ASPECTS OF ANTIBIOTIC PRODUCTION BY TWO STRAINS OF STAPHYLOCOCCI

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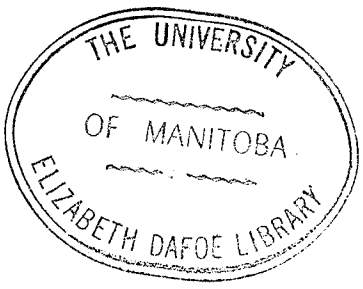

ABSTRACT

Antibiotic production by Staphylococcus epidermidis strains 29297 and 36534 was affected to the same extent by variations in oxygen tension. Strain 36534 produced much greater amounts of antibiotic when grown in a mixture of 10% carbon dioxide in air. Antibiotic production by strain 29297 was depressed in this mixture.

Dialyzable and heat-stable components of proteose peptone supported antibiotic production by the two strains. It was further shown that these components were amino acids and that L-tryptophan was essential for antibiotic production but not for growth.

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INTRODUCTION

Hsu (1966) investigated the production of antibacterial substances by the genus Staphylococcus. The properties of the antibiotics isolated from coagulase negative strains 29297 and 36534 were examined and it was suggested that they might be small molecular weight peptides.

The object of the present study has been to investigate in greater detail cultural conditions and nutritional factors which affect antibiotic production by strains 29297 and 36534. It is hoped that the information obtained will provide the basis for the development of a synthetic medium in which the mechanism of biosynthesis of these antibiotics may be studied.

LITERATURE REVIEW

Nutritional Requirements of Staphylococci

(a) Growth factors

The growth factor requirements of members of the genus Staphylococcus have been investigated. Hughes (1932) showed that a component present in meat extract allowed heavy growth of staphylococci when added either to a peptone medium or to a synthetic medium. Analysis of this component showed that it was a member of the group of substances known as natural bases. Knight (1935) prepared concentrates of this staphylococcal growth factor from yeast extract and tested its effect on the growth of selected strains of staphylococci in a chemically defined medium. The substance described by Knight was a weak base which yielded relatively soluble compounds with most base precipitants. Knight (1937a) also found that the Staphylococcus aureus growth factor concentrate prepared from yeast extract could be replaced by nicotinamide and vitamin B_1 (Thiamin) together. In the absence of either compound no growth occurred. Neither nicotinic acid nor its derivatives were as effective as nicotinamide in promotion of the growth of Staph. aureus. In further studies, Knight (1937b) demonstrated that maximum growth could be obtained when nicotinamide was added to the culture medium at a concentration of 10^{-6} M. when vitamin B_1 was held

constant at 2.0×10^{-7} M. Also, heavy growth was obtained when vitamin B_1 was added at a concentration of 10^{-8} M. with nicotinamide held constant at 10^{-5} M. Knight and McIlwain (1938) showed that vitamin B_1 and nicotinamide are highly specific for the growth of Staph. aureus, and they emphasized that growth activity cannot be maintained if compounds which diverge in structure from that of vitamin B_1 and nicotinamide are supplied to the organisms.

Porter and Pelczar (1941) attempted to increase the growth of Staph. aureus in the chemically defined medium prepared by Knight (1935) to a level comparable to that in glucose-meat infusion broth cultures. Their results indicated that the essential growth substance required by fastidious strains in a synthetic medium was biotin or some closely related compound. These workers concluded that since maximum growth equal to that in meat infusion medium was not obtained, essential stimulatory ingredients were either absent or there were physical differences in the medium.

Koser et al (1938) observed that a strain of Staph. albus grew in a medium of known composition which contained thiamin and nicotinic acid. They also showed that the growth rate of this strain was considerably faster in broth and in the chemically defined medium to which fractions of spleen preparations were added. It appeared that some other growth factor was required by this strain for optimum growth. Vilter

and Spies (1940) have shown that the addition of pyridoxine at a concentration of 0.3 to 1.2 µg/ml serves as an accessory growth factor for Staph. albus. However, since other groups had demonstrated that nicotinamide promoted better growth of Staph. aureus than nicotinic acid, Koser and his co-workers might have achieved faster growth rates for their strain with nicotinamide.

Gretler et al (1955) studied the vitamin requirements of 90 coagulase positive and 46 coagulase negative strains of staphylococci in a casein hydrolysate medium. Thiamin and nicotinic acid were essential for the growth of all the coagulase positive strains tested. Of the 46 coagulase negative strains tested, 38 possessed an absolute requirement for biotin. In addition, 12 coagulase negative strains required pantothenic acid for growth, and two of these required pyridoxine. Kogl and van Wagtendonk (1938) showed that the addition of 5×10^{-6} µg of biotin methyl ester stimulated the growth of Staph. aureus in the medium described by Knight (1937a). However, this growth factor was apparently not essential for their strain since it was able to grow in the basal medium without biotin.

The investigations noted demonstrate that there are wide variations among species and strains of staphylococci with respect to their vitamin requirements.

(b) Nitrogen requirements

Staphylococci require an organic source of nitrogen for growth. Members of this genus thrive best if amino acids, amides or purines are provided.

Evidence in the literature regarding the essential amino acid requirements for growth of staphylococci varies widely. Fildes et al (1936) described a nutrient medium suitable for the growth of a typical wild strain of Staph. aureus. This medium consisted of 14 amino acids, salts, glucose and the Staphylococcus factor described earlier by Hughes (1932) and Knight (1935). Serine, cystine and tryptophan were omitted from this medium. Serine inhibited the growth of the organism and cystine and tryptophan were unnecessary. Fildes and Richardson (1937) further showed that growth of Staph. aureus without cystine was possible only if organic sulphur compounds which contained a mercapto (-SH) group were accessible to the organisms for cystine synthesis.

Gladstone (1937) studied the effect of tryptophan on the growth of 26 strains of staphylococci in a complete amino acid medium. Only four of 25 strains able to grow in the complete medium were unable to grow in the absence of tryptophan. His studies indicate that some staphylococci differ in their ability to synthesize tryptophan de novo.

Gladstone has determined which amino acids are most necessary for the rapid growth of various staphylococcal

strains. These are cystine, leucine, valine, proline, glycine, aspartic acid, phenylalanine, and arginine. Histidine and methionine are less important. Tyrosine and lysine in most cases are not required. Two representative strains, a coagulase positive Staph. aureus Wood 46 (N.C.T.C. No. 3095) and a coagulase negative Staph. albus Wood 86 (N.C.T.C. No. 3094) showed no significant differences in terms of their essential amino acid requirements. These results suggest that all wild type members of the genus Staphylococcus have similar capabilities for the synthesis of cellular material although Gladstone has further shown that these same organisms can be trained to grow in the absence of an organic nitrogen source.

Mah et al (1967) have investigated the nutritional requirements of Staph. aureus S-6, a strain which produces enterotoxin. They found that 11 amino acids were necessary for optimal growth of the S-6 strain. These were glycine, valine, leucine, threonine, phenylalanine, tyrosine, cysteine, methionine, proline, arginine and histidine. The carbon source used was glucose which was added at a concentration of one per cent. Their conclusions agree with those of Gladstone that there are strain differences with respect to amino acid requirements. The S-6 strain needed three amino acids, cysteine, threonine and tyrosine, which were not required by the strains investigated by Gladstone. However, Gladstone's

strains required cystine and aspartic acid which were not necessary for growth of the S-6 strain.

(c) Mineral requirements

Little is known about the exact mineral requirements for the growth of staphylococci.

Shooter and Wyatt (1955, 1956) have studied the effect of certain ions on the growth of a strain of Staph. aureus. They found that magnesium and calcium were required for growth of their test strain. In further studies they showed that potassium ions were essential for growth of their strain of Staph. aureus. Clostridium welchii (Shankar and Bard, 1952), lactic acid bacteria (MacLeod and Snell, 1947), and Escherichia coli (Friedman and Fox, 1954), also require potassium ions for growth.

Young and Barker (1964) found that at a concentration of 10 mM magnesium, Staph. aureus (strain Duncan) ribosomes were almost completely dissociated into subunits. Mao (1967) showed that 50% of Staph. aureus 209P ribosomes were in 100S particles and 45% of the ribosomes were in 70S particles when the concentration of magnesium was 16 mM. These results suggest that in whole organisms, concentrations of magnesium ions sufficient to maintain structural and functional ribosomal particles are required for the synthesis of proteins. This may be more critical for the synthesis of extracellular proteins and enzymes than for structural intracellular proteins.

Weinberg (1964), in studies of secondary biosynthetic processes of Bacillus strains, found that concentrations of manganese in excess of the quantity required to maintain vegetative growth were necessary for the production of antibiotics, D-glutamyl peptides, endospores, bacteriophage, and protective antigens. This work is in agreement with the effect of magnesium ions on ribosomal integrity and protein synthesis by Staph. aureus as shown by Mao above.

