STUDIES ON THE GROWTH OF SPECIES OF *ACTINOMYCES*

I. Cultivation in a Synthetic Medium with Starch

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Various complex organic media such as "hormone" agar, brain heart infusion agar, blood agar, chopped meat, etc., have been used by different investigators to isolate and maintain cultures of *Actinomyces israeli* (Erickson, 1940; Holm, 1948; Miller and Drake, 1951; Rosebury *et al.*, 1944; Thompson and Lovestedt, 1951). Erickson and Porteous (1953) have pointed out that "Such media are amongst the richest used for the cultivation of microorganisms, 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. israeli*." It seems worthwhile, therefore, to present a synthetic medium containing purified potato starch which has been used for the successful cultivation and maintenance of cultures of species of *Actinomyces* that have been described as *A. israeli* and *A. naeslundi*, and a single strain described as *A. bovis*.

MATERIALS AND METHODS

Fifteen strains of the genus *Actinomyces* were employed, although not all strains were used in all experiments. Thirteen of these (#260, 261, 262, 263, 277, 278, 279, 281, 282, 283, 284, 286, and 287) were the same strains used in previous studies (Howell and Fitzgerald, 1953). The two additional strains, #295 and 296, were obtained from the American Type Culture Collection as strains #10048 and 10049, respectively. Strain #295 was isolated from human pleural fluid; #296 from the left ankle of a patient with an atypical case of actinomycosis. Both of the latter strains were isolated in 1946 (Emmons, 1955, *personal communication*).

Maintenance of stock cultures. Stock cultures of all strains were maintained by serial transfer at weekly intervals on a casitone-yeast extract medium (modified fluid thioglycolate) having the following composition: casitone (Difco), 15 g; yeast extract (Difco), 5 g; glucose (anhydrous), 5 g; sodium chloride, 2.5 g; L-cysteine hydrochloride, 0.75 g; K_2HPO_4, 1.2 g; KH_2PO_4, 1.2 g; disodium ethylene diamine tetra-acetic acid,[3] 0.25 g; agar, 0.72 g; water to make 1000 ml; final pH 7.3. The agar was suspended in 200 ml of water and sterilized separately by autoclaving at 120 C (15 lb pressure) for 20 min. The remaining fraction of the medium, or the medium without agar, was sterilized by Seitz filtration. This medium is henceforth designated as "CYE" medium.

Routine transfers were made by inoculating a tube containing approximately 7 ml of this medium to which 0.5 g powdered $CaCO_3$ had been added with 1 drop of the growth suspension from the previous culture. The tubes were then placed in a Brewer jar; the latter was evacuated to 1 to 2 cm Hg, flushed three times with 5 per cent CO_2-95 per cent N_2 and incubated under slight positive pressure with 5 per cent CO_2-95 per cent N_2 at 37 C.

Preparation of inoculum. Inoculum of all strains, except #278 and 284, for all experiments was obtained by growing the organisms at 37 C for one or two transfers in tubes containing 5 ml of a modification of the medium used by Kline and Barker for the cultivation of *Butyribacterium rettgeri* (Kline and Barker, 1950) (see table 1), for convenience designated as medium I. Anaerobiosis and a carbon dioxide atmosphere were obtained by placing 5 drops of 10 per cent Na_2CO_3 and 5 drops of a saturated solution of pyrogallol

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[3] Disodium ethylene diamine tetra-acetic acid was kindly supplied by the Alrose Chemical Company, Providence, Rhode Island, under the trade name "sequestrene NA 2".

on the cotton plug and then immediately sealing the tubes with a rubber stopper. Strains #278 and 284 were grown on brain-heart infusion broth, pH 7.4, incubated in a Brewer jar with 5 per cent CO_2-95 per cent N_2 as described above. After 2 to 3 days' incubation, the cultures were centrifuged, and the cells suspended in a 0.05 per cent aqueous solution of L-cysteine hydrochloride adjusted to pH 7.2 before sterilization.

