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The Cultivation of *Actinomyces israelii* in a Progressively Less Complex Medium

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SUMMARY: Six strains of *Actinomyces israelii* were trained to grow in continued subculture on a medium composed of 99 volumes of 1 % casein hydrolysate + 1 volume of heart broth, the mixture containing 0.5 % (w/v) glucose. Poor growth of limited viability was obtained in a medium consisting of 1 % casein hydrolysate + 0.5 % glucose (w/v) only. The addition of a wide variety of different amino-acids, fatty acids, growth factors and mineral salts to certain of the less complex media did not compensate for the partial or complete omission of the heart broth. Of the glucose supplied in relatively complex media about 40 % was utilized, and lactic acid equivalent to 30-60 % of the glucose utilized appeared in the medium.

Actinomyces israelii, the anaerobic organism which is the causal agent of actinomycosis in man and in some animals, has a well-established reputation for being difficult to maintain in laboratory culture. Alternation of media such as cooked meat + serum, nutrient agar + sterile horse blood, concentrated sheep heart broth + glucose, has been recommended (Erikson, 1940). Rosebury (1944) noted the advisability of a 'mixed diet', and found brain + heart infusion of especial value. Later workers (e.g. Thompson, 1950; Holm, 1950) used brain broth, 'hormone' agar, blood agar or broth supplemented with ascitic fluid. Brewer's (1940) thioglycollate (mercaptoacetate) medium, in common use for this and other anaerobes, contains pork infusion solids. Such media are amongst the richest used for the cultivation of micro-organisms, yet short life, frequent dying out and at best lessened activity attend the prolonged use of any one of these complex media for the cultivation of *A. israelii*. The following report describes an attempt to produce simpler media, and the growth characteristics obtained thereon.

METHODS

Organisms. Five human strains, typical *Actinomyces israelii*, and one porcine strain closely resembling these, were selected for detailed study. All six strains had been freshly isolated from cases of actinomycosis.

Apparatus. Except where otherwise mentioned all liquid media were dispensed into rimless test-tubes 12 × 75 mm., plugged with cotton-wool.

Automatic siphon-burettes of 2 or 10 ml. capacity which could be sterilized and also dismantled for cleaning were devised to fit 500 ml. (20 oz.) screw-capped 'medicine flats' and 25 ml. screw-capped bottles. These burettes were used for dispensing all media.

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All new glassware was given a preliminary cleansing in hot chromic + sulphuric acid mixture. For routine cleansing glassware was, wherever possible, boiled with soap and water, scrubbed with hot soapy water, rinsed with tap water, then with distilled water and finally dried in the oven. Apparatus not amenable to this treatment was cleaned in hot chromic + sulphuric acid mixture, drained, rinsed successively in tap water, dilute NaOH, tap water, distilled water, and then dried. Before contact with any medium all apparatus was sterilized by autoclaving at 20 lb./sq.in. for not less than 30 min., and thereafter dried in the oven.

All measurements of pH values were made electrometrically using a glass electrode.

Anaerobiosis. Culture tubes were stacked in two layers with an intervening wad of filter-papers in standard anaerobic jars. Jars were evacuated to 1 cm. Hg and filled with commercial compressed nitrogen (*c.* 99.8 % pure) which had been passed through three alkaline pyrogallol towers. This procedure was repeated twice.

Chemicals. D-Glucose (anhydrous A.R., British Drug Houses Ltd.); casein hydrolysate, 'Vitamin free for microbiological assay' (Allen and Hanbury); casein, light white soluble (British Drug Houses Ltd.); agar, New Zealand (Davis Gelatin Co. Ltd.); 2-amino-2-(hydroxymethyl)-1,3-propanediol (Eastman-Kodak); KH_2PO_4 (A.R., British Drug Houses Ltd.); NaOH (A.R.). All other inorganic chemicals mentioned in the text were of 'reagent' quality; other organic chemicals mentioned were the best available and were not further purified.

Media

The six strains of *A. israelii* have been carried in repeated subculture in each of several media for varying periods. The composition of these media is given in Tables 1 and 2.