(d) Carbohydrates

Strasters and Winkler (1963) have studied the pathways of carbohydrate oxidation in several strains of Staph. aureus. They found that strains grown in broth readily oxidized glucose, gluconate, ribose, succinate and lactate, which indicated that the pentose and citric acid cycles both operated in these cells. However, with cells grown on 0.1% glucose, they noted that oxidation of intermediates of the pentose and citric acid cycles was almost completely suppressed. With cells grown both in broth and glucose, carbon dioxide was produced from glucose. This indicated that the pentose cycle operates in cells grown in both media.

Ivler (1965) found that coagulase positive staphylococci differ from coagulase negative staphylococci with respect to their oxidative properties and endogenous respiration when grown on glucose. Coagulase negative cells

continued to respire in the presence of glucose. Coagulase positive cells, on the other hand demonstrated suppressed glucose oxidation. This effect was probably because of higher levels of endogenous respiration already present in coagulase positive cells. With regard to the metabolism of glucose, Ivler found that increased concentrations of glucose (from 0.5% to 1.0%) resulted in suppression of citric acid cycle enzymes in coagulase negative cells. In coagulase positive cells, aconitase or isocitric dehydrogenase activity was not observed when glucose was added at a 0.5% concentration. The addition of glucose at a concentration of 1.0% to a yeast extract and mineral salts medium increased the growth rate and total cell yield of coagulase positive cells.

In general, Ivler's results demonstrate that oxidative processes differ in coagulase positive and coagulase negative staphylococcal cells. However, both species are similar in one respect. In the presence of glucose, oxygen consumption is depressed. In the presence of amino acids such as alanine and glutamic acid, the ability to consume oxygen is completely eliminated. The demonstration that oxygen consumption is eliminated in the presence of glutamic acid does not agree with the work of Strasters and Winkler (1963). These authors showed that the rate of oxygen uptake in Staph. aureus cells grown with or without glucose was seven or eight times greater than the rate of oxygen uptake when glutamic acid was supplied as the exogenous substrate.

Antibiotic Production by Staphylococci

The activity and properties of antibiotics produced by staphylococci have been reviewed by Hsu (1966).

The production of antibacterial substances by members of the genus Staphylococcus is an uncommon phenomenon. Jennings and Sharp (1947) discovered that only 10% of 205 strains of staphylococci which they tested would inhibit the growth of Corynebacterium diphtheria, C. xerosis, and C. pseudodiphtheriticum. Hsu and Wiseman (1967) reported that only 8.5% of 1065 coagulase positive and 4.9% of 387 coagulase negative strains of staphylococci isolated from clinical specimens showed antibiotic activity when tested on solid medium against the Staph. aureus Oxford 209P indicator strain.

Gardener (1949) has described two media suitable for the production of a heat-stable, non-dialyzable antibiotic by a strain of Staph. aureus. One of these media consisted of 1% each of Lab Lemco and Eupeptone with 0.5% sodium chloride and tap water. The initial pH of this medium was 7.6. The other medium was composed of a casein digest, mono- and di-potassium hydrogen phosphate salts, ferrous sulphate, and 0.25% glucose. Su (1948) demonstrated that good yields of Micrococcin, a heat-stable, dialyzable antibiotic produced by Micrococcus varians, could be achieved in a peptone medium with added sodium chloride. The initial pH of this medium

was 7.4. Su also obtained good growth of this organism with yeast extract, acid-hydrolyzed gelatin, or acid-hydrolyzed casein. However, yields of antibiotic in these media were poor.

Murray and Loeb (1950) found that antibiotics from Staph. aureus and from β-hemolytic streptococci which were active against their Strept. pyogenes indicator strain could be produced in Ficin (a meat digest) supplemented with peptone at a pH of 7.0.

Barrow (1963) described the production of a heat-stable (in acid pH only), dialyzable antibiotic which was inactivated by trypsin but not by pepsin. The organism which produced this antibiotic was the Type 71 strain of Staph. aureus. This antibiotic was produced in a horse flesh tryptic digest broth with added 0.0002% (w/v) cystine·HCl. Barrow noted that increased yields of antibiotic were obtained when glucose or mannitol were added. The cultures were incubated overnight at 37°C, and antibiotic production paralleled growth closely.

Loeb et al (1950) showed that titres of a heat-stable, dialyzable antibiotic produced by a strain of M. epidermidis (now called Staph. epidermidis; Breed et al, 1957) were greater when 0.5% and 1.0% proteose peptone were added to a nutrient broth basal medium. Antibiotic production was greater with increased proteose peptone concentrations.

The proteose peptone appeared to provide some nutritional factor necessary for satisfactory production of the antibiotic. Loeb did not investigate the properties of this factor. Conditions for optimal antibiotic production are usually emperically defined by the authors.

No literature is available which describes the production of antibiotics from staphylococci in a completely synthetic medium. The wide variations in techniques and media employed by various workers make it difficult to draw conclusions about the mechanisms of synthesis of antibiotics by staphylococci. Such mechanisms can only be elucidated by the use of media of known chemical composition under different cultural conditions.

MATERIALS AND METHODS

Cultures

(a) Antibiotic-producing strains

Two coagulase negative strains of staphylococci, designated 29297 and 36534, were employed throughout this investigation. These strains, supplied by Mr. A. Hsu of this department, were characterized as Staph. epidermidis according to the criteria of Breed et al (1957).

(b) Indicator strain

Staph. aureus Oxford 209P was used exclusively as an indicator of antibiotic production by the producing strains.

The cultures were received in freeze-dried ampoules. These were broken open, suspensions of the dried strains were made in Brain Heart Infusion (BHI) broth and these were then subcultured onto BHI agar slants. The strains were plated out and their purity was checked by the Gram staining reaction. After 24 hours of incubation, growth from the slants was suspended in BHI broth and freeze-dried. A separate ampoule of each strain was used for each experiment.

Agitation of Cultures

Cultures were incubated in an Eberbach reciprocating water bath (Canadian Laboratory Supplies Ltd. Montreal, P.Q.).

For experiments with carbon dioxide, a New Brunswick Controlled Environment Incubator Shaker (Model R-25, New Brunswick Scientific Co. Inc., New Brunswick, New Jersey) was employed. The reciprocating stroke distance of both instruments was adjusted to one inch.

Assay Methods

(a) Growth

Optical density (O.D.) measurements were made in the Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York) with filter No. 69.

(b) Antibiotic production

Antibiotic production was assayed by the methods of Hsu and Wiseman (1967). Nine milliliters of solid medium contained in plates which measured 15 x 60 mm was stabbed in duplicate with a Pasteur pipette charged with a twice-washed culture of the test strain. The plates were incubated at 37°C for various lengths of time and were then sprayed with a suspension of the Oxford indicator strain which had been standardized to an O.D. of 0.1 in double-strength BHI broth. The plates were sprayed with this suspension which was contained in a glass atomizer. After further incubation overnight at 37°C, the inhibition zones adjacent to the test strain were measured and the mean of two estimates was recorded.

When liquid medium was used, the culture fluid was first centrifuged at 13,000 x g for 10 min in a refrigerated centrifuge. The pH of the supernatant was adjusted to 7.2 with concentrated HCl, and serial two-fold dilutions of this fluid were made in three ml volumes of distilled water. The tubes were autoclaved at 10 lb pressure for 10 min. Three milliliters of a suspension of the indicator strain adjusted to an O.D. of 0.1 in double-strength BHI broth was then added to each tube and initial O.D. readings were taken. After overnight incubation at 37°C, the tubes were shaken on a Vortex Jr. Mixer (Scientific Industries Inc.) and the optical densities of each tube were measured again. The difference between the two readings was plotted on graph paper against the $\log_2$ dilution of antibiotic. The end-point was taken as the $\log_2$ dilution of antibiotic which corresponded to the O.D. of the indicator strain at the mid-point of the linear portion of the growth curve.

(c) Protein

Protein was determined by O.D. measurements at 280 mμ in the Unicam SP800 double-beam recording spectrophotometer (Unicam Instruments Ltd., Cambridge, England).

(d) Phosphorus

Total phosphorus and inorganic phosphate were determined by the method of Fiske and Subbarow (1925).

Chromatography

(a) Gel filtration

A Sephadex K25/100 column (Pharmacia Canada Ltd., Montreal, P.Q.) was used in the fractionation of proteose peptone components which were responsible for antibiotic production. A single batch of Sephadex G-25 (medium grade), obtained from the same company, was heated at 60°- 80°C for six hours in distilled water, and was used in all experiments. The fines were decanted off four or five times, and a dilute slurry of the prepared Sephadex in distilled water was poured into the column to a height of 90 cm.