TABLE 1

Composition of medium of Kline and Barker for growth of Butyribacterium rettgeri (Kline and Barker, 1950), modified for growth of Actinomyces (Medium I)

	Per L
1. Salt solution, 4× strength	250 ml
KH_2PO_4	60 g/L
$(NH_4)_2SO_4$	4 g/L
$MgSO_4 \cdot 7H_2O$	0.8 g/L
$CaCl_2 \cdot 2H_2O$	0.08 g/L
Na-acetate $2H_2O$	1.2 g/L
2. L-Cysteine hydrochloride	1 g
3. Minor salts*	10 ml
$FeSO_4 \cdot 7H_2O$	0.4 g/L
$MnSO_4 \cdot 2H_2O$	0.015 g/L
$NaMoO_4 \cdot 2H_2O$	0.015 g/L
4. L-Tryptophan	0.04 g
5. Dextrose, anhydrous	10 g
6. Vitamin mixture	10 ml
Thiamin·HCl	20 mg/100 ml
Pyridoxine·HCl	10 mg/100 ml
Pyridoxamine·2HCl	5 mg/100 ml
Pyridoxal·HCl	5 mg/100 ml
Ca-pantothenate	20 mg/100 ml
Riboflavin	20 mg/100 ml
Nicotinic acid	10 mg/100 ml
Nicotinamide	10 mg/100 ml
p-Amino benzoic acid	1.0 mg/100 ml
Biotin	0.5 mg/100 ml
Folic acid	0.5 mg/100 ml
7. Purines and pyrimidines	10 ml
Adenine sulfate	200 mg/100 ml
Guanine·HCl·$2H_2O$	200 mg/100 ml
Uracil	200 mg/100 ml
Xanthine	200 mg/100 ml
Thymine	200 mg/100 ml
8. Coenzyme A	10 ml
5 mg/50 ml + 1 drop 50% Na_2S sol Stored under vacuum	
9. DL-Thioctic acid	
10 mg/100 ml 25% ethanol	1 ml
10. Casein hydrolyzate†	40 ml
11. Yeast extract, Difco	1 g

* Add 1 ml conc. HCl to water prior to addition of salts.

† Nutritional Biochemicals Corporation, vitamin free casein hydrolyzate (acid hydrolyzed) 10% solution.

Adjust to pH 6.5, make to 1000 ml and sterilize by filtration through a Selas 03 porcelain filter.

To insure uniformity of the inoculum the cell suspensions of all strains except those of #260, 278, 279, 284, 286, and 296 were then homogenized in a tissue grinder (A. H. Thomas #4288-B). The suspensions of strains #260, 279, 286 and 296 were easily homogenized by drawing the suspensions into, and then expelling them from, a 5-ml serological pipette at least ten times. Strains #278 and 284 grew diffusely in broth so that homogenization was unnecessary.

After homogenization, the suspensions were again centrifuged, the supernatant discarded and the cells resuspended in approximately 5 ml of the neutral cysteine solution. One ml of this suspension was then diluted 1:5 and the optical density determined in a Beckman Model B spectrophotometer at 660 mμ. Assuming a straight line relationship between optical density and cell concentration, the dilutions necessary to give an optical density of 0.5 were calculated; these dilutions were then prepared using the cysteine solution as diluent, and 0.05 ml used to inoculate each tube. In all cases, the optical density of the final inoculum was within the range of 0.500 ± 0.025. A straight line relationship between cell concentration and optical density is thus indicated.

General methods. The synthetic medium to be described is based upon a medium developed by the junior author for study of the vitamin requirements of *Histoplasma capsulatum* and, for convenience, is designated as medium II. It was prepared as indicated in table 2.

In all experiments the media were dispensed in 5-ml amounts into matched 20- by 150-mm colorimeter tubes. Tubes of media were inoculated within 2 hr after preparation. In those instances in which this was not possible nor feasible, they were stored under Na_2CO_3-pyrogallol seals.

