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AUTHOR DAVID KAHLER

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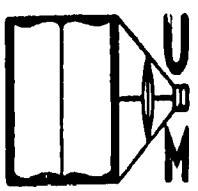

STUDIES IN PIGMENTATION IN *MICROCOCCUS PYOGENES* VAR. *AUREUS*

by

David Kahler

A THESIS

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INTRODUCTION

The fact that many microorganisms have exhibited some type of coloration has aroused the curiosity of mankind since its beginning. Many attempts have been made to explain why pigment production by a particular organism varies from time to time under supposedly the same conditions. As pigmentation is usually accompanied by good growth, some workers have stated that pigment is formed during cell proliferation and is the result of active bacterial metabolism. Such a theory discounts any idea that a particular metabolite directly affects pigmentation.

Other workers have favored a theory of inheritance of color and support their theory with the evidence of Bunting's work (3) with Serratia marcescens. Using a synthetic medium Bunting found that four types of colonies, namely: dark red, bright pink, pale pink, and white, grown under controlled conditions, all eventually came to an equilibrium with about 97.0 percent dark red and 3.0 percent bright pink colonies.

Most workers in this field have felt that there are particular growth factors or combinations of these factors which cause an organism to produce pigment. Evidence has indicated that these factors are different for each species of organism and may even vary within the species. Supporters of this third theory point out that such a hypothesis could

include the first theory as the organism forms products during good growth which serve as metabolites for pigmentation.

With staphylococci many attempts have been made to study the effect of various nutrilites upon pigmentation. However these studies have been made upon media which consist of components such as (1) meat extract, (2) evaporated milk, (3) extracts from or agglutinins prepared against staphylococci or (4) alcohol precipitated extracts of beef, brain, spleen, heart and kidney. The end result has been that the information has varied with the test conditions and very little agreement has resulted. Also methods of measuring pigmentation have been for the most part, crude and therefore not too reliable.

It was felt that if a method could be evolved by which the amount of pigment could be measured with at least a fair degree of accuracy, then various media, both natural and synthetic, could be used to study the effect of chemically defined compounds upon pigmentation in S. aureus (Micrococcus pyogenes var. aureus). With this object in mind this study was undertaken.

LITERATURE REVIEW

Pigmentation in microorganisms has at times been instrumental in guiding the destiny of mankind since the time before Christ. During the siege of Troy in 332 B.C. by Alexander and his fellow Macedonians, the appearance of blood-like droplets in bread was interpreted to mean destruction of those within the city (27). As a result, the Macedonians continued their assault and the city of Troy fell.

Since the above event, instances of "bloody bread" have altered the destiny of man until 1819 when a scientific explanation of the phenomenon was made by Bartolomea Bizio (27), a professor at the University at Padua. Bizio promptly recognized the fungus nature of the coloring matter and proceeded to infect other materials with the organism. "Bloody spots" appeared within twenty-four hours. He named the causative organism Serratia marcescens, the genus in honor of Serrafina Serratia, the first to operate a steamboat on the Arno river. The specific name means decaying or putrefying. In 1827 a complete report, including the chemical nature of the pigment, was made by Bizio.

The other carotenoid pigment producers have also had interesting genealogies although most of these organisms were investigated for their pathogenic significance before

extensive work was done on their chromogenic properties. Robert Koch (35) first noted a micrococcus in pus in 1878, and two years later Pasteur cultivated the organism in liquid medium. Rosenbach (34) made a thorough study of the organism and the basis of its pigment production, and divided the genus into two species -- Staphycoccus pyogenes aureus and Staphylococcus pyogenes albus. Passet (35) added a yellow variety, Staphylococcus pyogenes citreus. Since that time many attempts have been made to classify these micrococci using various criteria such as fermentation reactions, pathogenicity, hemolytic activity, etc., but the predominating characteristic has been the pigment as is evident by the present nomenclature -- Micrococcus pyogenes aureus.

Zopf (38) studied the pigment content of eight organisms and found lipoxanthins to be present in Bacterium egregium, Bacillus chrysoloia and Staphylococcus aureus. Gurd and Denis (15) reported that carotenoids were found in Mycobacterium leprae. Reader (26), using the chromatographic technique, identified carotene in Sarcina aurantiaca and Chargaff (7), working with the carotenoid pigments of Sarcinia lutea, Sarcina aurantiaca and Staphylococcus aureus, found sarinene, xanthophyll, carotene, and zeaxanthine to be present. Baumann et al (2) found that several species of Flavobacterium, Corynebacterium, Staphylococcus, and Mycobacterium synthesized

carotenes; however, attempts to transform the carotene to vitamin A by the use of microorganisms failed. However a carotenoid-like structure was thought by Skinner and Gunderson (31) to be the precusor of vitamin A in a species of Corynebacterium.

French (11) working with photosynthetic bacteria, liberated the cell contents by supersonic vibration. A water soluble protein which was attached to insoluble pigments, bacteriochlorophyll and carotenoid, were liberated. Reimann and Eckler (28), studying four variant types of Micrococcus tetragenus, isolated the following pigments: yellow variant - xanthophyll, pink variant - rhodaxanthin, brown variant - carotin, and mucoid pink variant - lycopene. Sobin and Stahly (30) extracted carotenoids with hot methanol from Flavobacterium, Sarcina, Micrococcus, Erwinia, Bacterium, Cellumonas and Staphylococcus and found that some species which are separated by biochemical reactions have identical pigments.

Ingraham and Baumann (16), using a synthetic medium, were able to demonstrate that the amount of carotene produced by a given organism varies with the medium, and that the content per gram of cells increases to a maximum after which it decreases. These workers suggested that such a carotenoid synthesis carried interesting biological implications since the organisms do not contain chlorophyll.

Pinner and Voldrich (24) reported that Staph. albus, Staph. citreus, and Staph. roseus are split off spontaneously from Staph. aureus in broth containing aureus agglutinins and that albus can be reverted to aureus by using media with a high concentration of anti-albus serum. Byatt, Jann and Salle (4) obtained an extract from ruptured cells of a highly chromogenic strain of Staph. aureus and this extract was used to induce pigmentation in white strain colonies. Nutini and Lynch (23) using an alcohol precipitated extract of beef brain, spleen, heart or kidney were able to convert the yellow S form of Staph. aureus to the white R form with altered biochemical characteristics. The findings of Goldworthy and Still (14) show that ox heart meat extract when added to nutrient agar inhibits pigment production in Bacillus prodigiosus and S. marcescens but enhances pigment production in Staph. aureus.

Two cultures of acid fast bacteria were found by Baker (1) which produced pigment in the presence of light and were devoid of pigment in the dark. Brief exposure to ultraviolet and sunlight and longer exposure to electric light conferred the ability to form pigment on fully developed unpigmented cultures. Kreitlow (19) exposed S. marcescens to a bright red light with the result that a bright orange color was produced at 20 and 27 C while under a blue light, a highly

chromogenic organism lost its pigmentation. Cultures exposed to white light formed intermediates.

Sullivan (34) reported the yellow pigments of Bacillus fucus, Micrococcus auranticus, M. citreus, M. tetragenus versatilis, and Sarcina lutea are formed very slowly in non-albuminous media but are quickly formed in a peptone solution plus salts. This worker found that magnesium sulfate and dipotassium phosphate favored pigmentation. Reid's (27) exhaustive study of 76 cultures representing 24 genera of chromogenic bacteria, showed that no pigment was produced when the medium contained no nitrogen or inorganic salts; however, amino acids as the sole source of nitrogen or metals found in pigments were not useful in stimulating pigmentation. Kharasch, Conway and Bloom (17) working with several carotenoid producers showed that large concentrations of biologically important metals (manganese, copper, iron) inhibit organism growth while smaller concentrations sometimes cause pigmentation loss. Turian's (36) studies with Mycobacterium phlei demonstrated the opposite to be true in that more carotenoids were produced when the medium contained traces of iron and manganese. Dewey and Poe (8) worked with minimum essentials for pigment production in S. marcescens and demonstrated that manganese, sulfate, and phosphate were necessary.