The void volume of the column was determined with a 0.1% solution of Blue Dextran 2000 (Pharmacia Canada Ltd.) in distilled water. Ten milliliters of proteose peptone factors were applied to the column and the components were eluted with distilled water at a flow rate of 50 ml/hr. The effluent was collected in 10 ml volumes in a Buchler fraction collector (Buchler Instruments Inc., Fort Lee, New Jersey) and the fractions were scanned at 280 mμ in the spectrophotometer. All chromatographic experiments were performed at room temperature.

Sodium azide at a concentration of 0.1% was used as a column preservative. Before fractionations were begun, the preservative was flushed from the column with three

liters of distilled water.

(b) Paper chromatography

One dimensional descending chromatography was performed on 23 x 57 cm sheets of Whatman 3 mm paper. Twenty-five microliters of sample at a concentration of 2 mg/ml were applied to the paper, dried, and chromatographed in butanol-acetic acid-water (4:1:5). The papers were then sprayed with a solution of 0.1% ninhydrin in butanol and heated at 160°C for five min. This procedure revealed ninhydrin-positive spots.

When hydrolysis of samples was necessary, the procedure was performed as follows. One milliliter of 6N HCl was added to four mg of sample contained in a glass ampoule. This was sealed and heated at 120°C for 18 - 20 hr when it was opened and neutralized with 4N NaOH.

EXPERIMENTAL RESULTS

Cultural and Environmental Factors Which Affect Antibiotic Production

(a) Temperature

The effect of incubation temperature on antibiotic production in staphylococcal strains 29297 and 36534 has been investigated. For these experiments, growth from overnight BHI agar slant culture of the test strains was washed, centrifuged twice in sterile 0.01M phosphate-buffered saline (Cruickshank, 1965) and adjusted to an optical density of 0.350. To 50 ml of modified Dolman-Wilson medium (see Appendix) in 250 ml Erlenmeyer flasks was added 0.1 ml of these suspensions. The flasks were incubated for three days over the temperature range of 26 - 40°C in the Eberbach reciprocating water bath with an aeration rate of 110 strokes/min. Antibiotic activity was measured as described in Materials and Methods. The results of these experiments are shown in Fig. 1.

Optimal antibiotic production for strain 29297 was achieved at an incubation temperature of 28°C. Strain 36534 produced slightly higher titers at 37°C than at 28°C, but the difference was not judged significant. For practical reasons therefore, all further liquid medium cultures in the various experiments were incubated at 28°C. For both strains, little difference in growth yield was observed after three

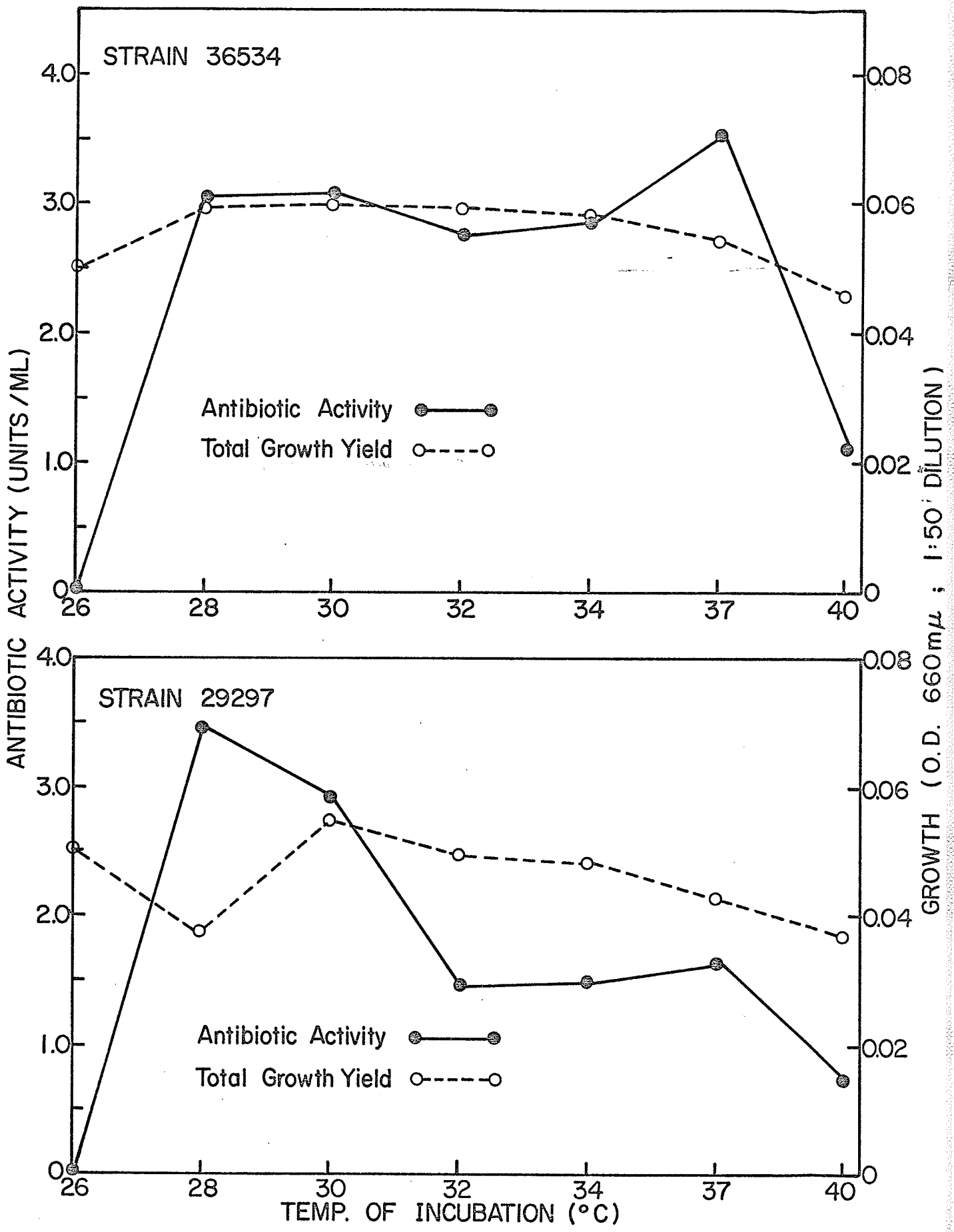


Fig. I The effect of incubation temperature on total growth yield and antibiotic production by staphylococci.

days of incubation. It should be pointed out that the growth curves in Fig. 1 reflect maximal yield and not growth rate.

(b) Aeration

In a study of the effect of aeration on antibiotic production, cultures of the test strains were incubated in the reciprocating water bath at shaking speeds which ranged from 40 - 125 strokes/min. Overnight BHI agar slant cultures of the test strains were prepared as described above. After three days of incubation at 28°C, antibiotic activity was measured.

Figure 2 demonstrates that optimal antibiotic production was achieved by both strains at a shaking speed of 75 strokes/min. Growth as reflected by O.D. reached a maximum between 75 - 100 strokes/min. It is evident that growth yields are less strikingly affected by aeration rates than is antibiotic production. A stroke rate of 75 strokes/min was used in all subsequent experiments in which liquid medium was required.

(c) Addition of carbon dioxide

Wiseman (1963) showed that certain strains of Staph. aureus produced greater yields of β-hemolysin when grown in an atmosphere which contained 25% carbon dioxide in air. Staphylococcal strains 29297 and 36534 were tested for their ability to produce antibiotic in the presence of 10% or 25% carbon dioxide in air.