After inoculation each tube was sealed as described and incubated at 37 C on a rotary shaker (New Brunswick Scientific Company, Model V) set at approximately 228 cycles per minute. Two to 4 tubes of each medium were inoculated with each strain of actinomycetes to be tested in each experiment except in the first. In this experiment, 16 tubes of each medium were inoculated with

TABLE 2

Composition of synthetic medium with starch used for Cultivation of Actinomyces (medium II)

	Per L
1. Salt solution, 4× strength (see table 1, item 1)	250 ml
2. Casein hydrolyzate*	40 ml
3. L-Tryptophan	40 mg
4. L-Cysteine hydrochloride	1.0 g
5. Glutathione	0.5 g
6. L-Asparagine	0.1 g
7. Minor salts (see table 1, item 3)	10 ml
8. Purines and pyrimidines (see table 1, item 7)	10 ml
9. Group 1 vitamins	10 ml
p-Amino benzoic acid	20 mg/100 ml
Folic acid	5 mg/100 ml
Vitamin B_{12}	10 μg/100 ml
Hemin†	2 mg/100 ml
Citrovorum factor‡	10 μg/ml
10. Group 2 vitamins	10 ml
Thiamin·HCl	20 mg/100 ml
Riboflavin	20 mg/100 ml
Inositol	20 mg/100 ml
11. Group 3 vitamins	10 ml
Nicotinic acid	10 mg/100 ml
Nicotinamide	10 mg/100 ml
12. Group 4 vitamins	10 ml
Pyridoxal·HCl	10 mg/100 ml
Pyridoxamine·2HCl	10 mg/100 ml
13. Group 5 vitamins	10 ml
Biotin	1 mg/100 ml
Pimelic acid	1 mg/100 ml
Oleic acid	10 mg/100 ml
14. Ca-pantothenate	20 mg/100 ml 10 ml
15. Coenzyme A	10 ml
Pabst, 70%, 10 mg/100 ml + 2 drops 50% Na_2S	
Stored under vacuum	
16. DL-Thioctic acid	1 ml
10 mg/100 ml, 25% ethanol	
17. Dextrose, anhydrous	10 g

* Nutritional Biochemicals Corporation, vitamin free, casein hydrolyzate (acid hydrolyzed), 10% solution.

† Dissolve in dilute NH_4OH.

‡ In 30% ethanol.

Adjust to pH 6.5 with 20% KOH and make to 500 ml. Immediately sterilize by filtration through Selas 03 porcelain filter. After sterilization, add 500 ml 0.1% starch solution sterilized previously by autoclaving at 15 lb pressure (120 C) for 30 min. Starch suspension prepared by adding 10 g of starch (Baker's purified potato starch, further purified by extraction with methanol in a Soxhlet apparatus for 48 hrs) to 90 ml cold distilled water which is then poured rapidly into approximately 10 volumes of boiling distilled water. Make volume to give a final concentration of 0.1%.

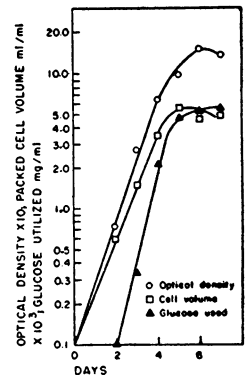

Figure 1. Rate of growth of *A. israeli*, Strain #277 on medium II at pH 6.5 as determined by measurements of optical density, packed cell volume and glucose utilized.

each strain so that two replicate tubes could be harvested daily for 8 days. In this instance growth was measured by determinations of optical density, packed cell volume, and glucose utilized. When plotted, data obtained from optical density measurements and packed cell volume gave similar curves. Data obtained from glucose utilization in some instances showed a short lag period, after which the curve was parallel with those obtained from the other determinations (figure 1). Therefore optical density determinations alone were used in all subsequent experiments as an index of the amount of growth present at any given time.

Optical density determinations were made on a Beckman Model B spectrophotometer at 660 mμ at zero time and at daily intervals thereafter. As the majority of strains studied grew as distinct granules of variable size, the tubes were opened daily, homogenized with a 5-ml serological pipette as described above, and resealed with Na_2CO_3-pyrogallol seals. When an optical density reading of 1.5 or greater was obtained on any tube, appropriate dilutions were made and the growth recorded in growth units (= O.D. × 1/dil). Values given represent the mean of all determinations made for a single strain on a given medium at a given time.