Table 1. *The composition of liquid media for the maintenance of Actinomyces israelii in continuous subcultivation*

Components	Media			
	S	A ₁	A ₂	A ₃
Heart broth	1 volume	1 volume	1 volume	1 volume
Casein tryptic digest	1 volume	—	—	—
Casein hydrolysate (1 %, w/v)	—	1 volume	1 volume	1 volume
Glucose	0.5 % (w/v)	0.5 % (w/v)	—	—
Glycerol	—	—	0.5 % (w/v)	—
Starch	—	—	—	0.04 % (w/v)

Table 2. *The composition of the dilution media A–O used for training Actinomyces israelii to grow in progressively less complex media*

Glucose at 0.5 % (w/v) was added to each medium.

Components	Dilution media										
	A	B	C	D	E	F	G	K	L	M	O
Heart broth (% v/v)	50	40	30	20	10	4	2	1	0.5	0.25	0.0
Casein hydrolysate, 1 % solution (% v/v)	50	60	70	80	90	96	98	99	99.5	99.75	100.0

Preparation of components of media

Heart broth. Beef hearts were used according to the method of Wright (Mackie & McCartney, 1938) with the following modifications: addition of NaCl, peptone and glucose was omitted; initial hot extraction of the mince was accompanied by vigorous mechanical stirring; subsequent steaming was performed in small batches (not more than 500 g. mince and 1 l. distilled water); filtration was through a closely packed wad of glass wool surmounted by the extracted meat. The final water-clear filtrate was adjusted by addition of N-NaOH to pH 7.2.

Casein digest. Casein (200 g.) was homogenized with distilled water (2 l.) in an Atomix (Measuring and Scientific Equipment Ltd., 14/28 Spenser St, London, S.W. 1) homogenizer. The suspension obtained was adjusted to pH 8.2 by addition of 10N-NaOH. The preparation otherwise proceeded according to Cole & Onslow (1916). The final digest was adjusted to pH 7.2.

Casein hydrolysate solution. Casein hydrolysate powder (5 g.) dissolved in distilled water (450 ml.) was adjusted by addition of N-NaOH to pH 7.2. The water-clear solution was made to 500 ml. with distilled water.

Sterilization of components of media. The heart broth, casein digest and casein hydrolysate solutions were separately placed in 500 ml. quantities in sterile screw-capped 'medicine flats' (20 oz.) and sterilized by autoclaving for not more than 10 min. at 20 lb./sq.in. The two casein media invariably remained water-clear. In the few instances where the heart broth did not stay clear it was centrifuged, the supernatant solution removed by decantation and resterilized as before, when it remained clear.

Mixing and dispensing of complete media. Media S, A₁, A₂ and A₃ (Table 1) were prepared in 500 ml. volumes as required by mixing aseptically their respective sterile components in sterile screw-capped bottles which were then steamed for not more than 5 min.; this steaming was repeated each time a bottle was opened. These media were dispensed aseptically in 2.5 ml. amounts via sterile burettes into sterile tubes which were then steamed for not more than 5 min. In every case a water-clear medium was obtained which had little or no tendency to give a precipitate on storage or incubation, except where the pH value decreased during growth. The amount of growth in these media was far superior to that in media of the same initial composition but which had been autoclaved after mixing the constituents. Repeated steaming of the completed media caused a slight diminution in growth. A sufficient volume of medium for immediate use was therefore made up at one time. Between 200 and 400 tubes of medium have been dispensed in this way each week during the past two years. The number of contaminated tubes had been negligible.

The dilution media A to O (Table 2) were compounded by aseptic dispensing of each sterile component solution in proper proportion via sterile burettes into sterile culture tubes (final volume of medium, 2.6 ml.) which were then steamed for not more than 5 min. Thus the completed media were steamed only once before inoculation; they were superior to media of the same initial

composition prepared as described for media S, A₁, A₂, and A₃. The tubes of dilution media remained water-clear, except when growth of the organism caused a marked decrease in pH value, and were practically colourless. This dispensing technique was too slow for routine work. Fifty to 100 tubes/week have been dispensed in this way over the last 18 months with a negligible number of contaminated tubes.

Methods of cultivation

Stock cultures were carried in the liquid media described above. Transfers were made by Pasteur pipette at intervals of not more than 7 days. The inhomogeneity of growth of the organisms made it impossible to use a measured volume of cell suspension as inoculum. Accordingly, three or four small (*c.* 1 mm. diam.) colonies or fragments of colonies were used as inoculum in transfers to fresh medium.

In the initial work organisms washed with sterile 0.85 % NaCl before subculture appeared to multiply more rapidly than unwashed organisms; later results were inconsistent, and an improvement in growth was again obtained by brief washing with sterile distilled water. It subsequently became clear that the frequency of subcultivation was the important factor; the inoculum is not now washed.