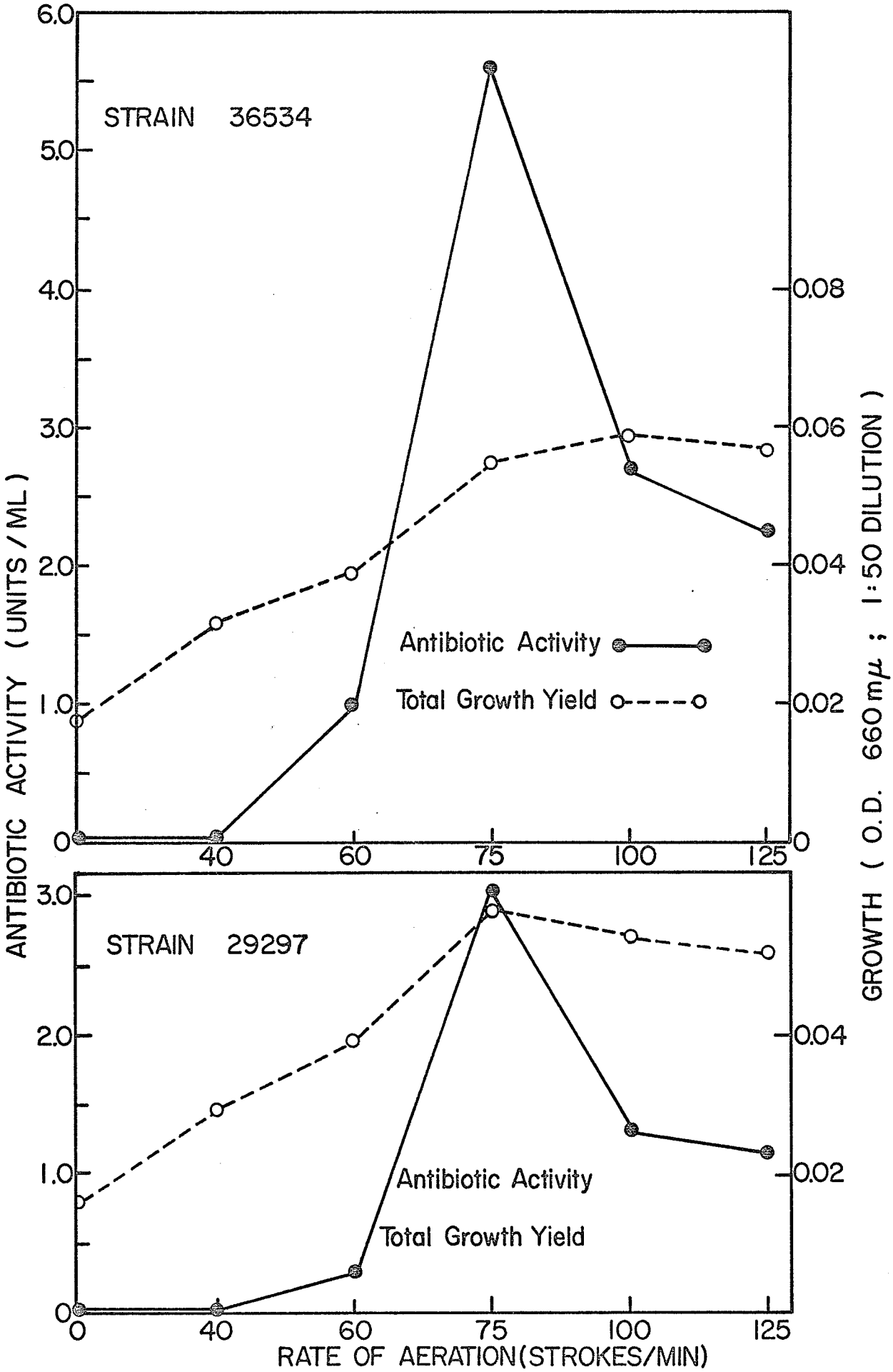


Fig.2 The effect of aeration on total growth yield and antibiotic production by staphylococci

The test strains were prepared and flasks were inoculated as described previously. Ten flasks per strain were then gassed for one minute each with a mixture of 10% or 25% carbon dioxide in air. The gaseous mixture was first passed through a sterilized tube filled with cotton before it entered the flasks. An equal number of cultures, to which no carbon dioxide was added, was also incubated. The concentration of carbon dioxide which entered the flasks was checked initially with the Burrell "Kwik-Chek" Gas Analyzer (Burrell Corp., Pittsburgh, U.S.A.). Antibiotic production was measured every two days for a period of 10 days. At each interval two cultures (per strain) which contained the carbon dioxide-in-air mixture and two control cultures were assayed for antibiotic activity.

The results obtained are shown in Tables I and II. With regard to strain 29297, antibiotic titers within the 10 day period at concentrations of 10% and 25% carbon dioxide in air did not reach the levels attained in the control cultures during this period. The pH of the control cultures was slightly alkaline in contrast with the carbon dioxide cultures which were acidic.

Strain 36534 showed different results. In this case maximal yields of antibiotic were observed in cultures incubated in an atmosphere of 10% carbon dioxide in air in contrast with the controls or 25% carbon dioxide-in-air

TABLE I

The Effect of Carbon Dioxide In-Air Mixtures on Antibiotic Production by Strain 29297.

Time (Days)	Control (no CO_2)			10% CO_2			25% CO_2		
	pH	Growth[1] (O.D.)	Activity[2] (units/ml)	pH	Growth (O.D.)	Activity (units/ml)	pH	Growth (O.D.)	Activity (units/ml)
2	7.7	0.035	1.1	6.7	0.020	1.2	6.5	0.022	0.9
4	8.1	0.068	4.1	6.8	0.027	2.8	6.5	0.024	3.4
6	8.5	0.072	4.3	6.7	0.025	1.4	6.4	0.022	2.4
8	8.7	0.068	4.6	6.7	0.024	1.45	6.4	0.024	1.2
10	8.8	0.062	2.1	6.5	0.019	3.8	-	--	-

1. The culture fluid was diluted 50 times. Values given are the means of duplicate measurements.
2. Values given are the means of duplicate measurements.

TABLE II

The Effect of Carbon Dioxide-In-Air Mixtures on Antibiotic Production by Strain 36534.

Time (Days)	Control (no CO_2)			10% CO_2			25% CO_2		
	pH	Growth[1] (O.D.)	Activity[2] (units/ml)	pH	Growth (O.D.)	Activity (units/ml)	pH	Growth (O.D.)	Activity (units/ml)
2	7.7	0.035	3.4	6.8	0.021	3.0	6.5	0.019	0
4	8.1	0.070	5.6	6.8	0.028	6.8	6.5	0.024	4.2
6	8.5	0.071	6.2	6.8	0.030	10.7	6.4	0.020	3.9
8	8.7	0.067	5.3	6.7	0.035	15.4	6.5	0.024	5.5
10	8.7	0.056	8.1	6.6	0.026	9.6	-	--	-

1. The culture fluid was diluted 50 times. Values given are the means of duplicate measurements.
2. Values given are the means of duplicate measurements.

cultures. On the other hand, flasks grown in the carbon dioxide environment were acidic compared with the alkaline controls and, as with strain 29297, growth was depressed by carbon dioxide.

(d) Manganese and ferrous ions

Hendlin (1949) showed that manganese ions were essential for the production of Bacitracin, a peptide antibiotic produced by a strain of Bacillus licheniformis. He also found that ferrous ions appeared to supplement the Bacitracin-producing capacity of manganese ions, but were unable to replace manganese ions. Hsu (unpublished observations) has suggested that the antibiotics produced by staphylococcal strains 29297 and 36534 are peptides.

For the experiments reported here, M/1,000 Mn^{++} (as $MnCl_2 \cdot 4H_2O$), and M/4,000 Fe^{++} (as $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) were added to the modified Dolman-Wilson medium in order that their effect on antibiotic production in the two staphylococcal strains might be studied. The cultures were incubated for three days.

Table III gives the results of these experiments. Manganese ions at the concentration tested completely inhibited antibiotic production by strain 29297 and caused significantly reduced antibiotic production by strain 36534. Ferrous ions did not stimulate antibiotic production in either

TABLE III

The Effect of Manganese and Ferrous Ions on Antibiotic Production

Addition to Dolman-Wilson Medium	Test Strains 29297 Growth[1] (O.D.)	29297 Activity[2] (units/ml)	36534 Growth (O.D.)	36534 Activity (units/ml)
No Addition	0.042	2.1	0.064	2.9
Manganese ions (M/1,000)	0.054	0	0.061	1.1
Ferrous ions (M/4,000)	0.043	2.2	0.053	2.1
Manganese ions (M/1,000) and Ferrous ions (M/4,000)	0.049	0	0.059	1.0

1. Culture fluid was diluted 50 times. Values given are the means of duplicate measurements.
2. Values given are the means of duplicate measurements.

strain. When both ions were added to the medium, inhibition in 29297 was complete and production was reduced in the case of 36534. Growth of the two strains was not significantly affected by the addition of either salt alone or in combination.

Isolation of Proteose Peptone Factors Necessary for Antibiotic Production

(a) Dialysis of Proteose Peptone

In the modified Dolman-Wilson medium, organic nitrogen is supplied to the test strains by proteose peptone (Difco Laboratories, Detroit, U.S.A.). As an initial step in the isolation of factors in proteose peptone necessary for antibiotic production by staphylococcal strains 29297 and 36534, proteose peptone (40 mg/ml) was dialyzed in Visking dialysis tubing against distilled water for 70 hr at 4°C. The ratio of proteose peptone to distilled water was 1:4. The diffusate (material which passed through the tubing) was concentrated to the original volume of the proteose peptone solution by flash evaporation. The diffusate and the material retained by the dialysis tubing were then used as Part A of the modified Dolman-Wilson medium and were added to Part C of the same medium. Antibiotic production by the test strains in these

TABLE IV

Stimulation of Antibiotic Production by Proteose Peptone Factors

Medium	Test Strains 29297		36534	
	Growth[1] (O.D.)	Activity[2] (units/ml)	Growth (O.D.)	Activity (units/ml)
Modified Dolman-Wilson (control)	0.066	4.3	0.072	5.9
Diffusate	0.074	4.5	0.076	4.2
Material retained by Dialysis tube	0.030	0	0.051	1.0

1. The culture fluid was diluted 50 times. Values given are the means of duplicate measurements.
2. Values given are the means of duplicate measurements.

media was measured after five days incubation.