Products of glucose fermentation were determined by essentially the same methods as those described previously (Pine and Barker, 1954). Ethanol was determined qualitatively and quanti-

tatively by diffusion into dichromate solution (Winnick, 1942), and the resulting acetic acid was identified and determined by Duclaux distillations.

All amino acids and vitamins employed were obtained from Nutritional Biochemicals Corporation; oleic acid from the Hormel Foundation; thioctic acid from Lederle Laboratories Division of the American Cyanamid Company; coenzyme A (70 per cent pure) from Pabst Brewing Company; citrovorum factor was kindly supplied by Dr. M. Silverman (National Institute of Arthritis and Metabolic Diseases).

RESULTS

Preliminary experiments, conducted over a period of 9 months, indicated that the CYE medium described above, when compared qualitatively with the usual media used to maintain cultures of the genus *Actinomyces*, supported better growth of all 15 strains studied than any other medium employed. Furthermore, when used for serial transfer of stock cultures, it generally produces the type of growth characteristic of each strain. Using this medium, then, without agar, as a baseline, media I and II were studied for their ability to support growth of 7 strains. It was found that the amount of growth produced by all strains tested after 7 days' incubation on either medium I or medium II, both adjusted to pH 6.5, was in most cases equal to or greater than that obtained on the CYE medium incubated 8 days (table 3). Additional experiments showed that medium II, at pH 6.5, supported excellent growth of all remaining strains except #278 and 284. Further experiments, therefore, were confined to studies on medium II since, except for the starch and casein hydrolyzate, its composition is defined.

TABLE 3

Relative amount of growth of specified strains of Actinomyces on three different media

Strain	Medium*		
	I	II	CYE
	Growth Units		
277	2.063	1.345†	2.188
281	1.353	3.000	0.995
287	3.375	0.830	1.250
283	—	0.390†	1.450
295	3.750	3.750	1.438
279	3.688	2.283	1.125
286	4.750	2.813	1.563

* Medium I: Modification of Kline and Barker's medium for *Butyribacterium rettgeri;* incubated 7 days. Medium II: Modification of Pine's synthetic medium for *Histoplasma capsulatum;* incubated 7 days. CYE Medium: Casitone yeast extract medium (without agar) containing 0.025% disodium ethylene diamine tetra-acetic acid; incubated 8 days.

† Very large aggregates still present after homogenization.

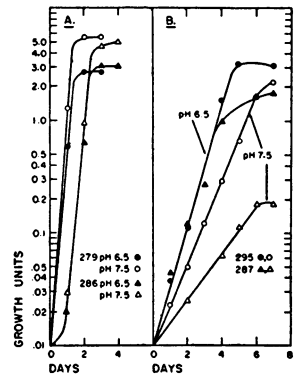

Figure 2. Response of strains #279, 286, 287 and 295 on medium II at pH 6.5 and 7.5.

However, when the pH of medium II was adjusted to pH 7.0 or 7.5 instead of 6.5, the response varied, depending on the type of organism. For example, with strains #279 and 286 neither the rate nor the amount of growth was considered to be influenced significantly by the change in pH (figure 2A). In the group of strains represented by #295 and including strains #260, 261, 282, 283, and 296, the rate of growth was decreased but the amount of growth obtained after 6 to 7 days' incubation was not significantly altered by raising the pH to 7.0 or 7.5 (figure 2B). In a third group, represented by strain #287, and including strains #262, 277, and 281, both the rate and amount of growth were markedly decreased at pH 7.5 (figure 2B).

Some growth of strain #278 was obtained on medium II at pH 6.5, though its rate of growth was slower and the amount of growth obtained after 6 days incubation less than that obtained with any of the other strains tested except 284. The addition of 0.1 per cent beef extract or bovine albumin (Fraction V) or the substitution of 0.4 per cent casitone (Difco) for the casein hy-