Chapman (5) seeking a selective medium for the isolation of staphylococci, reported that a 7.5 percent sodium chloride added to a Bacto phenol-red agar provided a good isolation medium and enhanced chromogenesis. Fagracus (9) found that the staphylococcus grew luxuriously with abundant pigmentation when the sodium chloride concentration was carried as high as 10 percent.

Kharasch, Conway and Bloom (17) observed that the development of pigment in S. marcescens does not require glucose but depend upon substances with available aldehyde or ketone groups. Sevag and Green (29) investigated the role of several breakdown products of glucose in the pigmentation of Staph. aureus and concluded that there is some unknown product of their intermediate metabolism which is required.

The work of Kliger, Grossowicz and Bergner (18) showed that both nicotinic acid and thiamin must be present for the full utilization of available carbohydrates in staphylococci but these workers did not mention the extent of pigmentation. However the improved Chapman-Stone (6) medium the yeast extract of which contains the above mentioned vitamins, produces a deep pigment. Gladstone (13) reported that 25 strains of Staph. aureus grew well on a medium which included 16 amino acids with the following acids being essential;

cystine, leucine, valine, proline, glycine, aspartic acid, phenylalanine, arginine and tryptophane. Porter and Pelzer (25) were unable to grow many strains of Staph. aureus in Gladstone's basal medium and reported that biotin, in addition to nicotinic acid, and thiamin was necessary for growth. The findings of Moller, Wey, and Wacker (21) demonstrated that about one-fourth of the staphylococci studied needed pantothenic acid and about one-half required purines or uracil. These last two groups of workers did not mention the effect of these growth factors on pigmentation.

EXPERIMENTAL METHODS

The organisms which were selected for this study were three strains of Staphylococcus aureus (Micrococcus pyogenes var. aureus). Two of the cultures No. 2 and 5 were isolated from the throats of students in the Department of Bacteriology and Public Health at Michigan State College. The third culture which will be designated 6 is 6538 from the American Type Culture Collection. Cultures 2 and 5 were selected because of their marked ability to form pigment on most solid media which supported their growth. Preliminary work was done with culture 5 as of the three strains used, this organism was the best pigment producer.

The first attempts to study pigment formation in these organisms were made on solid media. Four media were compared, namely (1) proteose peptone No. 3 agar plus 10 percent evaporated milk, (2) proteose lactose agar, (3) dextrose agar and (4) Chapman's No. 110 agar. An attempt was made to measure pigment production by visual observation at the end of a 10 day incubation period. However the amount of pigment could not be measured by observing the growth on the agar plates because the degree of pigmentation in isolated colonies was markedly different from that in heavy confluent growth.

A method of measuring the amount of light reflected off the pigmented cells was tried but little success resulted

because the differences of pigmentation on various media were not sufficient for differentiation. Even when cells were removed from the growing surface and placed on various backgrounds, no marked improvement in differentiation resulted.

The method of Stahly et al (33), for measuring the amount of pigment produced by cells grown on a solid medium with a photoelectric colorimeter, met with more success than the ones previously tried. The following formula was used to determine the amount of pigment:

$$\text{Amount of pigment} = \frac{\text{Measured pigment} \times 0.1000}{\text{Measured turbidity}}$$

However it was felt that a more accurate method could be devised if the pigment was extracted from the cells with an organic solvent.

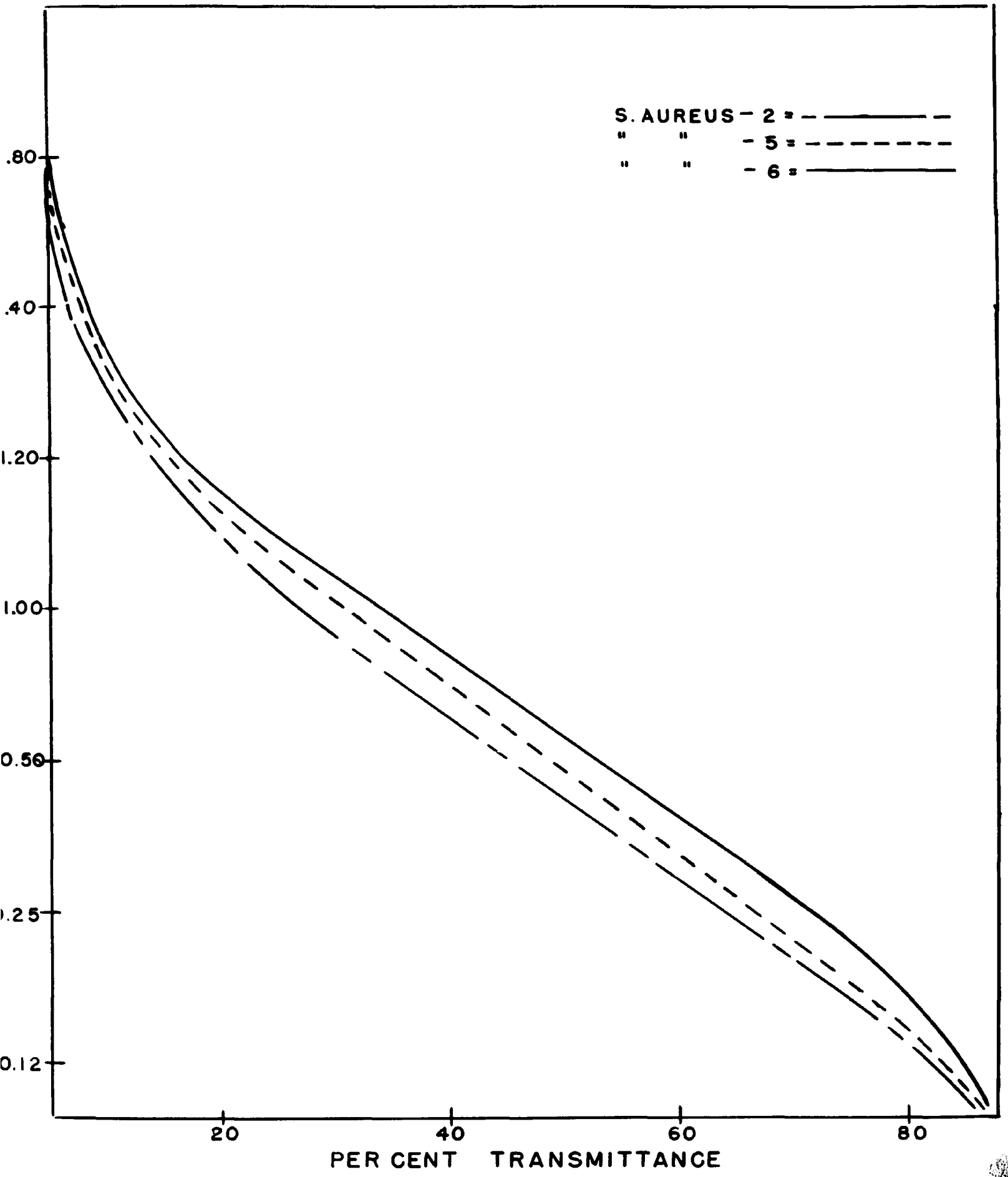
The following method was devised: 200 ml portions of the test medium, prepared with double distilled water, were placed in 500 ml Erlenmeyer flasks, the pH was adjusted to 7.00 and the medium was autoclaved for 15 minutes at 15 pounds (121 C). Three ml aliquots amounts of the medium were seeded from a 24 hour nutrient agar slant. After 12 hours incubation at 30 C, transplants were made to a second set of aliquots which were also incubated 12 hours. Four drops of these 12 hour cultures were added to the 200 ml portions of the test medium. These flasks were shaken during incubation for 24 hours on a Burrell shaker at 30 C to promote maximum growth by bringing oxygen and nutrients to the growing cells.