The results are shown in Table IV. Antibiotic production by strain 29297 was stimulated only in the diffusate medium when compared to material retained by the dialysis tubing. In the case of strain 36534 much greater amounts of antibiotic were produced in the diffusate medium than in the material retained by the dialysis tubing. Growth of both strains in the diffusate medium was slightly better than in the modified Dolman-Wilson controls.

Although dialysis initially proceeded for 70 hr, it was found that antibiotic titers after 34 hr were almost as high and subsequently all further experiments used material dialyzed for this length of time.

(b) Sephadex Chromatography of Diffusate

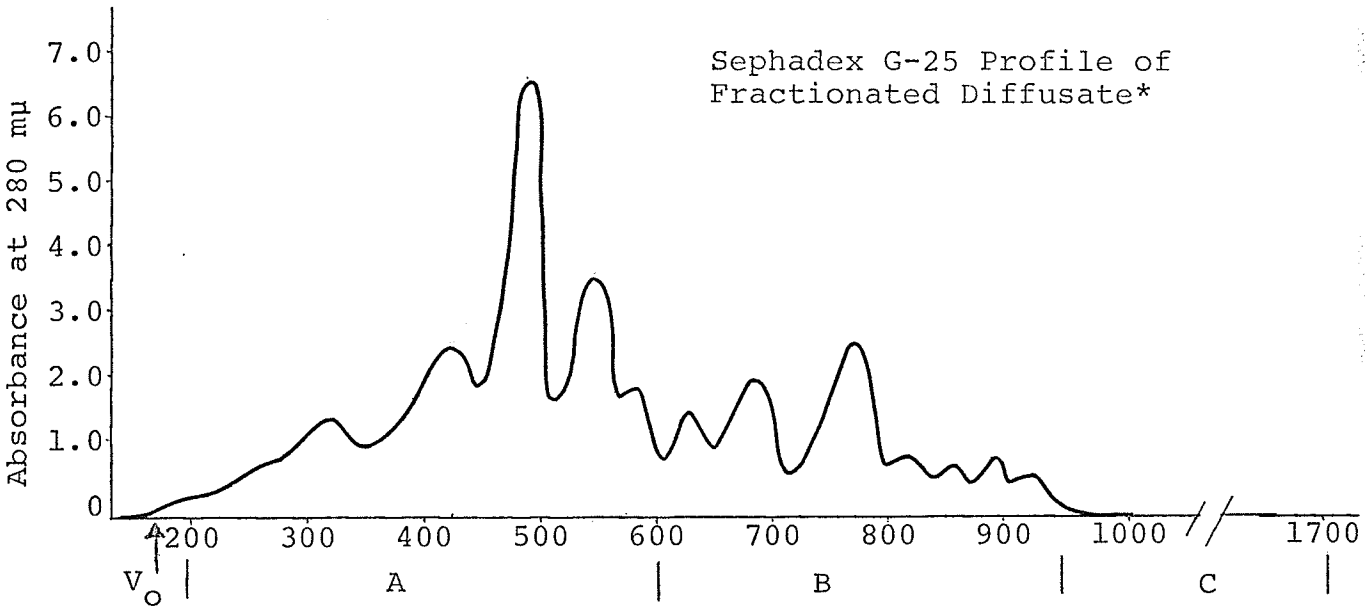
Factors necessary for antibiotic production which are contained in the proteose peptone diffusate were further purified after dialysis by gel filtration with Sephadex G-25.

A volume of 100 ml of diffusate was reduced by flash evaporation to 20 ml. The Sephadex was prepared as indicated in the Materials and Methods section.

Effluent from the Sephadex G-25 column was arbitrarily divided into sections A, B and C (Fig. 3). All fractions from each section were pooled, concentrated by flash evaporation and adjusted to the same protein values

Fig. 3

Sephadex Chromatography of Factors which Stimulate Antibiotic Production



Effluent Volume (ml). Sections shown above.

Medium	Average Inhibition Zones (mm) Strain 29297	Strain 36534
1. Modified Dolman-Wilson	6.5	5.0
2. Complete Diffusate	5.5	3.0
3. Casamino Acids	0	0
4. Casamino Acids + section A	2.5	3.0
" " + section B	0	0
" " + section C	0	0
" " + sections A,B,C.	6.0	5.5
" " + sections A,C.	6.0	5.5
" " + sections B,C.	0	0

*Fluid was diluted if optical density was greater than 1.5.

V_o is void volume of column.

by measurement of optical densities at 280 mμ. Section C contained no material which absorbed at 280 mμ. It was thought, however, that material in this section might contain required trace elements or growth factors.

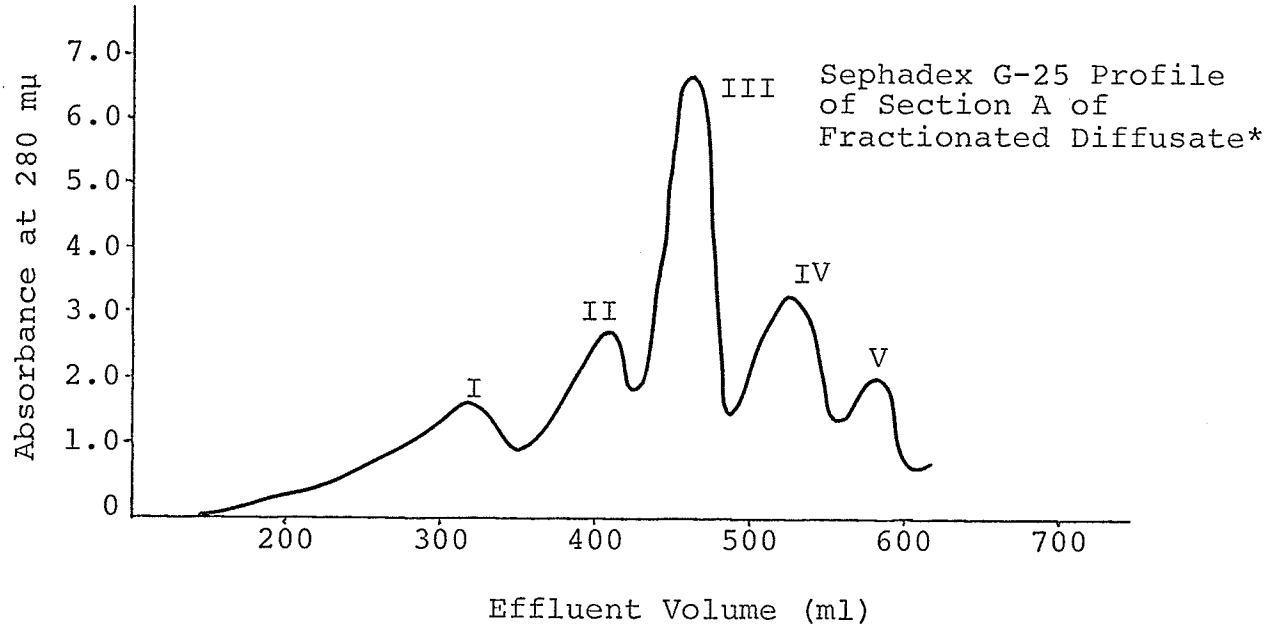
Volumes of each section which contained equal amounts of material which absorbed at 280 mμ were added to a basal casamino acids agar medium (see Appendix). This medium alone supported growth of the test strains, but no antibiotic production. Measurements of antibiotic production were performed as described in Material and Methods.

Results shown in Fig. 3 indicate that section A of the fractionated diffusate contained nutrients necessary for stimulation of antibiotic production by the test strains. Components of sections B and C did not stimulate antibiotic production, although those in section C appeared to enhance the ability of section A to stimulate antibiotic production.

Since components in section A alone were required for antibiotic production, it was necessary to test each of the five peaks in section A for stimulating ability. Effluent fractions from each of the five peaks in section A which absorbed at 280 mμ were pooled and concentrated by flash evaporation. Volumes of each fraction adjusted to equal amounts of material according to their absorption at 280 mμ were added to the casamino acids medium. Antibiotic production on this solid medium was measured as described in Materials and Methods.