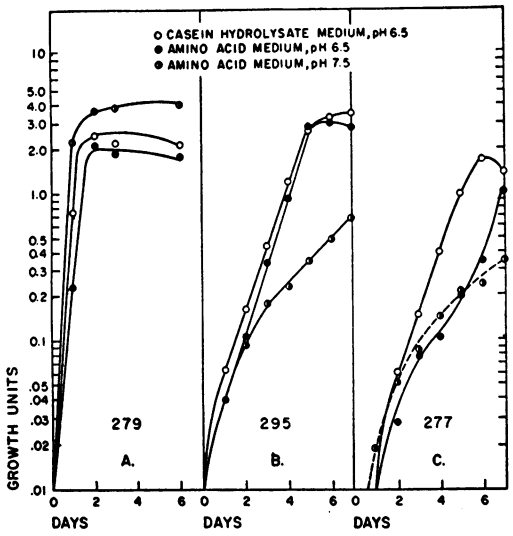

Figure 3. Comparison of rates of growth of strains #279, 295 and 277 on medium II prepared with casein hydrolyzate and prepared with amino acids.

drolyzate did not significantly alter either the rate or amount of growth of this strain.

Repeated attempts to grow strain 284 on medium II at either pH 6.5 or 7.5 were unsuccessful. However, excellent growth of this strain was obtained when 0.4 per cent casitone (Difco) was substituted for the casein hydrolyzate.

Following the successful cultivation of 13 of the 15 strains of actinomycetes on medium II, attempts were made to substitute a known mixture of amino acids for the casein hydrolyzate. Ten grams of an amino acid mixture,[4] based upon the percentage composition of casein as given by Hawk, Oser and Summerson (1951), with the addition of 0.3 per cent L-hydroxyproline, was suspended in 250 ml warm distilled water (4 per cent solution). This solution was then used in preparing medium II in place of the casein hydrolyzate. Cysteine was omitted from the amino acid mixture and added separately as indicated in table 2. Growth curves obtained with medium II prepared with casein hydrolyzate were compared with those on the same medium prepared with the amino acid mixture. It is obvious from the sample curves of figure 3 that the amino acid mixture can be substituted successfully for casein hydrolyzate, although the rate of growth of the group of strains represented by #277, (277, 262, 281 and 287) was slightly retarded. Strains #263, 278, 282, 283, and 284 were not tested on the amino acid medium.

As previously indicated, all cultures in all experiments were incubated under constant shaking. It was believed initially that under such conditions a smoother type of growth might be obtained. Shaking would also impose a more rigorous test of the ability of a medium to allow growth of small inocula. Furthermore, with acid media, it was felt that shaking would allow more carbon dioxide to dissolve in the medium, since some strains have been reported to require carbon dioxide for growth (Holm, 1948; Miller and Drake, 1951). In a single experiment, the rates of growth obtained with 6 strains incubated on the shaker were compared with those obtained from duplicate tubes incubated under stationary conditions. The results indicated that there was no significant difference in the growth rates in some instances; in others, slightly higher rates were obtained from the stationary cultures.

In preliminary experiments with strains 286, 287, and 295, an attempt was made to assess the role of the starch. It was found that starch was essential for growth of all three strains at pH 6.5. It was stimulatory for strains 286 and 295 and required for 287 at pH 7.5.

In order to test the nutritional value of medium II adjusted to pH 6.5 for the continued cultivation of strains of actinomycetes, serial transfers of 10 strains were made twice weekly for 3 to 4 transfers. Transfers were made at these intervals since it was found that the pH of the medium dropped from 6.5 to 4.0 or 4.5 after incubation for 6 to 7 days. In making the transfers the cultures were homogenized as described above and approximately 0.05 ml (1 drop) of the culture suspension transferred to a new tube containing approximately 5 ml of medium. All strains in all subcultures gave excellent growth and showed no evidence of decreased vitality or morphological change.

Analyses of the fermentation products of strains 277, 279, 281, 286, and 287 on medium II

[4] Composition of amino acid mixture: L-alanine, 0.55 g; L-arginine, 0.43 g; L-aspartic acid, 0.61 g; L-glutamic acid, 2.33 g; glycine, 0.05 g; L-histidine, 0.21 g; L-hydroxyproline, 0.03 g; L-isoleucine, 0.63 g; L-leucine, 0.97 g; L-lysine·HCl, 0.76 g; DL-methionine, 0.34 g; L-phenylalanine, 0.50 g; L-proline, 0.80 g; DL-serine, 0.77 g; L-threonine, 0.38 g; L-tryptophan, 0.12 g; L-tyrosine, 0.67 g; DL-valine, 0.65 g.

showed that only 37 to 58 per cent of the glucose was fermented. Carbon recoveries of the fermented glucose ranged from 96 to 104 per cent. Mixtures of acetic acid, formic acid, and ethanol accounted for 2 to 8 per cent of the carbon recovered, the residual being lactic acid. Carbon dioxide was not formed in significant amounts. The formation of lactic acid by six strains of *A. israeli* has been reported by Erickson and Porteus (1953).