Shake and slope cultures on nutrient media (equal volumes of heart broth and casein digest without carbohydrate) were inoculated at each subculture; after maximum growth was attained the tubes were sealed with paraffin wax and stored at 2°. These cultures were used as additional checks on the purity of cultures, and, when necessary, as a source of renewed subcultures. The incubation temperature was 37°.

RESULTS

Two strains (X and Y) of the six selected for study had originally been cultivated for several months in various complex media prepared by the more usual techniques. When repeatedly subcultured in medium S (Table 1) growth was at first slight and it was only possible to maintain the cultures by inoculating large numbers of tubes of medium (liquid, slopes and shakes). Subcultivation was necessary at intervals of not more than 7 days. Originally the liquid medium was dispensed in 10 ml. amounts in 18 × 150 mm. tubes. To conserve medium and to obtain a larger number of inoculated tubes, the smaller tubes mentioned previously (under *Apparatus*) were adopted. Much better mass of growth, in proportion to the volume of medium, then resulted, presumably due to more rapid attainment of equilibrium conditions between gas and liquid phases. Good growth in medium S which was continuously reproducible on subcultivation, was only obtained after 7–15 subcultures with strains X and Y and with other strains tested which had received similar initial treatment. The remaining four strains were taken into medium S immediately after isolation in pure culture; good growth, continuously reproducible on subcultivation in medium S, was obtained after three or four subcultures. Medium S was therefore particularly suitable for recently isolated

strains; patience and persistence were required to bring old stock cultures into a reliable vigorous state of growth.

Paper chromatographic analysis (Consden, Gordon & Martin, 1944) of the amino-acid content of the casein digest and the casein hydrolysate suggested that replacement of the casein digest in medium S by the casein hydrolysate should be the first step towards the attainment of a defined medium. The casein digest and casein hydrolysate contained approximately the same proportions of those amino-acids which were present in both. The casein digest lacked proline and hydroxyproline and contained only traces of glycine and aspartic acid; the casein hydrolysate lacked tryptophan and contained only traces of hydroxyproline and cystine. Accordingly media A_1 , A_2 and A_3 (Table 1) were prepared. As the next step the heart broth was progressively eliminated from medium A_1 and replaced by similar volumes of casein hydrolysate (1%, w/v) to give the series of dilution media A-O shown in Table 2. The cultural characteristics of the six selected strains of *A. israelii* in any one of the media listed in Tables 1 and 2 were remarkably similar. In what follows these cultural characteristics are described.

Cultures in media A_1 , A_2 , A_3 and S

All strains were grown initially in medium S. It has been possible in every case to maintain healthy growth in this medium for an apparently indefinite period by weekly transfers of fresh medium; one strain has been in continuous cultivation in this medium for one year, the other five for periods up to two years. Once good reproducible growth was obtained in medium S transfers were made to media A_1 , A_2 and A_3 and four parallel cultures are now maintained. Growth in medium A_1 has always been good and it has been possible to maintain continuous cultures for 18 months by weekly transfers. Growth has not, however, been as prolific as in medium S (Pl. 1, fig. 1 and Table 3). In media A_2 and A_3 repeated subcultures maintained in the same way tended to decrease in vigour during the first ten weeks. Subsequent cultivation during 6 to 9 months has shown no diminution in vigour of the cultures.

In media A_1 , A_2 and S all strains grew as discrete, compact, medium-sized 'bread-crumbs', 'cauliflower' or 'puffball' colonies which settled in the bottom of the culture tubes. Clouding of the medium did not take place. In medium A_3 the colony form was frequently the same as in media A_1 , A_2 and S but the colonies were almost invariably accompanied by much greater numbers of minute puffball colonies, even in cases where, as in one of the glucose media, it could be claimed that abnormally large cauliflower colonies were typical; occasionally only minute colonies appeared in medium A_3 . Apart from colony form, growth in this (0.04%) starch medium was usually accompanied by a slight but distinct opalescent haze in the immediate vicinity of the colonies, the medium as a whole remaining clear.

In medium S the pH value fell as low as 4.6 during 7 days growth; the medium remained water-clear. In medium A_1 the pH value fell as low as in medium S, but below pH 5.2 a flocculent precipitate usually appeared. In medium A_2 the pH value decreased only slightly during growth; the maximum

decrease recorded was from pH 7.2 to 5.5 during 7 days. The more usual terminal pH value was 6.5; the medium remained clear. In medium A₃ the usual terminal pH value was 6.5.