Fig. 4

Sephadex Chromatography of Section A of Proteose Peptone Factors which Stimulate Antibiotic Production



	Medium	Average Inhibition Zones (mm) Strain 29297	Strain 36534
1.	Modified Dolman-Wilson	10.0	6.5
2.	Complete Diffusate	7.5	5.0
3.	Casamino Acids	0	0
4.	Casamino Acids + Peak I	0	0
	" " + Peak II	2.3	0
	" " + Peak III	0	2.0
	" " + Peak IV	0	0
	" " + Peak V	0	0

* Fluid was diluted if optical density was greater than 1.5.

Figure 4 shows that test strains 29297 and 36534 may differ with respect to substances necessary for antibiotic production. Strain 29297 required nutrients which were present in peak II of section A of the fractionated diffusate in contrast with strain 36534 which required substances present in peak III. No antibiotic was produced in the casamino acids medium. Good separation of peaks II and III was not obtained and consequently it is difficult to be certain whether the nutrients required are in fact located in peak II or in peak III. Since the substances which are required for antibiotic production originated from the diffusate of a dialyzed protein hydrolysate, it is probable that the nutrients which stimulate antibiotic production are either amino acids or small molecular weight peptides.

Analysis of Proteose Peptone Factors which Stimulate Antibiotic Production

(a) Paper Chromatography

One dimensional chromatography was performed on the fractions which stimulated antibiotic production by the test strains. The procedures for application of samples to the papers and for hydrolysis of the fractions are described in the Materials and Methods section. The active fractions were first lyophilized. To

each paper was applied 25 µl of whole fractions and hydrolyzed fractions, both at a concentration of two mg/ml. Equal amounts of known amino acids were also applied to the papers. The solvent system used was butanol-acetic acid-water (4:1:5). The spots were revealed with a solution of 0.1% ninhydrin in butanol. The Rf values of the spots were calculated and the tentative amino acid identifications are given in Table V.

Four ninhydrin-positive spots were present in stimulating fractions II and III. When the ninhydrin-positive spots of hydrolyzed samples were compared with the original smaples, identical Rf values were observed. There was no increase in the number of ninhydrin-positive spots in the hydrolyzed samples. Chromatography of hydrolyzed and unhydrolyzed vitamin-free casein showed an increase in the number of ninhydrin-positive spots in the hydrolyzed samples. It was therefore concluded that fractions II and III contained mixtures of simple amino acids. Amino acid possibilities for each spot are recorded within an experimental error of 5% or less. Within the sensitivity range of the colour reaction with ninhydrin, fraction II, which stimulates antibiotic production by strain 29297, contains a possible nine amino acids. Fraction III, which stimulates antibiotic production by strain 36534, contains a possible 12 amino acids.

TABLE V

Comparison of Rf Values of Fractions which Stimulate Antibiotic Production with Those of Known Amino Acids

Amino Acid (L-form)	Rf Value	Stimulating Fraction		Rf Value	Possible Amino Acids*
Leucine	0.683	II (Strain 29297)			
Methionine	0.525	Spot	a)	0.683	Leucine, Isoleucine
Alanine	0.315				
Aspartic Acid	0.230		b)	0.536	Methionine, Valine, Tryptophan
Serine	0.234				
Glutamic Acid	0.273		c)	0.307	Alanine
Lysine	0.171				
Glycine	0.251		d)	0.229	Aspartic Acid, Serine, Hydroxy-prolin
Phenylalanine	0.578				
Proline	0.353				
Hydroxy-proline	0.231	III (Strain 36534)			
Histidine	0.192	Spot	a)	0.646	Leucine, Isoleucine
Valine	0.519				
Threonine	0.275		b)	0.504	Methionine, Valine, Tryptophan
Cystine	0.117				
Tyrosine	0.384		c)	0.234	Aspartic Acid, Serine, Hydroxy-prolin
Tryptophan	0.525				
Arginine	0.193		d)	0.184	Arginine, Lysine Histidine
Isoleucine	0.645				

* Possible amino acids were determined within a 5% experimental error or less.

(b) Amino Acid Analysis

It was possible that amounts of some amino acids in fractions II and III which stimulated antibiotic production were too low for detection with ninhydrin on the paper chromatographs. Therefore, lyophilized samples of the stimulating fractions were analyzed for amino acid content with the Technicon Auto Analyzer (Technicon Corp., Ardsley, New York). Since there was no increase in the number of ninhydrin-positive spots revealed by paper chromatography, the samples were not hydrolyzed before they were subjected to analysis. The samples (0.01 - 0.1 mg) were first dissolved in 0.5 ml of 0.1N HCl, and were then applied to the auto analyzer. The standards used were 0.2 μM each of norvaline and norleucine. The percentages of amino acids in each of the fractions which stimulate antibiotic production are recorded in Table VI. The fractions contained more amino acids than could be shown with paper chromatography. It was also possible to quantitate the amino acids with the auto-analyzer.

It must be remembered that fraction II stimulates antibiotic production by strain 29297, whereas fraction III stimulates antibiotic production by strain 26534. Since many of the same amino acids appear in both fractions, different concentrations of the same amino acids may be required to stimulate each strain. For example, 2.7% of fraction II and 14.7% of fraction III are methionine.

TABLE VI

Amino Acid Analysis of Antibiotic Stimulating Fractions Based on the Technicon Auto-Analyzer

Amino Acid	Percentage of Amino Acid in Fraction*	
	II (Strain 29297)	III (Strain 36534)
Aspartic Acid	8.8	1.4
Threonine	6.9	4.2
Serine	3.9	8.4
Glutamic Acid	9.7	0.0
Proline	5.3	0.0
Glycine	4.0	12.6
Alanine	12.5	5.6
Valine	6.9	2.1
Methionine	2.7	14.7
Isoleucine	4.2	1.4
Leucine	28.7	16.8
Phenylalanine	1.2	23.1
Lysine	3.0	0.0
Histidine	0.0	5.6
Arginine	4.2	1.8

* Total amount of amino acids present in fraction II 0.0143 mg
fraction III 0.0568 mg

Leucine is present in the largest amounts in both fractions, although there is about 40% more leucine in fraction II. Neither cysteine nor tryptophan were detected in fractions II and III.

(c) Phosphorus Analysis

Fractions II and III were analyzed for total and inorganic phosphorus according to the method of Fiske and Subbarow (1925). It was found that the values obtained for total phosphorus in both fractions were approximately equal to those obtained for inorganic phosphate. Consequently, little or none of the phosphorus present was derived from organic sources.

Replacement of Proteose Peptone Factors which Stimulate Antibiotic Production

Fractions II and III, which were obtained after Sephadex chromatography of the proteose peptone diffusate, were shown to stimulate antibiotic production by the test strains 29297 and 36534. It was subsequently shown by amino acid analysis that these fractions contained various amounts of up to 14 amino acids. These amino acids were added to the basal casamino acids medium so that specific amino acid requirements for antibiotic production might

TABLE VII

Essential Nutrients Required for Antibiotic Production by Staphylococcal Strains 29297 and 36534.

Medium[1]	Strain 29297		Strain 36534	
	Growth[2]	Inhibition Zones (mm)	Growth	Inhibition Zones (mm)
1. Modified Dolman-Wilson (control)	++++	>20	++++	>20
2. Casamino Acids	++	0	++	0
3. Casamino Acids + Amino Acids: Fraction II	++	0		
Fraction III			++	0
4. Casamino Acids + Amino Acids (II or III) + Tryptophan and Cysteine	+++	4.0	+++	3.0
5. Casamino Acids + Amino Acids (II or III) + Tryptophan	+++	2.5	+++	1.5
6. Casamino Acids + Amino Acids (II or III) + Cysteine	+++	0	+++	0
7. Casamino Acids + Amino Acids (II or III) + Tryptophan and Cysteine + Nucleotides and Bases	+++	4.5	+++	2.0

1. See Appendix for medium constituents and concentrations.

2. Growth: ++ Moderate; +++ Heavy; ++++ Very Heavy

be identified. It was noted that tryptophan and cysteine were not recorded by the auto-analyzer. These amino acids were also not given in the typical analysis of Bacto Casamino Acids provided by Difco Laboratories. Tryptophan and cysteine were therefore added to the medium at concentrations of 2×10^{-4}M and 1×10^{-4}M respectively. Various medium combinations were prepared as described in the Appendix. The plates were inoculated by the stab technique and were incubated at 37°C for eight days. At this time the plates were sprayed with the indicator strain and were re-incubated overnight at 37°C.

Table VII shows that tryptophan is required for antibiotic production by both strains although the organisms were able to grow in its absence. Neither cysteine nor the nucleotides and bases which were added were necessary for antibiotic production. However the appearance of colonies on the plates suggested that cysteine enhanced the growth of the test strains.

