ASPECTS OF ANTIBIOTIC PRODUCTION BY TWO STRAINS OF STAPHYLOCOCCI

A Thesis
Presented to
The Department of Medical Microbiology
Faculty of Medicine
The University of Manitoba

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
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October 1969

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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.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Mr. A. Hsu of this department who supplied the strains of staphylococci and entered into many useful discussions during the course of this project.

He wishes particularly to thank Dr. G. M. Wiseman for inspiration in many facets of the project and for his patient guidance during the writing of this thesis.

He is indebted to the Medical Research Council for arranging financial assistance.

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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 x 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 <u>Bacillus</u> 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 <u>Staph</u>. <u>aureus</u> as shown by Mao above.

(d) <u>Carbohydrates</u>

Strasters and Winkler (1963) have studied the pathways of carbohydrate oxidation in several strains of <u>Staph</u>. <u>aureus</u>. 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