Better growth was obtained by buffering medium A₁. The casein hydrolysate moiety of this medium was compounded in Sorensen's m/15 phosphate buffer, pH 7.2 (Clark, 1928) instead of in distilled water, with adjustment of pH value and sterilization as before. Similarly, a casein hydrolysate solution (1%, w/v) containing m/15 2-amino-2-(hydroxymethyl)-1,3-propanediol was prepared and adjusted to pH 7.2. Medium A₁ which contained either of these buffered casein hydrolysate solutions gave amounts of growth which approached that obtained in medium S (Pl. 1, figs. 1, 2; see also Table 3).

Cultures in dilution media A-D

Serial cultivation of strains X and Y were made: from medium A₁ to dilution medium A→B→C→D. In the first few subcultures growth was only obtained in media A and B. Twelve subcultures were necessary before minimal growth was obtained in medium D; another fifteen subcultures were necessary to train the organism to grow in medium F. With the four freshly isolated strains growth in medium F was established in half the time. By proceeding in this manner the proportion of heart broth in the medium was progressively decreased (Table 2) until five of the six strains were trained to grow consistently on medium K (heart broth, 1 vol.; 1% casein hydrolysate, 99 vol.; glucose, 0.5% w/v). The exceptional strain has grown well only in dilution media A-D. Some growth was obtained with all strains in all dilution media but below the limits mentioned growth was slight and of limited viability. Growth in the series of dilution media occurred in graded amounts; with the inocula mentioned, the amount of growth in medium K (medium D in the case of the exceptional strain) was sufficient to provide viable inocula for twelve fresh tubes of medium (see Pl. 1, fig. 3, and Table 3).

Cultures in supplemented dilution media D and F

When reproducible growth had been established in media D and F the effect of adding defined compounds to these media was tried. All supplements were adjusted to pH 7.2, made to volume and sterile 0.1 ml. samples added aseptically to the media. Except where otherwise stated no consistent enhancement or inhibition of growth was obtained in repeated trials during 6 to 12 consecutive subcultures with any of the following compounds at the concentrations stated:

Thiamin, riboflavin, nicotinic acid, nicotinamide, pyridoxine, pantothenic acid, *p*-aminobenzoic acid, inositol, choline, ascorbic acid (each 10 µg./ml.), folic acid (0.4 mµg./ml.), biotin (80 mµg./ml.), vitamin B₁₂ (1 µg./ml.), tested singly or together.

Glycine, DL-alanine, DL-serine, DL-threonine, DL-valine, DL-norvaline, DL-leucine, DL-isoleucine, DL-norleucine, DL-aspartic acid, DL-glutamic acid, L-asparagine, DL-glutamine, L-arginine, L-lysine, L-histidine, DL-ornithine, DL-citrulline, DL-methionine, L-phenylalanine, L-tyrosine, DL-iodotyrosine,

DL-tryptophan, L-proline, sarcosine (100 $\mu\text{g./ml.}$) tested individually; the addition, singly or together, of L-cystine, L-hydroxyproline and DL-tryptophan at the same concentrations to medium O had no effect.

Table 3. *Typical yields of Actinomyces israelii harvested after 7 days anaerobic growth in the various media shown*

Media A₁, A₂, A₃, S and A-O: see Tables 1 and 2.

Medium a: medium A₁ containing M/15 2-amino-2-(hydroxymethyl)-1,3-propanediol, pH 7.2.

Medium b: medium A₁ containing M/15 phosphate buffer, pH 6.0.

Medium c: medium A₁ containing M/15 phosphate buffer, pH 7.2.

Medium d: medium A₁, pH 7.2—no buffer added.

Wet weights determined after washing cells three times with distilled water; dry weights determined after drying over H₂SO₄ *in vacuo*; total N by Kjeldahl digestion.