DISCUSSION

A direct relationship between aeration rate and antibiotic production in the test strains was not observed. Maximum antibiotic production took place in each case at an aeration rate of 75 strokes/min. At higher or lower rates little or no activity was observed. It is not clear whether oxygenation at the higher rates inactivates the antibiotic or whether the low levels of activity are due to a metabolic effect. Growth yield was less affected by variations in oxygen tension over the range tested. Since the inherent error in the measurement of antibiotic activity is greater than the estimation of growth in terms of O.D., it is hard to rule out the possibility that differences in the effect of aeration rate on growth and antibiotic production observed in Fig. 2 may in fact be somewhat artificial. This is unlikely since the experiment was repeated and similar results were obtained.

Carbon dioxide (10% by volume) brought about a two to three fold increase in antibiotic production by strain 36534 but inhibition was observed with strain 29297. The pH of cultures which had been incubated in the presence of carbon dioxide was slightly acidic and it could be said that acid conditions are responsible for enhancement of antibiotic production in the case of strain 36534. By contrast, strain 29297 appeared to require alkaline conditions for optimal antibiotic production.

With regard to the effect of incubation time on antibiotic production, maximal titers were observed with strain 29297 in the absence of carbon dioxide between four and eight days. With strain 36534 in the presence of carbon dioxide, highest titers were observed on the eighth day. These data conflict with those of Hsu (1966) which showed that maximal antibiotic production was achieved on solid media by both strains after three or four days of incubation. It should be pointed out that his work was done on solid media upon which oxygen tension in relation to growth and antibiotic production was entirely different.

The presence of factors in the diffusate of dialyzed proteose peptone which stimulate antibiotic production by strain 29297 and 36534 suggests that they are amino acids or small peptides. It was subsequently shown that the stimulating factors are unlikely to be peptides in view of the fact that acid hydrolysis of the factors revealed no increase in the number of ninhydrin-positive spots on a paper chromatogram. The Technicon Auto-Analyzer confirmed the identity of the ninhydrin-positive spots as amino acids. It is of interest to note that the auto-analyzer appeared to be a more sensitive tool than paper chromatography in the detection of amino acids.

The possibility that the antibiotic stimulating factors were nucleotides was ruled out on the basis of their phosphorus content, all of which was inorganic. Furthermore,

results given in Table VII showed that adenosine-5'-phosphate, cytosine-5'-phosphate, uridine-5'-phosphate and guanosine-5'-phosphate were not required for antibiotic production.

We have shown that L-tryptophan although not required for growth, is essential for antibiotic production by both strains 29297 and 36534. This would first appear to be at variance with results expressed in Fig. 4 in which it was shown that peaks II and III stimulated antibiotic production by 29297 and 36534 respectively. However, both peaks were in close proximity to each other and while it is probable that both contained some tryptophan, it is possible that the concentrations were not equal in both peaks. Tryptophan concentration is undoubtedly important in the stimulation of antibiotic production. Furthermore, it could be suggested that the amino acids other than tryptophan which are contained in peaks II and III are unnecessary for antibiotic production, although this was not in fact tested. The better production of antibiotic on modified Dolman-Wilson medium might be explained by the presence of optimal concentrations of tryptophan in contrast with those contained in the effluents after fractionation on Sephadex. It would be valuable to determine whether the addition of different concentrations of tryptophan to the basal casamino acids medium would support increased antibiotic production which would match that

in the modified Dolman-Wilson medium.

The apparent enhancement of antibiotic production by section C of the fractionated diffusate (Fig. 3) suggests that factors other than amino acids stimulate antibiotic production. However, their chemical nature remains obscure.

In conclusion, antibiotics produced by staphylococcal strains 29297 and 36534 are undoubtedly of different chemical composition on the basis of the following observations: (1) different temperature optima were observed in the production of each antibiotic, (2) carbon dioxide enhanced production by strain 36534 but not by strain 29297, and (3) Mn^{++} salts completely inhibited antibiotic production by strain 29297 in contrast with only partial inhibition by strain 36534. Stimulation of antibiotic production in both strains by L-tryptophan does not necessarily conflict with these observations.

SUMMARY

1. Temperature optima for maximal antibiotic production by strains 29297 and 36534 were different. Maximum yields by 36534 were obtained at an incubation temperature of 37°C in contrast with 29297 in which a temperature of 28°C was best. Agitation of liquid medium cultures of both strains at a rate of 75 strokes/min was critical for maximal yields. Antibiotic production by strain 36534 was significantly increased by the addition of a mixture of 10% carbon dioxide in air. In the case of strain 29297 the addition of carbon dioxide-in-air mixtures depressed antibiotic production. Neither strain required manganese or ferrous ions for antibiotic production. In fact, manganese ions completely inhibited antibiotic production by strain 29297 and significantly depressed antibiotic production by strain 36534.

2. Nutrients in proteose peptone necessary for antibiotic production by the test strains were dialyzable and could be autoclaved.

3. Fractions from the diffusate of dialyzed proteose peptone which stimulated antibiotic production contained amino acids with no detectable peptide material as shown by paper chromatography and auto-analysis. No nucleotides were contained in the antibiotic stimulating fractions.

4. Staphylococcal strains 29297 and 36534 produced antibiotic in a semi-synthetic medium composed of casamino acids (Difco) supplemented with L-tryptophan. L-tryptophan was required for antibiotic production but not for growth. Cysteine was not essential for antibiotic production but appeared to enhance growth of the two strains on the semi-synthetic medium.

BIBLIOGRAPHY

Barrow, G.I. 1963. The nature of inhibitory activity by *Staphylococcus aureus* Type 71. J. Gen. Microbiol. 32:255-261.

Breed, R.S., Murray, E.G.D. and Smith, R.N. (eds.). 1957. *Bergey's Manual of Determinative Bacteriology*, 7th ed., The Williams and Wilkins Co., Baltimore, U.S.A.

Cruickshank, R. (ed.). 1965. *Medical Microbiology*, 11th ed., E. and S. Livingstone Ltd., London, England.

Fildes, P. and Richardson, G.M. 1937. The nutrition of *Staphylococcus aureus*; Sulphur requirements. Brit. J. Exp. Path. 18:292-304.

Fildes, P., Richardson, G.M., Knight, B.C.J.G. and Gladstone, G.P. 1936. A nutrient mixture suitable for the growth of *Staphylococcus aureus*. Brit. J. Exp. Path. 17:481-484.

Fiske, C.H. and Subbarow, Y. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66:375-400.

Friedman, S. and Fox, C.L. Jr. 1954. Studies on the relationship of potassium to metabolism and purine biosynthesis in *Escherichia coli*. J. Bacteriol. 68:186-193.

Gardener, J.F. 1949. An antibiotic produced by *Staphylococcus aureus*. Brit. J. Exp. Path. 30:130-138.

Gladstone, G.P. 1937. The nutrition of *Staphylococcus aureus*; Nitrogen requirements. Brit. J. Exp. Path. 18:322-333.

Gretler, A.C., Mucciolo, P., Evans, J.B., and Niven, C.F. Jr. 1955. Vitamin nutrition of the staphylococci with special reference to their biotin requirements. J. Bacteriol. 70:44-49.

Hendlin, D. 1949. Nutritional requirements of a Bacitracin-producing strain of Bacillus subtilis. Arch. Biochem. 24:435-446.

Hsu, C.Y. 1966. Antibacterial substances produced by the genus Staphylococcus. M.Sc. Thesis, University of Manitoba, Winnipeg, Man.

Hsu, C.Y. and Wiseman, G.M. 1967. Antibacterial substances from staphylococci. Can. J. Microbiol. 13:947-955.

Hughes, T.P. 1932. Growth requirement of staphylococci. J. Bacteriol. 23:437-447.

Ivler, D. 1965. Comparative metabolism of virulent and avirulent staphylococci. Ann. N.Y. Acad. Sci. 128:62-80.

Jennings, M.A. and Sharp, A.E. 1947. Antibacterial activity of the Staphylococcus. Nature 159:133-134.

Knight, B.C.J.G. 1935. An essential growth factor for Staphylococcus aureus. Brit. J. Exp. Path. 16:315-326.

——— 1937a. XCVII. The nutrition of Staphylococcus aureus; Nicotinic acid and vitamin B_1. Biochem. J. 31:731-737.

——— 1937b. CXXVIII. The nutrition of Staphylococcus aureus; The activities of nicotinamide, aneurin (vitamin B_1) and related compounds. Biochem. J. 31:966-973.

Knight, B.C.J.G. and McIlwain, H. 1938. CLXVI. The specificity of aneurin and nicotinamide in the growth of Staph. aureus. Biochem. J. 32:1241-1251.