DISCUSSION

The application of simple, practical quantitative methods for the estimation of the rates of growth of cultures of the genus *Actinomyces* and and the successful cultivation of at least two types of these organisms on a liquid synthetic medium containing starch affords an opportunity for critical studies of the growth requirements of these organisms.

The role of the starch in the medium is uncertain. Direct coenzyme A determinations of the starch, as given by Brown and Snell (1954), have shown that it is free of coenzyme A or its precursors. Results with Histoplasma have indicated that the product is free of thiamin, nicotinic acid, inositol, biotin, oleic, and thioctic acids (Pine, 1955, *unpublished data*). It is believed therefore that the sole function of the starch in this medium is to maintain nontoxic concentrations of oleic acid. Further studies on the role of starch and oleic acid are in progress.

The strains of actinomycetes studied have been isolated from various sources and carried in artificial culture for variable periods of time. Two, #279 and 286, were originally isolated from, respectively, a sinus following a tooth extraction and gingival scrapings. On the basis of their colony form on brain-heart infusion agar or the "hormone" agar of Bailey (1925), their growth in fluid thioglycolate, and their ability to grow as well aerobically as anaerobically on hormone agar or brain-heart infusion blood agar (Howell, 1955, *unpublished data*), both strains appear to be typical of the smooth type of *A. naeslundi* described by Thompson and Lovestedt (1951). Three strains, #262, 263 and 287, were originally isolated from human cases of cervico-facial actinomycosis; #277 from a brain abscess (human); and #281 from an abscess of the cheek (human). All five produce the rough, breadcrumb or cauliflower-like colonies described by Negroni and Bonfiglioli (1937), Erickson (1940), Rosebury *et al.* (1944) and Thompson (1950) as being characteristic of *A. israeli*. Two strains, #278 and 284, on the basis of colony form and growth in broth, appear to be typical of *A. bovis* as described by Erickson (1940) and Thompson (1950). The remainder of the strains were originally isolated from various human sources such as gingival scrapings, mandibular abscesses, etc., and are somewhat variable in their gross morphological characteristics, though in general resembling *A. israeli*. Regardless of the length of time all have been carried in continuous subculture by the senior author, in some instances up to 18 mo, there has been no evidence of any permanent gross or microscopic morphological change, evidence of colonial mutation, or change from a rough to a smooth form. Therefore, while it is possible that more recently isolated strains might have given slightly different results, this possibility seems unlikely.

As yet the fact that an initial pH of 7.0 or 7.5 inhibits the rates and amounts of growth of several strains is unexplained. However, it would appear that some essential growth factor available at pH 6.5 becomes unavailable to strains #277, 281, 287, 262, and 263 at pH 7.0 or 7.5, since the amount of growth of these strains is substantially decreased at either of these higher pH levels.

In attempts to obtain growth of strain 284, medium II was fortified with bovine albumin (Fraction V), beef extract, or casitone (Difco). Although some growth was obtained by the addition of 0.1 per cent beef extract, the substitution of the casein hydrolyzate with casitone (pancreatic digested U.S.P. casein) allowed much better growth. The albumin appeared to have no effect.

SUMMARY

Thirteen of 15 strains of the genus *Actinomyces*, representing at least two different types, including strains that have been described as *A. israeli* and *A. naeslundi* have been successfully grown and maintained in a liquid synthetic medium containing starch. The rates or amounts of growth or both of certain strains were significantly decreased by raising the pH from 6.5 to 7.0 or 7.5. Lactic acid and traces of ethanol, acetic and formic acids were formed from glucose.

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