	Stock media				Buffered media			
	A ₁	A ₂	A ₃	S	a	b	c	d
Wet weight (mg./ 10 ml. medium):	29.1	10.3	30.6	79.9	60.0	20.0	55.6	37.6
Dry weight (mg./ 10 ml. medium):	1.7	0.5	1.4	3.9	2.8	—	2.8	2.0
Total N (μg./10 ml. medium):	166	53	118	283	252	52	268	152
Wet weight } Dry weight }	17.1	20.6	21.8	20.5	21.4	—	19.9	18.8
Dry weight } Total N }	10.2	9.4	11.0	13.8	11.1	—	10.4	13.1

	Dilution media											
	A	B	C	D	E	F	G	K	L	M	O	
Wet weight (mg./ 10 ml. medium):	30.9	35.8	27.6	31.1	22.8	7.8	2.8	6.2	1.3	0.4	0.0	
Dry weight (mg./ 10 ml. medium):	2.2	2.3	1.7	2.0	1.8	0.5	0.2	0.4	0.1	0.0	0.0	
Total N (μg./10 ml. medium):	223	194	147	154	110	41	15	26	5	—	—	
Wet weight } Dry weight }	14.0	15.6	16.2	15.5	12.7	15.6	14.0	15.5	13.0	—	—	
Dry weight } Total N }	9.8	11.8	11.5	13.0	16.3	12.2	13.3	15.4	20.0	—	—	

Acetic, propionic, butyric, valeric, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, linoleic, undecylic, traumatic, behenic acids (10, 100 and 1000 $\mu\text{g./ml.}$), tested individually; acids with molecular weights greater than that of caprylic acid tended to inhibit growth at 1000 $\mu\text{g./ml.}$; malonic, succinic, malic, fumaric, citric acids (0.01 M).

Thymine, uracil, adenine, guanine, xanthine (each 100 $\mu\text{g./ml.}$), creatine, creatinine, cholesterol, taurocholic acid (100 $\mu\text{g./ml.}$).

Chlorides of Na, K, Ca, and Mg were added singly, in certain pairs (Na + K) and (Ca + Mg), and all four combined to give final concentrations simulating those in whole blood, serum, blood cells, or muscle; supplements of the following salts: KI, (NH₄)₂SO₄, MgSO₄, FeSO₄, KClO₃, (0.01 M).

Sodium fluoride (0.01 M) and NaCN (> 0.0002 M) inhibited growth.

Longevity of cultures in the presence of various carbohydrates

The basal medium chosen for fermentation tests and for longevity tests in presence of various carbohydrates was heart broth (1 vol.) + casein digest (1 vol.), with the addition of 1 ml. Andrade's indicator/100 ml. complete medium. Each carbohydrate was added to a final concentration of 0.5 % (w/v) except starch which was added to final concentration 0.04 % (w/v).

Table 4. *The relative amounts of growth, and of acid production, and relative longevity of cells in cultures of Actinomyces israelii under various conditions*

Cultures were subcultivated at weekly intervals and also kept in continuous incubation in unchanged media in the presence of various carbohydrates.

Supplement	Relative amount of growth	Relative acid production	Longevity		
			14 days	21 days	35 days
Glucose	+++	AAA	LLL	LL	L
Fructose	+++	AAA	LLL	L	0
Sucrose	+++	AAA	LLL	L	L
Maltose	+++	AAA	LLL	0	0
Raffinose	+++	AAA	LLL	L	L
Xylose	++	AA	LLL	0	0
Rhamnose	++	AA	LLL	LLL	LL
Glycerol	+++	0	LLL	LLL	LLL
Sorbitol	+	0	LLL	LLL	LLL
Mannitol	+	0	LLL	LLL	LLL
Starch	+++	0	LLL	LLL	LLL
Dextrin	+++	0	LLL	LLL	LLL
Glycogen	+++	0	LLL	LLL	LLL
Nil	+++	0	LLL	LLL	LLL

+++ Excellent growth in 7 days, increasing markedly during 35 days.

++ Fair growth in 7 days, increasing slightly during 35 days.

+ Slow growth in 7 days, increasing very slightly during 35 days.

AAA rapid acid production in 7 days, continuing over 35 days.

AA slow acid production in 7 days, increasing slightly in 35 days.

Viability: LLL excellent, LL fair, L poor viability after successive weekly subcultures and under continuous incubation in unchanged medium at the times noted.

Strains X and Y were used and inoculations made initially from medium S. The tubes so inoculated were incubated continuously except for brief examination at weekly intervals for growth and acid production. At the end of each week of incubation, small inocula were transferred to fresh tubes of medium S to test viability. At the end of the first week of incubation of the initially inoculated tubes of carbohydrate media, inocula were removed to fresh tubes of similar carbohydrate media; at the end of a further week these last cultures were again transferred to fresh carbohydrate media and repeated serial subcultures maintained in this way. The results (Table 4) showed that the rate and quantity of growth roughly paralleled the rate and quantity of acid production; conversely the organism remained viable longest in those media containing supplements from which least acid was produced.