Following the shaking period the cells were separated from the supernatant fluid by centrifugation and washed twice with 0.85 percent saline. Next 10 ml of 0.85 percent saline plus glass beads were added to the cell mass and the suspension was shaken for one-half hour in order to break up existing clumps. Further to insure a homogeneous suspension, 40 ml of 0.85 percent saline was added to the cell suspension and the resulting suspension was passed through six layers of cheese cloth.

The number of organisms was determined photometrically. Population curves for the three test organisms were made by plotting microscopic counts against light transmission using a Cenco Sheard Photometer Serial No. 2003 equipped with a blue Wratten Filter No. 49. (Figure 1) This filter has a maximum adsorption between 4400-4600 Angstrom Units. As shown in Figure 2 the maximum absorption of the methanol extracted pigment was found to be at 4500 Angstrom Units; therefore, little error was introduced when a 1-10 dilution of the suspension was adjusted to give a reading between 30 and 45 percent light transmission.

Following the determination of the cell count, 35 ml of the cell suspension was centrifuged, the clear supernatant fluid was decanted and 15 ml of methanol was added to extract the pigment from the cells. To insure a complete extraction the alcoholic cell mass was kept at 7 C for 24 hours. The

FIGURE 1


S. AUREUS - 2 =
" " - 5 =
" " - 6 =
.80
.40
1.20
1.00
0.50
0.25
0.12
20
40
60
80
PER CENT TRANSMITTANCE

extracted pigment was then separated from the cells by centrifugation, and passed through a Swinney filter if the extracted pigment solution was not absolutely clear. The amount of pigment was determined with a Beckman Spectophotometer Model B, Serial No. 20302, by plotting the percent light transmission against the dry weight of pigment per ml of extracted pigment. (Figure 3) Readings obtained from the Beckman were made at the point of maximum adsorption which proved throughout the study to be 4500 Angstrom Units.

The amount of pigment per cell was calculated in the following manner:

$$\text{Amount of pigment per cell} = \frac{\text{Wt. of pigment in 15 ml methanol}}{\text{No. of cells extracted}}$$

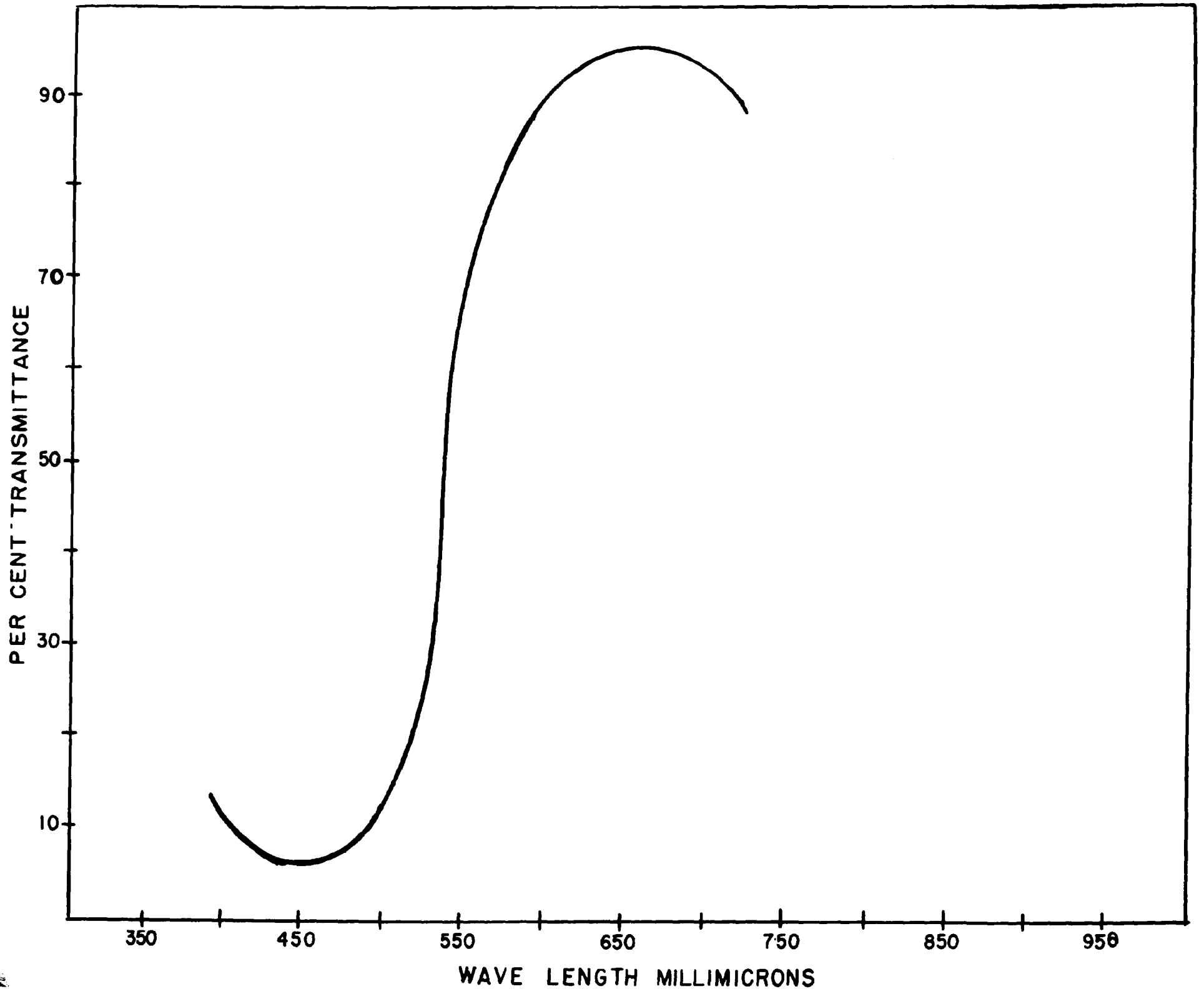
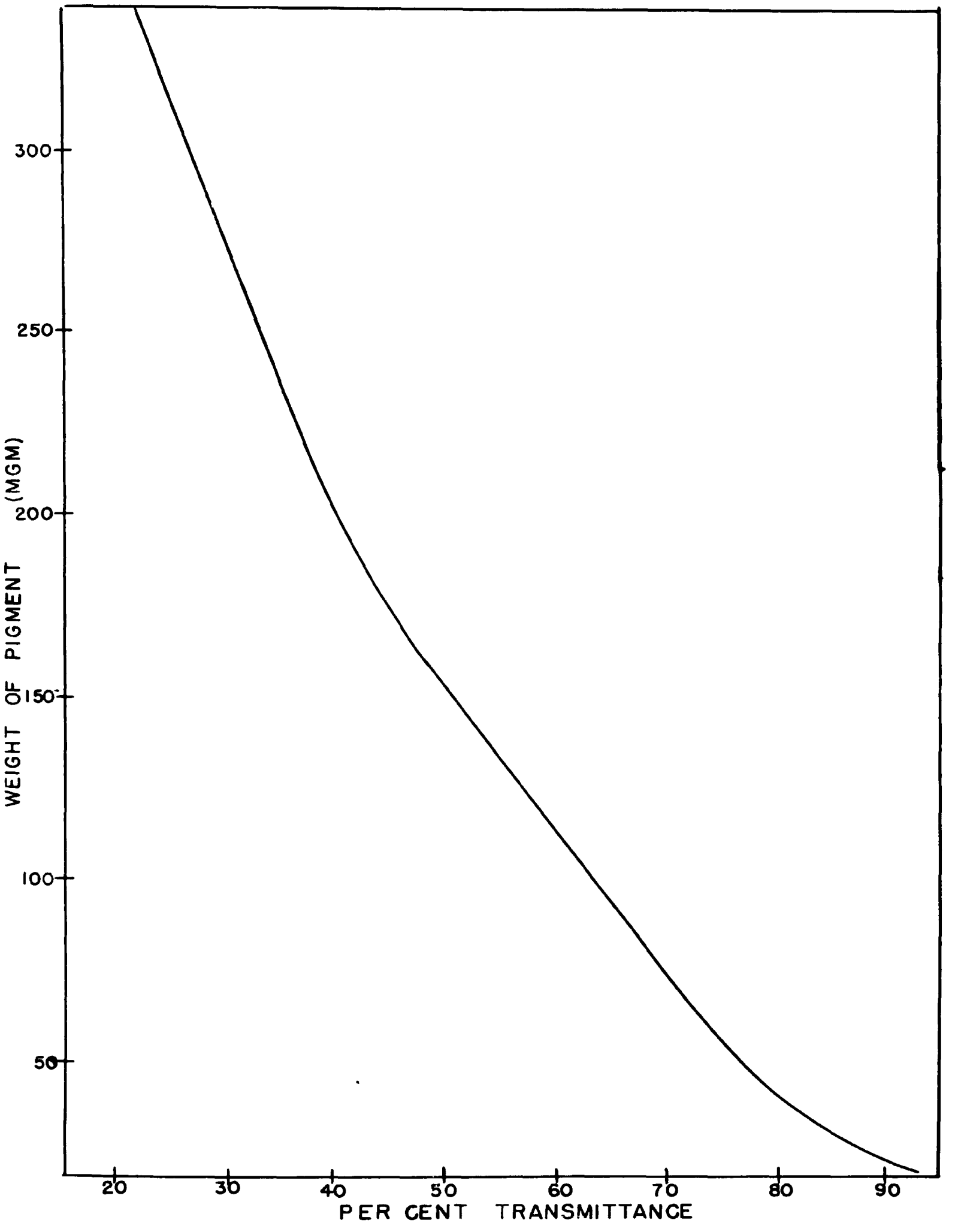