Kögl, F. and van Wagtendonk, W.J. 1938. Cited in Porter, J.R. 1948.

Koser, S.A., Finkle, R.D., Dorfman, A., Gordon, M.V. and Saunders, F. 1938. Studies on bacterial nutrition. A comparative study of the growth promoting properties of various substances. J. Infect. Dis. 62:209-218.

Loeb, L.J., Moyer, A. and Murray, R.G.E. 1950. An antibiotic produced by Micrococcus epidermidis. Can. J. Res. 28E: 212-216.

MacLeod, R.A. and Snell, E.E. 1947. Some mineral requirements of the lactic acid bacteria. J. Biol. Chem. 170:351-365.

Mah, R.A., Fung, D.Y.C. and Morse, S.A. 1967. Nutritional requirements of Staphylococcus aureus S-6. Appl. Microbiol. 15:866-870.

Mao, J.C.H. 1967. Protein synthesis in a cell-free extract of Staphylococcus aureus. J. Bacteriol. 94:80-86.

Meynell, G.G. and Meynell, E. 1965. Theory and Practice in Experimental Bacteriology. Cambridge University Press, pp. 36-37.

Murray, R.G.E. and Loeb, L.J. 1950. Antibiotics produced by micrococci and streptococci that show selective inhibition within the genus Streptococcus. Can. J. Res. 28E:177-185.

Porter, J.R. 1948. Bacterial Chemistry and Physiology. John Wiley and Sons, Inc., N.Y. page 699.

Porter, J.R. and Pelczar, M.J. Jr. 1941. The nutrition of Staphylococcus aureus; The influence of biotin, bios II_B, and vitamin H on the growth of several strains. J. Bacteriol. 41:173-192.

Shankar, K. and Bard, R.C. 1952. The effect of metallic ions on the growth and morphology of Clostridium perfringens. J. Bacteriol. 63:279-290.

Shooter, R.A. and Wyatt, H.V. 1955. Mineral requirements for growth of Staphylococcus pyogenes; Effect of magnesium and calcium ions. Brit. J. Exp. Path. 36:341-350.

——— 1956. Mineral requirements for growth of Staphylococcus pyogenes; Effect of potassium ions. Brit. J. Exp. Path. 37:311-317.

Strasters, K.C. and Winkler, K.C. 1963. Carbohydrate metabolism of Staphylococcus aureus. J. Gen. Microbiol. 33:213-229.

Su, T.L. 1948. Micrococcin. An antibacterial substance formed by a strain of Micrococcus. Brit. J. Exp. Path. 29:473-481.

Vilter, S.P. and Spies, T.D. 1940. Vitamin B_6 as an accessory growth factor for Staphylococcus albus. Science 91:200-201.

Weinberg, E.D. 1964. Manganese requirements for sporulation and other secondary biosynthetic processes of Bacillus. Appl. Microbiol. 12:436-441.

Wiseman, G.M. 1963. Studies with the β-hemolysin of Staphylococcus aureus. Ph.D. Thesis, University of Edinburgh, Edinburgh, Scotland.

Young, R.J. and Barker, G.R. 1964. The ribosomes of Staphylococcus aureus (strain Duncan). Biochem. J. 91:22C-23C.

APPENDIX

Modified Dolman-Wilson Medium

Part A: Dissolve 20 gm of Difco Proteose Peptone in one liter of distilled water. Adjust the pH to 7.8 with 4N NaOH, and autoclave at 15 lb pressure for 15 min.

Part B: Add 14 ml of 0.1M $NaH_2PO_4 \cdot H_2O$ (13.8 gm/l) to 36 ml of 0.1M K_2HPO_4 (17.42 gm/l).

Part C: Dissolve the following salts in Part B and bring the volume to 100 ml with distilled water. Sterilize by filtration through a Millipore HA (0.45 μ) membrane. The pH of this solution should be about 5.8.

NaCl	5.0 gm
Ammonium lactate (80% syrupy solution)	6.0 ml
$CaCl_2$	0.11 gm
$MgSO_4 \cdot 7H_2O$	0.2 gm
(Tri) sodium citrate	0.2 gm

Complete medium: Add 5.0 ml of sterile Part C to 45 ml of sterile Part A. The final pH will be 7.2 - 7.3. This medium is ready to inoculate.

Media for the Determination of Requirements for Antibiotic Production

Amino acid stock solutions (L-forms) were dissolved in distilled water. L-aspartic acid and L-glutamic acid were dissolved in 0.3N NaOH. L-tryptophan was dissolved in 0.3N HCl. So that nutritional requirements of antibiotic production by Staph. epidermidis strains 29297 and 36534 could be determined, it was necessary to know the nature and concentration of the amino acids contained in the two Sephadex fractions of the complex Dolman-Wilson medium which stimulated antibiotic production. This information was taken from Table VI. The molar concentrations of amino acids were determined by multiplication of the percentages of amino acids contained in each auto-analysis sample (Table VI) by the dry weight of peak II and III samples (0.033 gm and 0.035 gm respectively) which stimulated antibiotic production. Adjustments were made to take into account the volume of medium used in these experiments. The concentrations of cysteine and tryptophan used were based on the recommendations of Fildes et al (1936) and Gladstone (1937).

Four separate components were made up as follows:

Part 1

Basal Medium Control for both strains: Difco Casamino Acids were dissolved in distilled water to a con-

centration of 13.5% w/v. A volume of 2.0 ml of this solution was added to all media such that the final casamino acids concentration was 3%. This basal medium was also used for initial experiments in which Sephadex fractions were tested for their ability to stimulate antibiotic production.

Part 2

For Strain 29297:

L-Amino Acid	Conc. (M)	Vol. added (ml)
Aspartic acid	4.3×10^{-3}	0.2
Threonine	3.8×10^{-3}	0.2
Serine	2.4×10^{-3}	0.2
Glutamic acid	4.3×10^{-3}	0.2
Proline	3.0×10^{-3}	0.2
Glycine	3.6×10^{-3}	0.2
Alanine	9.2×10^{-3}	0.2
Valine	3.9×10^{-3}	0.2
Methionine	1.2×10^{-3}	0.2
Isoleucine	2.1×10^{-3}	0.2
Leucine	1.4×10^{-2}	1.0
Phenylalanine	4.9×10^{-4}	0.2
Lysine	1.4×10^{-3}	0.2
Arginine	6.7×10^{-4}	0.2
Total volume added		3.6 ml

For Strain 36534:

L-Amino Acid	Conc. (M)	Vol. added (ml)
Aspartic acid	6.0×10^{-4}	0.2
Threonine	2.3×10^{-3}	0.2
Serine	5.5×10^{-3}	0.2
Glycine	1.2×10^{-2}	0.2
Alanine	4.2×10^{-3}	0.2
Valine	1.1×10^{-3}	0.2
Methionine	6.8×10^{-3}	1.0
Isoleucine	6.0×10^{-4}	0.2
Leucine	8.8×10^{-3}	1.0
Phenylalanine	9.6×10^{-3}	1.0
Histidine	2.4×10^{-3}	0.2
Arginine	1.6×10^{-3}	0.2
Total volume added		4.8 ml

Part 3

For both strains:

Cysteine·HCl: A volume of 0.2 ml of a 1.0×10^{-4}M solution was added to the basal casamino acids medium.

Tryptophan: A volume of 0.2 ml of a 2.0×10^{-4}M solution was added to the basal casamino acids medium.

In the experimental procedure, these two amino acids were added either separately or in combination.

Part 4

For both strains:

A stock solution of nucleotides and bases was prepared as follows: To one liter of 0.1N HCl was added 0.1135 gm each of adenosine-5'-phosphate, cytosine-5'-phosphate, uridine-5'-phosphate, guanosine-5'-phosphate, adenine sulphate, and guanine hydrochloride. A volume of 0.8 ml of this solution when added to the basal casamino acids medium gave a final concentration of approximately 10 µg/ml of each nucleotide and base according to the recommendations of Meynell and Meynell (1965).

The four parts were used in various combinations as follows:

1. Part 1 alone.
2. Part 1 + Part 2 (different for each strain).

3. Part 1 + Part 2 + Part 3 (cysteine and/or tryptophan).

4. Part 1 + Part 2 + Part 3 (cysteine and/or tryptophan) + Part 4.

These media were each made up to a total volume of 8.0 ml with distilled water in 25 ml screw-capped tubes. The pH of each test medium was adjusted to 7.8 with 4N NaOH before it was autoclaved. Difco Agar (0.135 gm) was added to each tube. The tubes were then autoclaved at 10 lb pressure for 10 min when they were poured into 15 x 60 mm petri plates each of which contained 0.9 ml of Part C of the modified Dolman-Wilson medium. The total volume of medium in each plate was 9 ml and the final pH was 7.2 - 7.3.