Glucose utilization and acid production during cultivation

Cells of all six strains were collected after the usual period of cultivation in media S, A₁, A₂, A₃ and dilution media F and G (Tables 1 and 2). The corresponding culture filtrates were analysed for glucose (method of Nelson, 1944) and for lactic acid (method of Barker & Summerson, 1941). Uninoculated tubes incubated under the same conditions served as controls. The results showed that of the glucose supplied *c.* 40 % was utilized and lactic acid equivalent to *c.* 30–60 % of the glucose utilized appeared in the medium. Lactic acid was not formed in the glycerol medium (A₂) or in the starch medium (A₃).

The effect of reducing substances on growth

Sodium mercaptoacetate, benzenesulphinic acid or mercaptoethanol at final concentrations from 0.1 to 2.5 % were added to medium S. Under anaerobic conditions the addition of these substances at 0.1 % made no difference; under aerobic conditions only very slight growth occurred as thin wispy colonies of limited viability. Under aerobic or anaerobic conditions these substances at 1.0–2.5 % were toxic. The addition of glutathione or cysteine under anaerobic conditions had no effect.

DISCUSSION

In earlier work the complex media used may have been beneficial in improving the reducing conditions, thereby counteracting, in part at any rate, poor anaerobic techniques. On the other hand such rich media may contain substances which are inhibitory to growth as well as those which are necessary for the organism.

In the present work we used an anaerobic technique which involved repeated evacuation and refilling of a vessel containing the culture tubes with nitrogen gas which has been passed through alkaline pyrogallol. This ensured removal of oxygen from the gas phase and also a considerable loss of dissolved oxygen from the fluid phase.

This enabled adequate anaerobic conditions to be established by an easy, repeatable technique and considerable simplification of the culture medium did not prove impossible.

Continuous subcultivation in the media described appears to depend on: separate sterilization of the components of media; sterilization under adequate but milder-than-usual conditions in previously sterilized containers; aseptic dispensing of the sterile components of the media; the use of the simplest possible medium, e.g. casein digest or hydrolysate with minimal supplements of broth + glucose, glycerol or starch; frequent subcultivation; good anaerobic conditions during cultivation. Our technique of progressive elimination of the heart broth moiety of a broth + casein hydrolysate + glucose medium has undoubtedly been justified, but we have failed to obtain a completely defined medium for *A. israelii*. Repeated attempts to eliminate the broth completely yielded organisms of only very limited viability.

The growth of our strains of *A. israelii* in each of our media during two years has remained typical: in liquid culture colonies were discrete, compact, hard, 'bread-crumbs', 'cauliflowers' or 'puffballs' which remained at the bottom of the culture tubes, the rest of the medium remaining clear. Surface colonies on solid media were abruptly raised, nodular, and appeared wet and glistening. No softening of the colonies, sudden increases in amounts of growth, development of an aerophilic or micro-aerophilic phase, or pigmentation of colonies has been observed in pure cultures; such changes we regard as indicative of contamination. The microscopic appearance of the cells remained characteristic throughout. It appears therefore that *A. israelii*, if treated according to our techniques soon after isolation in pure culture, is not difficult to maintain in culture for protracted periods.

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EXPLANATION OF PLATE

- Fig. 1. Cells of *Actinomyces israelii* harvested into microcentrifuge tubes after anaerobic growth for 7 days in media A₁, A₂, A₃ and S (Table 1). Four culture tubes containing a total of 10 ml. medium were harvested in each case.
- Fig. 2. Cells of *Actinomyces israelii* harvested into microcentrifuge tubes after anaerobic growth for 7 days in buffered and unbuffered medium A₁ (Table 1 and p. 469): (a) buffered with m/15 2-amino-2-(hydroxymethyl)-1,3-propanediol, pH 7·2; (b) buffered with m/15 phosphate buffer, pH 6·0; (c) buffered with m/15 phosphate buffer, pH 7·2; (d) no buffer added. One culture tube containing 2·5 ml. medium was harvested in each case.
- Fig. 3. Cells of *Actinomyces israelii* harvested into microcentrifuge tubes after anaerobic growth for 7 days in media A–O (Table 2). Four culture tubes containing a total of 10 ml. medium were harvested in each case.

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D. ERIKSON & J. W. PORTEOUS—THE CULTIVATION OF *ACTINOMYCES ISRAELII*. PLATE 1