90
70
50
30
10
PER CENT TRANSMITTANCE
350
450
550
650
750
850
950
WAVE LENGTH MILLIMICRONS

FIGURE 2

FIGURE 3


300
250
200
150
100
50
WEIGHT OF PIGMENT (MGM)
20
30
40
50
60
70
80
90
PER CENT TRANSMITTANCE

RESULTS

Attempts to promote pigmentation in S. aureus have resulted in the formulating of a wide variety of media. In recent years such media as dextrose agar, proteose peptone No. 3 agar plus 10 percent evaporated milk, proteose lactose agar, and Chapman's No. 110 agar have been most frequently used for studying pigmentation in staphylococci. As workers in this field have not agreed that any one of these media are superior to the others in causing staphylococci to pigment, it was felt that a comparison of these media would be profitable. Also such preliminary work would offer some leads for further investigation as to the specific factors and medium components which are instrumental in causing pigmentation.

The constituents of the above media were as follows:

Dextrose Agar

Water	1000 ml
Beef extract	3.0 gm
Tryptose	10.0 gm
Dextrose	10.0 gm
Sodium chloride	5.0 gm
Agar	15.0 gm

Proteose Lactose Agar

Water	1000 ml
Proteose peptone	20.0 gm
Dextrose	0.5 gm
Lactose	10.0 gm
Disodium acid phosphate	5.0 gm
Sodium chloride	5.0 gm
Agar	15.0 gm

Proteose Peptone No. 3 Agar

Water	1000 ml
Proteose peptone No. 3	20.0 gm
Dextrose	0.5 gm
Sodium chloride	5.0 gm
Disodium acid phosphate	5.0 gm
Agar	15.0 gm

Chapman's No. 110 Agar

Water	1000 ml
Yeast extract	2.5 gm
Tryptone	10.0 gm
Mannite	10.0 gm
Lactose	2.0 gm
Dipotassium acid phosphate	5.0 gm
Sodium chloride	75.0 gm
Gelatin	30.0 gm
Agar	15.0 gm

Petri plates were prepared of the above media and quadruplicate seedings were made using washed cells of S. aureus No. 5 taken from a nutrient agar slant. Two plates of each medium were incubated at 20-22 C and the other two plates were incubated at 37 C. One plate at each incubating temperature was sealed with parafilm tape in order to retain the moisture of condensation so that the moist condition would be maintained throughout the 10 day growing period. The other plate of each medium was left unsealed and as a result the agar and cells lost considerable moisture after 10 days incubation. A comparison of the pigmentation produced under these conditions was made.

The results of these tests are shown in Table 1. The data show that none of the media which was kept in a moist

condition contained cells that were pigmented. Therefore it could be argued that dry conditions are necessary for pigmentation; however, it should be pointed out that, although the parafilm tape is porous material, it may have some influence upon oxygen tension and thus affect pigment production. It is a well known fact that an adequate oxygen supply is necessary for pigmentation.

As far as incubation temperatures were concerned, the data show that, at the end of ten days, there was very little difference in the amount of pigment produced. A fair amount of pigment was produced on dextrose agar but pigmentation on proteose peptone No. 3 agar and proteose lactose agar was rather inconsistent in that the former medium gave better pigmentation at 37 C and the latter at 20 C. Chapman's No. 110 agar medium gave the most consistent results in that good pigmentation was obtained at both incubation temperatures. The results at the end of five days were much the same as those shown in Table 1 in that the greatest amount of pigment and most consistently pigmented cells were obtained on Chapman's No. 110 medium. In the light of the above results Chapman's No. 110 medium merited further investigation. Also several of the chemical complexes in the medium could be replaced by known chemical compounds, thus offering an opportunity to detect the compounds instrumental in inducing pigmentation.

In some recent work by Chapman (6), it was reported that with medium No. 110, pathogenic staphylococci showed greater pigmentation at 30 C than at 20 or 37 C. An attempt was made to confirm these findings using culture 5. Thirty plates were seeded with one drop each of a saline suspension from a 24 hour nutrient agar slant and 10 of these plates were incubated at each temperature for 10 days. The results were rather inconsistent in that plates at each incubating temperature showed greater pigmentation than other plates at the two other incubating temperatures. However one fact was apparent, more plates showed good pigmentation at 30 C than at the two other incubating temperatures thus confirming Chapman's findings.

As these inconsistencies in pigmentation occurred to some extent on all media and among colonies on the same petri dish, an attempt was made to grow the cells in a liquid medium. One hundred ml portions of the modified liquid medium of Chapman were placed in 250 ml Erlenmeyer flasks. These flasks were seeded with two drops of a saline suspension from a 24 hour nutrient agar slant. Two flasks were incubated at each temperature, namely, 20, 30, and 37 C for a 10 day period. Following incubation the cells were separated from the supernatant fluid by centrifugation. The results are presented in Table 2. When visual observations of the cell masses were

made, it can be seen that the results at 30 and 37 C were much the same in that a good pigmentation was obtained at both temperatures. However when the cells were extracted with methanol according to the method of Stahly et al (33), the cells which were grown at 30 C showed greater pigmentation than those grown at 37 C. As a result of these tests an incubation temperature of 30 C was used in subsequent studies.

In order to accelerate growth by maintaining an adequate supply of oxygen and nutrients to the cells, the incubating flasks in subsequent tests were shaken for 24 hours instead of leaving them standing still for a 10 day growing period as was done previously. Approximately the same amount of pigment was produced using these two periods of growth.

The Effect of Various Constituents of Chapman's No. 110 Liquid Medium

The next step in the investigation of the factors which are instrumental in causing pigmentation was to study the effect on pigmentation when one of the constituents was withheld from Chapman's No. 110 liquid medium. For instance one medium contained all the constituents with the exception of mannite, another all the components with the exception of sodium chloride etc. The amount of pigment produced was determined by the method described in Experimental Methods.

The results of these tests are reported in Table 3. In this table the number of organisms per ml of medium, the number of organisms extracted, and the weight of pigment per cell are given. There are several interesting observations which can be noted from these data. First that growth did not occur when gelatin was left out of the medium despite the presence of tryptone to serve as a source of nitrogen, mannite and lactose to serve as a source of carbohydrate, and yeast extract to serve as a source of B complex vitamins. Perhaps gelatin could have served as a neutralizing agent to some toxic end-product, however, this is unlikely as demonstrated by subsequent data. The most reasonable interpretation is that one or several of the essential amino acids were (1) absent, (2) present but in a very limited quantity, or (3) destroyed in the preparation of the tryptone with the result that gelatin served as the sole or main source of this essential metabolite. Therefore in the absence of gelatin, the metabolite was not present in sufficient amount to induce growth.

Another fact to note is that the number of cells per ml of medium varied from 1.1 to 2.6 x 10^{9} while pigment production varied over a much wider range, it being from 0.95 to 5.37 x 10^{-10} mgm. In four out of the five media the amount of pigment did not show a direct correlation with the

number of cells produced when one of the following were lacking: mannite, lactose, sodium chloride or tryptone. When mannite was lacking, 1.8×10^{9} organisms per ml of medium and 3.56×10^{-10} mgm of pigment was found while the lack of tryptone resulted in 2.2×10^{9} organisms and 1.79×10^{-10} mgm of pigment. The lowest bacterial count, 1.1×10^{9} organisms per ml of medium and the poorest pigment production, 0.95×10^{-10} mgm, were found when yeast extract was absent from the medium. The complete medium showed a pigment production of 4.70×10^{-10} mgm.

Some of the subsequent data show that a direct correlation occurs between the number of organisms and the amount of pigment. The reason that such a correlation is not found in the data given above is that such components as yeast extract, gelatin, or tryptone have sufficient amount of the supposedly lacking constituent to contaminate the medium. As a result the organisms have all the essentials for growth and the removal of one of the constituents simply alters the amount which is available. It is obvious that such a method gives only a rough indication of the role of the individual constituents to pigmentation. However one fact does seem to stand out in these data, it being that when yeast extract was lacking from the medium, growth was poor and the amount of pigment produced was considerably less than when the other components

were withheld. As the most important components of the yeast extract are the B complex vitamins, the next logical step was to substitute such a complex for the yeast extract and to observe the effect upon growth and pigmentation.

The Effect of the B Complex Vitamins Upon Pigmentation

The same medium as in the previous test was used with the exception that the B complex supplement was substituted for the yeast extract. The supplement contained the following vitamins:

(amount per ml of vitamin solution)

Thiamin	100 γ
Pyridoxine	200 γ
Calcium pentothenate	100 γ
Riboflavin	100 γ
Niacin	100 γ
Para amino benzoic acid	100 γ
Inositol	100 γ
Biotin	100 γ

The vitamin supplement was added in the amount of 0.25 ml per 100 ml of medium, the concentration of each vitamin in the finished medium being 0.25 per ml of medium with the exception of pyridoxine. This vitamin was added in twice the amount of the others. Also each of the vitamins was made up in separate solution in the same concentration except for biotin. This solution contained 0.5mγ per ml as the biotin requirement of the test organisms was considerably less than that of any of the other B complex vitamins.

The determination of the effect of these vitamins as a complex and as separate units was made in the following manner: the medium which contained the entire B complex was used as a control. Then a run was made in which one vitamin was left out of each medium and the number of cells and the amount of pigmentation were determined.

It is apparent that the data (Table 4) are much more consistent in these tests than those shown in the previous table. This would be expected as in this case only one factor, the vitamin requirement, was being considered. The amount of pigment produced ranged from 0.48 to 4.31 x 10^{-10} mgm and the number of organisms varied from 1.9 to 5.3 x 10^{9} per ml of medium. The interesting observation under these circumstances was that in the two cases where the amount of pigment was small, the number of organisms produced was correspondingly low. When the B complex was left out of the medium, 0.48 x 10^{-10} mgm of pigment was produced with a bacterial population of 1.9 x 10^{9} organisms. The absence of thiamin in the medium resulted in 0.51 x 10^{-10} mgm of pigment and a bacterial count of 2.7 x 10^{9} organisms. When the remaining vitamins were withheld from the medium, the following results were obtained:

pyridoxine lacking - 4.14 x 10^{-10} mgm of pigment with 5.1 x 10^{9} organisms

riboflavin lacking - 4.06 x 10^{-10} mgm of pigment with 5.2 x 10^{9} organisms

biotin lacking - 4.31×10^{-10} mgm of pigment with 5.5×10^{9} organisms

niacin lacking - 3.71×10^{-10} mgm with 4.3×10^{9} organisms

para amino benzoic acid lacking - 3.77×10^{-10} mgm of pigment with 4.4×10^{9} organisms

calcium pentothenate lacking - 4.08×10^{-10} mgm with 5.0×10^{9} organisms

inositol lacking - 3.68×10^{-10} mgm of pigment with 3.8×10^{9} organisms.

The complete medium contained 3.81×10^{-10} mgm of pigment and a bacterial count of 4.9×10^{9} organisms.

A comparison of the results, when the B complex or thiamin was lacking with the remaining data in the table, shows that thiamin was essential for maximum pigment production and for good growth. The data indicate that under these conditions the bacterial growth must be over 3.0×10^{9} organisms per ml of medium in order to attain good pigmentation. The fact that the amount of pigment produced by the complete medium was less than when pyridoxine, riboflavin, biotin, and calcium pentothenate were left out of the medium is not significant as the differences are well within the limits of error of the testing method. When the various vitamins of the B complex were substituted for the yeast extract in Chapman's modified liquid medium, good pigmentation did not occur unless thiamin was included in the medium.

However as previously mentioned, there were other components in the medium such as gelatin and tryptone which may have appreciable amounts of B complex, and so a medium of a more chemically defined nature was investigated. This was especially true in the light of the work of Kliger, Grossowicz, and Bergner (18) who reported that both thiamin and niacin were required for the full growth of staphylococci.

The Role of the B Complex Vitamins in Pigmentation Using a Semi-synthetic Medium

The medium which was made for further study was of a semisynthetic nature and contained the following components:

Water	1000 ml
Vitamin-free casamino acids	5.0 gm
Tryptophane	10.0 mgm
Cystine	10.0 mgm
Lactose	2.0 gm
Mannite	2.0 gm
Dipotassium acid phosphate	5.0 gm
Sodium chloride	10.0 mgm
Magnesium sulfate 7 H_2O	20.0 mgm
Ferrous sulfate 7 H_2O	10.0 mgm
Manganese sulfate H_2O	10.0 mgm

The B complex vitamins were added to the above medium in the same concentration (0.25 ml per 100 ml of medium) as used in the previous modification of Chapman's No. 110 medium. The complete vitamin supplement was added except for the particular vitamin under test. The test medium was dispensed in 200 ml amounts into 500 ml Erlenmeyer flasks, otherwise the test procedure was the same as that used before.

The test results are presented in Table 5. The most significant fact demonstrated was that the test organism, S. aureus 5, would not grow in a medium which did not contain thiamin or niacin and that growth was poor in the absence of biotin. The data show that there was definitely a niacin and possibly a biotin contamination from the gelatin or tryptone in the medium used in the previous test. These findings, thus, confirm the work of Kliger et al. as far as the thiamin and niacin requirements of staphylococci are concerned. With respect to biotin, it has been reported by several workers that various cultures of staphylococci differ in their need for this vitamin. The fact that growth did occur demonstrates one of three things, namely; (1) the organisms can grow to a very limited extent and thus produce a very small amount of pigment in the absence of biotin, (2) the organism can synthesize a very limited amount of biotin which is required for meager growth, or (3) there is enough contaminating biotin in the so-called vitamin free casamino acid to sustain limited growth.

The first possibility is quite probable as the amount of biotin required for growth is very small as compared to that of the other vitamins. The second theory does not seem to be sound for the following reason: if the organisms were able to synthesize biotin at all, they would be able to

synthesize a sufficient amount for good growth. The third hypothesis could also be a logical explanation although microbiological tests with the casamino acids have shown it to be biotin free to the extent of 0.025*mγ*.

The fact that when biotin was not added to the medium poor growth and no pigmentation resulted, and when it was incorporated into the medium good growth and good pigmentation resulted, demonstrates that biotin is an essential vitamin. This is true for the test organism S. aureus 2 and 6, as well as for 5.

The remaining vitamins, namely; pyridoxine, riboflavin, para amino benzoic acid, calcium pentothenate, and inositol, were either synthesized by the organism or were not required for pigmentation as the amount of pigment varied from 3.40 to 4.20×10^{-10} mgm when any of the above vitamins were left out of the medium. However it is interesting to note that the low figure of 3.40×10^{-10} mgm was paralleled by the comparatively low bacterial count of 2.7×10^{9} organisms per ml of medium and occurred when the medium lacked riboflavin. The greatest production of pigment, 4.20×10^{-10} mgm, was obtained when the bacterial count was comparatively high, and occurred when inositol was withheld from the medium. In the absence of pyridoxine 3.55×10^{-10} mgm of pigment were found in the presence of a bacterial population of 3.5×10^{9} organisms.

When para amino benzoic acid or calcium pentothenate was lacking, the pigment production was approximately the same (3.54 and 3.42 x 10^{-10} mgm respectively) with the same bacterial population (4 x 10^{9} per ml of medium). These data show once again that the amount of pigment produced by the organisms was closely allied with the bacterial count and this relationship was apparent even when the differences in pigmentation were not great.

Although the amount of pigment which was produced when each of the vitamins was withheld from the medium, was not determined with cultures 2 and 6, preliminary tests showed that thiamin and niacin were also required by these organisms.

In order to see if the close correlation between the amount of pigment and the number of organisms continued to exist when the concentration of thiamin and niacin was decreased, a series of tests using 25.0, 12.5, 5.0, and 2.5γ per 100 ml was tried. The three test organisms were used. The data for thiamin are presented in Table 6. With culture 2 the amount of pigment dropped from 2.32 to 2.20 x 10^{-10} mgm and bacterial counts from 1.66 to 1.20 x 10^{9} organisms when the concentration of thiamin was decreased one half. With one-fifth the original concentration of thiamin, 1.52 x 10^{-10} mgm of pigment and 520 x 10^{6} organisms were found, while one-tenth the original concentration of this vitamin resulted in 0.77 x 10^{-10} mgm of pigment and 350 x 10^{6} organisms.

Culture 5 showed a greater drop in the amount of pigment produced than did culture 2 over the same thiamin range. With this culture the weight of pigment decreased from 4.42 to 4.32 x 10^{-10} mgm with the number of organisms dropping from 3.5 to 3.3 x 10^{9} with half the normal amount of thiamin present. When the concentration of thiamin was reduced to one-fifth the original amount, 2.40 x 10^{-10} mgm of pigment was present with a bacterial population of 2.61 x 10^{9} organisms. The highest dilution of thiamin, one-tenth of the original, gave 2.03 x 10^{-10} mgm of pigment with a bacterial count of 2.0 x 10^{9} organisms.

The decrease in the amount of pigment in culture 6 was as follows: the original concentration of thiamin gave 3.62 x 10^{-10} mgm of pigment with 2.9 x 10^{9} organisms, one half of the original concentration gave 2.87 x 10^{-10} mgm of pigment with 2.0 x 10^{9} organisms, one-fifth of the original concentration gave 0.78 x 10^{-10} mgm of pigment and 680 x 10^{6} organisms and one-tenth of the original concentration gave 0.88 x 10^{-10} mgm of pigment with 600 x 10^{6} organisms.

The amount of pigment produced followed very closely the bacterial population except where 5.0 or 2.5γ of thiamin was used with culture 6. Here slightly more pigment was produced with the lower concentration of thiamin than with the higher, although the bacterial counts of the former were slightly greater than those of the latter. This difference is best

explained by the fact that there is some error when a small amount of pigment was produced due to the inaccuracy of the spectophotometer readings above 90.0 percent light transmission. In this case the readings were 91.0 and 92.0 percent respectively

A much more significant fact is that there appears to be a definite drop in the amount of pigment produced in all three test organisms when the thiamin concentration dropped, below 12.5γ . In other words the amount of thiamin in the test medium could be halved without seriously affecting the amount of pigment produced but below this point pigment production dropped sharply. Again the weight of pigment produced followed very closely the number of organisms, the bacterial counts fell off considerably when 12.5γ was used. This was also well exemplified with culture 6 when 25.0γ were compared with 12.5γ . Here the slight drop in pigmentation from 3.65 to 2.85 x 10^{-10} mgm was accompanied by a correspondingly slight drop in bacterial count, 2.8 to 1.9 x 10^{9} organisms.

The decrease in pigment production when the amount of niacin was decreased from 25.0 to 2.5γ per 100 ml of medium is demonstrated in Table 7. Again the concentrations tested were the same as in the previous tests, namely 25.0, 12.5, 5.0, and 2.5γ per 100 ml.

Upon examination of the data using culture 2, it was demonstrated that pigment production dropped from 3.30×10^{-10} mgm with 25.0γ of niacin to 3.11×10^{-10} mgm with 12.5γ of niacin and finally to 1.28×10^{-10} mgm of pigment with 5.0γ of niacin. No growth occurred when 2.5γ of niacin was employed. Bacterial counts dropped from 2.31 to 2.20×10^{9} organisms when the concentration of niacin was one-fifth of the original.

When the concentration of niacin was reduced one-half, using culture 5, the weight of pigment decreased from 3.32 to 3.28×10^{-10} mgm while the bacterial population increased from 3.4 to 3.7×10^{9} organisms. Further reductions in the amount of niacin in the medium resulted in the following: one-fifth of the original concentration gave 1.70×10^{-10} mgm of pigment with 2.8×10^{9} organisms, and one-tenth of the original concentration gave 0.90×10^{-10} mgm of pigment with 1.9×10^{9} organisms.

Culture 6 showed a range of pigment production from 4.32×10^{-10} mgm with a niacin concentration of 25.0γ to 0.98×10^{-10} mgm with a niacin concentration of 2.5γ. The intermediate concentrations of 12.5 and 5.0γ of niacin showed 4.07 and 1.32×10^{-10} mgm of pigment respectively. Bacterial counts ranged from 4.8×10^{9} with the greatest concentration of niacin to 900×10^{6} organisms with the lowest concentration of niacin.

The results were comparable to those obtained with thiamin in that reducing the concentration of niacin by one-half did not seem to decrease either the amount of pigment formed or the number of organisms to any marked degree. However when the concentration of niacin was reduced to one-fifth or one-tenth of the original concentration, the response of the organisms was considerably different for niacin from what it was for thiamin. Pigment production dropped below 1.0×10^{-10} mgm for all three organisms when 2.5γ of thiamin was in the medium, while with niacin the results were irregular in that no growth occurred with culture 2, with culture 5, 1.88×10^{-10} mgm of pigment was obtained and 0.98×10^{-10} mgm of pigment was demonstrated with culture 6. Such data would indicate that the critical concentration of thiamin is more constant among the species of staphylococci than the critical concentration of niacin.

The possibility of niacin being a precursor of the carotenoid molecule is difficult to visualize although the 5-carbon ring which makes up the niacin molecule might serve as a precursor for the ionone ring of the carotenoid molecule. The most probable role of niacin is that which it plays a part with tryptophane in the formation of coenzyme I and coenzyme II. These coenzymes are among the most important "hydrogen carriers" within the bacterial cell and are required

for many aerobic transformations. Therefore niacin in this role is but indirectly related to pigmentation by serving as a precursor for enzymes which are necessary for the well-being of the bacterial cell.

Relationship of Cocarboxylase to Thiamin in Pigmentation of Staphylococci.

Through phosphorylation thiamin is converted to the coenzyme cocarboxylase. This coenzyme functions in the metabolism of many organisms by permitting decarboxylation of certain ketoacids (e.g. pyruvic acid). Snell (32), in discussing the chemical aspects of bacterial nutrition, reported that Streptococcus salivarius and Lactobacillus fermenti grow when either thiamin or cocarboxylase is supplied while S. aureus grows if either thiamin or its two moieties, thiazole and pyrimidine, are present. However nothing was stated about the role of cocarboxylase in staphylococci.

With the above work in mind it was thought wise to see if cocarboxylase could be substituted for thiamin, and if it could, what effect it would have on the pigmentation of the three test organisms. The test medium was the same as that used for the thiamin titration with the exception that 25.0 γ per 100 ml of medium of cocarboxylase was substituted for thiamin.

The data which are presented in Table 8 show that there was very little difference in the results when either thiamin

or cocarboxylase was present in the medium. With culture 2, 2.33×10^{-10} mgm of pigment was produced with thiamin and 2.58×10^{-10} mgm with cocarboxylase. Culture 5 gave 3.88×10^{-10} mgm of pigment with both thiamin and cocarboxylase while the results with culture 6 showed 4.27×10^{-10} mgm of pigment with thiamin and 4.68×10^{-10} mgm of pigment with cocarboxylase. Bacterial populations were very similar under the two test conditions.

Such data would indicate that one of the main functions of thiamin is to serve as a precursor for cocarboxylase. Some work has also indicated that thiamin is incorporated into the carotenoid molecule during its formation. From these two facts several theories of pigment formation can be postulated: (1) thiamin may serve as a precursor for cocarboxylase and enter into the formation of the carotenoid molecule at the same time, (2) thiamin may function during the logarithmic stage of growth as a precursor for cocarboxylase and then when this requirement is satisfied, thiamin enters into the formation of the carotenoid or (3) both thiamin and cocarboxylase can be incorporated into the pigment molecule.

Effect of Salts on the Pigmentation of Staphylococci

Reports have occurred in the literature which state that various salts besides the phosphate buffers are required by staphylococci when it is grown in a semisynthetic medium.

The salts generally required for growth are sodium chloride, ferrous sulfate, manganese sulfate, and magnesium sulfate. The effect of these salts on pigmentation in staphylococci has never been reported although Turian (35) stated that traces of iron and manganese are necessary for pigmentation in Mycobacterium phlei. The salts were added in the following concentrations per 100 ml of medium:

Sodium chloride ---------- 1.0 mgm.
Ferrous sulfate $7H_2O$ ---------- 1.0 mgm.
Manganese sulfate H_2O---------- 1.0 mgm.
Magnesium sulfate $7H_2O$--------- 2.0 mgm.

The basal medium was the same as that used to determine the vitamin requirements with the exception of the salts under test. Test procedure was the same as that used previously.

The weight of pigment produced by the three cultures when the various salts were added individually to the medium are presented in Table 9. In the first column of the table are the data for culture 2. When the four salts were added, thus giving a complete medium, 3.93×10^{-10} mgm of pigment was produced but if the four salts were absent from the medium, a sharp drop in pigment production resulted, the amount being 1.29×10^{-10} mgm. The addition of sodium chloride, ferrous sulfate or magnesium sulfate gave the following amount of pigment: 1.26, 1.44, and 1.12×10^{-10} mgm respectively. It is apparent that with these salts in the medium, there is no increase in pigmentation. However upon the addition of

manganese sulfate, pigment production increased to 2.32×10^{-10} mgm. The number of organisms varied from 1.5×10^{9} when none of the salts was included to 2.2×10^{9} organisms when sodium chloride, ferrous sulfate or manganese sulfate was added. The complete medium showed a bacterial count of 1.8×10^{9} organisms.

The results obtained with culture 5 are seen in the second column of the table. Once again when the four salts were included in the medium, the maximum pigmentation was obtained and when the four salts were left out of the medium there was a decided decrease in the amount of pigment produced. With this culture the former conditions gave 4.06×10^{-10} mgm. of pigment and the latter conditions 1.52×10^{-10} mgm of pigment. When the four salts were added separately to the medium, the following amounts of pigment were obtained: with sodium chloride - 2.42×10^{-10} mgm, with ferrous sulfate - 2.48×10^{-10} mgm, with magnesium sulfate - 2.26×10^{-10} mgm, and with manganese sulfate - 3.93×10^{-10} mgm. The data show that with this culture a composite of the salts increased pigment production considerably as compared to the medium lacking these salts. Again when the salts were added one at a time, the greatest increase in amount of pigment produced occurred when manganese sulfate was incorporated in the medium.

In all cases with culture 5 the bacterial populations were slightly over 4.0×10^9 organisms per ml of medium.

The third column of the table shows data obtained with culture 6. The inclusion of the four salts in the medium gave a pigment production of 4.16×10^{-10} mgm. The medium without the four salts showed a pigment production of 2.86×10^{-10} mgm. For the third time the data demonstrate that a greater amount of pigment was produced when the salts were included in the medium than when they were withheld. However with this strain the addition of the separate salts did not result in increased pigmentation. The addition of sodium chloride or ferrous sulfate gave 2.27×10^{-10} mgm of pigment, the addition of magnesium sulfate gave 2.45×10^{-10} mgm of pigment while the addition of manganese sulfate gave 2.58×10^{-10} mgm of pigment. The number of organisms per ml of medium with this culture was approximately 2.0×10^9 under all test conditions.

From the above data it is evident that the three organisms varied in their pigment response to the four salts. However the one common factor which they possess is that all cultures responded with a greater output of pigment when all four salts were present in the medium. It would be expected that the bacterial population would be considerably greater when pigment production was higher; in other words, when the four salts were in the medium. This did not occur with cultures 2 and 5.

In each case the bacterial counts of the media with and without the salts were approximately the same (culture 2 - 1.8 and 1.5 x 10^9 organisms, culture 5 - 4.6 and 4.0 x 10^9 organisms). The expected pattern is followed with culture 6 as there were approximately twice as many organisms when the salts were included in the medium as when they were left out. From these data it appeared as though one of the salts, namely magnesium sulfate, had an effect upon the amount of pigment produced in cultures 2 and 5 but had very little effect upon the number of organisms produced. On the other hand, with culture 6 the salts seemed to have an effect upon the number of organisms as well as on the extent of pigmentation.

The data that show an increase in pigment production upon the addition of manganese sulfate are very interesting, especially in the light of Turian's (35) findings with Myco. phlei. The role played by the manganese sulfate in the metabolism, and therefore in the pigmentation of the organism, is not understood and calls for some speculation. As manganese is not found in the carotenoid molecule, its probable role is of catalytic nature. This has been found to be true in the case of chlorophyll formation in plants and hemoglobin formation in animals. Manganese serves as an activator for the phosphotases, arginases, dipeptidases, and laccases and therefore may be in demand in several instances in the formation of the carotenoid pigments.

The role of the dipotassium phosphate in the growth and pigmentation of the three cultures of staphylococci is treated separately due to its many metabolic functions. The effect of this salt directly upon pigmentation has never been reported; however, the need of the phosphate salts for the general well being of most organisms shows that at least indirectly it is related to pigment production. One of the most important functions is the buffer capacity. Another function is the part played by phosphates in the sugar metabolism of the cells. Five-tenths gm per 100 ml of dipotassium acid phosphate was added to the test medium. This was sufficient to buffer the medium above pH 6.0 during the 24 hour growing period. The test medium was the same as that used in the previous test with the exception of the phosphate salt.

The contents of Table 10 compare the results obtained with the three organisms when they were grown with and without the phosphate salt. As would be expected the absence of the phosphate resulted in much poorer growth and much less pigmentation than when the salt was in the medium. With culture 2 the differences were not as great as with the other two cultures, the weight of pigment falling from 2.33 to 1.72 x 10^{-10} mgm and the number of organisms dropping from 1.8 x 10^{9} to 600 x 10^{6} per ml of medium. With culture 5 the loss in

pigment production was from 3.88 to 1.90 x 10^{-10} mgm which is no doubt the result of the bacterial population decreasing from 3.6 x 10^{9} to 560 x 10^{6} organisms per ml of medium. The greatest loss in pigmentation is seen with culture 6 where the drop was from 4.27 to 1.36 x 10^{-10} mgm. With this organism the number of bacteria per ml of medium decreased from 2.95 x 10^{9} to 700 x 10^{6} when the phosphate buffer was left out of the medium.

Also shown in the table are the pH values of the supernatant fluid after centrifuging. The test medium was adjusted to a pH of 7.00 before seeding. After growth the pH of the test medium containing the phosphate salt had dropped approximately one unit, the pH values being 5.90 for culture 2, 6.30 for culture 5 and 6.18 for culture 6. When the phosphate buffer was left out of the medium, the pH drop was over two units, it being 4.70 for culture No. 2, 4.75 for culture 5 and 4.97 for culture 6.

The lower pH which resulted when the buffer was not included in the medium was probably the principal reason for the poor growth and low pigment production. When the bacterial populations were approximately 2.0 x 10^{9} organisms for culture 2, around 4.0 x 10^{9} organisms for culture 5 and 3.0 x 10^{9} organisms for culture 6, pigmentation was good and when the number of organisms fell below these levels, pigmentation decreased noticeably.

The Role of Carbohydrates in Pigmentation

The significance of the carbohydrate content of a medium in relation to the pigmentation of staphylococci has been investigated to some extent by Chapman (6). He found that the addition of evaporated milk and subsequently lactose, enhanced pigmentation markedly. In a medium which was reported in 1948, Chapman (6) included mannite as well as lactose, the former serving as a differentiating sugar. In the light of Chapman's experiences and as Sevag and Green (29) had reported inconclusive results as far as pigmentation was concerned when carbohydrates or their intermediate products were added to the medium, it was felt that the effect upon pigmentation of the most common laboratory sugars should be investigated.

The sugars which were included in this phase of the study were lactose, mannite, dextrose and sucrose. Each sugar, when used separately, was added in the amount of 0.4 gm per 100 ml. This is the total amount of sugar previously used in the media when 0.2 gm of lactose and 0.2 gm of mannite were added. This was done to maintain a constant amount of sugar in all media tested and thus to eliminate any possible chance that greater growth or pigmentation would be obtained in either medium due to the one containing more carbohydrates than the other.

In Table 11 the data are presented for this experiment. The pigment production varied from 2.79 to 4.09 x 10^{-10} mgm while the bacterial populations were fairly constant in that they ranged between 4.0 to 4.6 x 10^{9} organisms per ml of medium. The greatest amount of pigment, 4.09 x 10^{-10} mgm, was obtained when both mannite and lactose were the carbohydrate source, followed by 3.60 x 10^{-10} mgm when sucrose alone was used. With lactose as the carbohydrate source in the medium, 3.50 x 10^{-10} mgm was obtained and with dextrose 2.79 x 10^{-10} mgm of pigment was found. The bacterial populations were as follows: 4.6 x 10^{9} organisms when mannite or lactose was in the medium, 4.4 x 10^{9} organisms with sucrose as the source of sugar, 4.1 x 10^{9} organisms with dextrose and 4.0 x 10^{9} organisms when lactose and mannite were included. Such differences in bacterial populations were not great enough to be significant.

The differences in the amount of pigment produced when the two sugars, lactose and mannite or the single sugars, lactose, mannite or sucrose were added to the medium were also not great enough to be important. However when dextrose served as the source of carbohydrate, the low figure of 2.79 x 10^{-10} mgm of pigment merits some consideration. As the bacterial populations were not consistent with the amount of pigment produced; that is, the tests with the greatest number of

organisms did not give the greatest amount of pigment, interpretation is difficult. However when lactose, mannite or sucrose was added separately to the medium, the pigment production and bacterial populations were approximately the same and all three showed more pigmentation and more organisms than when dextrose was present in the medium. Such findings allow one to conclude that those sugars were superior to dextrose in affecting pigmentation. The possible explanation of these findings might be found in the products which were formed from these sugars during autoclaving.

According to Lewis (20) products deleterious to growth of some organisms including staphylococci, were formed when phosphates in a concentration above 0.3 percent and dextrose in a concentration above 0.5 percent were autoclaved together. On the other hand lactose had to be added in a slightly higher concentration (0.6 percent) before these deleterious effects were apparent. As pigmentation is closely allied with good growth, it is possible that Lewis' findings offer a partial explanation of the differences in the amount of pigment production with the various sugars.

Other workers have obtained varied results when growth responses to autoclaved and filtered sugars have been compared. Some data such as Lewis', show that it is necessary to filter sugars in order to get a true picture of the carbohydrate

metabolism. Other work indicates that this is necessary for only a few of the more susceptible sugars while still other data, Fulmer et al (12), show that all sugars can be autoclaved as this process produces intermediates which are beneficial for growth. There are innumerable reasons for these discrepancies, such as the pH and amino acid content of the medium, the various salts present in the medium, the time and pressures used in autoclaving and the wide variety of organisms which have been used for testing.

A search of the literature revealed that nothing had been reported on the effect of filtered as compared to autoclaved sugars upon pigmentation. If there are some detrimental or advantageous breakdown products derived from sugars which are autoclaved and these products affect growth, as some workers have reported, there might also be some effect upon pigmentation.

With the above in mind, a comparison between filtered mannite and lactose and autoclaved mannite and lactose was made. A combination of these two sugars was used as it had been shown in preceding tests that these sugars gave maximum pigmentation. Tests were run using the three test organisms. Mannite and lactose were added to the regular semisynthetic medium in the concentration of 0.2 gm per 100 ml of medium.

The data from this test are presented in Table 12. It is apparent that with all three organisms there was definitely more pigment produced per cell when they were grown in a medium using filtered sugars than when autoclaved sugars were employed. With culture 2 the medium containing the filtered sugars gave 3.42×10^{-10} mgm of pigment with 3.4×10^{9} organisms while the medium with the autoclaved sugars gave 2.04×10^{-10} mgm of pigment with 2.0×10^{9} organisms per ml of medium. Using culture 5 the filtered sugars showed 5.72×10^{-10} mgm of pigment and the autoclaved sugars 4.68×10^{-10} mgm of pigment with bacterial counts of 4.1 and 4.7×10^{9} organisms respectively. Culture 6 showed the greatest difference in the amount of pigment produced, 6.24×10^{-10} mgm were found when the medium contained the filtered sugars and 4.39×10^{-10} mgm when the medium included the autoclaved sugars. With this culture the bacterial population under both test conditions was practically the same; 2.9×10^{9} organisms with the filtered sugars and 2.8×10^{9} organisms with the autoclaved sugars.

These data offer some interesting information. One interpretation of the results could be that when these two sugars were autoclaved, some of the intermediates which were formed were detrimental to the formation of the carotenoids in staphylococci. Another interpretation of the results is

that with the breakdown of some of the sugar during autoclaving, there was not as much of the original sugar left in the medium as when the filtered sugars were used. As this sugar combination seems to give good pigmentation, an altering of these sugars in any way such as autoclaving, results in less pigment being produced. On the other hand it does seem quite plausible that with some other sugar or sugar combination, products more favorable for pigmentation would be formed from the autoclaved sugar than from the unaltered, filtered sugar.

The relationship of the amount of pigment to the number of cells produced calls for some discussion. In much of the previous data which have been given there was a direct relationship between the amount of pigment and the number of cells produced. When counts were high, pigmentation was good and when the counts were low, the amount of pigment dropped off considerably. However with the altering of the salts, and now with the filtered and autoclaved sugars, this does not seem to be the case. In both instances pigmentation seemed to become a factor independent from growth. