medium (total-N)	182	179	149	— 30
Medium (α -amino-N)	130	122	93	— 29
Medium (ammonia-N)	0	2	2.5	+ 0.5

The absolute yield of organism in medium HP7 was however low. Medium HP6/B was therefore further modified to contain half the concentration of each of the amino acids in mixture 'B' (Table 4). A yield of organism equivalent to 80 $\mu\text{g. total-N/ml. medium}$ was obtained after 6 days incubation in this medium HP8 containing initially 335 $\mu\text{g. total-N/ml. medium}$. This may be compared (Table 6) with the yield equivalent to 55 $\mu\text{g. total-N/ml. of medium HP1}$ which contained initially 948 $\mu\text{g. total-N/ml. (Fig. 1, Table 3)}$.

Miscellaneous experiments with modifications of medium HP

In an experiment reported above a partial requirement for L-asparagine in the nutrition of *A. israelii* strain Wills was noted. Addition of L-glutamine to medium HP5 (Table 6) at 100, 200 and 300 $\mu\text{g./ml.}$ had no significant effect on growth rates or on yields of organism. Addition of ascorbic acid at 200 $\mu\text{g./ml.}$ to medium HP4 (Table 6) made no significant difference to the yield of organism although a slight decrease in the length of the lag phase was noted.

DISCUSSION

The most significant recent advance in our knowledge of the nutritional requirements of *Actinomyces israelii* and related organisms was made by Howell & Pine (1956) who showed that a complex but essentially chemically defined medium, originally devised for the culture of *Histoplasma capsulatum*, also supported several *Actinomyces* species. By determining the growth curves for a single strain (Wills) of *A. israelii* in systematic modifications of the medium of Howell & Pine it has now been possible to assess the nutritional value of the major nitrogen-containing components of the original medium. It has been found that all the ammonium sulphate and the purine and pyrimidine bases of the original medium may be

Table 6. *Cumulative modifications to the Howell & Pine (1956) medium, together with the calculated total-N content of each medium and the yields of Wills strain of Actinomyces israelii grown in each medium*

Medium and cross-reference	Cumulative modifications to medium HP	Calculated total-nitrogen content of medium ($\mu\text{g. N/ml. medium}$)	Maximum yield of organism as cell-N ($\mu\text{g. N/ml. medium}$)
HP (Tables 1, 2, 3)		948	—
HP1 (Tables 1, 2, 3)	Coenzyme A and citrovorum factor omitted	948	55
HP2 (Table 3)	(NH_4) ₂ SO ₄ omitted	736	65
HP3 (Table 3)	L-cysteine .HCl concentration decreased to 1/5 concentration in medium HP	664	75
HP4 (Table 3, Fig. 2)	Glutathione concentration decreased to 1/5 concentration in medium HP	610	80
HP5 (Table 3)	Purine and pyrimidine bases omitted	578	80
HP6/A (Tables 1, 3, 4)	Starch omitted; casein hydrolysate replaced by amino acid mixture (A)	603	52
HP6/B (Table 4)	As HP6/A but amino acid mixture (A) replaced by amino acid mixture (B)	613	70
HP7 (Tables 4, 5)	Glutamic acid concentration decreased to 1/10 and concentrations of other amino acids of mixture (B) decreased to 1/4 the concentrations in medium HP6/B	182	29
HP8 (Table 4)	Concentration of all amino acids of mixture (B) decreased to 1/2 the concentrations in medium HP6/B	335	80

omitted. The initial concentration of cysteine in the medium was decreased to one-fifth of its original concentration without affecting the final yield of organism and under certain circumstances glutathione could be omitted. Casein hydrolysate was successfully replaced by a mixture of twenty amino acids. Of the eighteen growth factors present in the original Howell & Pine medium, coenzyme A and citrovorum factor were found to be unnecessary for the cultivation of the Wills strain of *A. israelii*. These modifications to the original medium, together with the elimination of starch from it, led to the formulation of a simplified defined medium (HP6/B, Table 6) which has supported the Wills strain of *A. israelii* in continued subculture over a period of 6 months. The medium contains approximately 65% of the total-N of the Howell & Pine medium and consistently gives a better yield of the organism (Table 6). Less extensive tests have shown that the Wills strain of *A. israelii* can survive at least five serial cultures in medium HP8 (Table 6), containing only 35% of the total-N of medium HP, without diminution in the growth yield. Of the twenty amino acids present in media HP6/B and HP8, cysteine and tryptophan are essential for the Wills strain of *A. israelii*.

The results depicted in Fig. 2 indicated that glutathione was not an essential component of the growth medium but that this compound was effective in decreasing the prolonged lag exhibited when an inoculum taken from a mother culture which had entered the stationary phase was used. Inocula taken from a rapidly developing mother culture gave no lag whether or not glutathione was present in the medium. Glutathione might fulfil either or both of two functions; it might act as a redox buffer ($E'_0 = 40$ mV.; pH 7) and/or as a nutrient for growth. Addition of ascorbic acid ($E'_0 = 50$ mV.; pH 7) to medium HP 4 (Table 6) containing 100 μ g. glutathione/ml. did slightly decrease the lag in a controlled experiment. This result suggests that the redox potential of the medium might be critical especially in the initial growth phase of *Actinomyces israelii* strain Wills but does not prove that glutathione acts exclusively or even mainly as a redox buffer. Glutathione may be required for metabolism within the cell and the actively growing organism is perhaps able to synthesize glutathione at a rate adequate for continued rapid growth, while the organism taken from a stationary phase culture is perhaps unable initially to synthesize the peptide sufficiently fast for rapid growth. It may be significant that L-cysteine proved to be an essential amino acid in the nutrition of the organism and it may be recalled that Erikson & Porteous (1953) emphasized the need for frequent subcultivation in the successful maintenance of *A. israelii*.

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REFERENCES

- CONWAY, E. J. (1950). *Microdiffusion analysis and volumetric error*, 3rd ed., p. 95. London: Crosby Lockwood and Son, Ltd.
- CHRISTIE, A. O. & PORTEOUS, J. W. (1959). The nutritional requirements of *Actinomyces israelii*. *Biochem. J.* **73**, 47 P.
- CHRISTIE, A. O. & PORTEOUS, J. W. (1960). A technique for obtaining uniform inocula of *Actinomyces israelii*. *J. gen. Microbiol.* **23**, 261.
- ERIKSON, D. (1940). Pathogenic anaerobic organisms of the Actinomyces group. *Spec. Rep. Ser. med. Res. Coun., Lond.* no. 240.
- ERIKSON, D. (1949). The morphology, cytology and taxonomy of the actinomycetes. *Annu. Rev. Microbiol.* **3**, 23.
- ERIKSON, D. (1953). Variation of mycelial pattern in sporogenous and asporogenous Actinomycetes. *6th Congr. int. Microbiol. Symp. Actinomycetales*, p. 102.
- ERIKSON, D. & PORTEOUS, J. W. (1953). The cultivation of *Actinomyces israelii* in a progressively less complex medium. *J. gen. Microbiol.* **8**, 464.
- ERIKSON, D. & PORTEOUS, J. W. (1955). Commensalism in pathogenic anaerobic Actinomyces cultures. *J. gen. Microbiol.* **13**, 261.
- HOLM, P. (1948). Some investigations into the penicillin sensitivity of human pathogenic actinomycetes. *Acta path. microbiol. scand.* **25**, 376.
- HOLM, P. (1951). Studies on the aetiology of human actinomycosis. II. Do the 'other microbes' of actinomycosis possess virulence? *Acta path. microbiol. scand.* **28**, 391.

- HOWELL, A. & PINE, L. (1956). Studies on the growth of species of *Actinomyces*. *J. Bact.* **71**, 47.
- POPE, C. C. & STEVENS, M. F. (1939). The determination of amino-nitrogen using a copper method. *Biochem. J.* **33**, 1070.
- PORTEOUS, J. W. (1960). An electrically heated micro-digestion apparatus. *Lab. Pract.* **9**, 795.
- ROSEBURY, T., EPPS, L. J. & CLARK, A. R. (1944). A study of the isolation, cultivation and pathogenicity of *Actinomyces israelii* recovered from the human mouth and from Actinomycosis in man. *J. Infect. Dis.* **74**, 131.
- THOMSON, L. & LOVESTEDT, S. A. (1951). An *Actinomyces*-like organism obtained from the human mouth. *Proc. Mayo Clin.* **26**, 169.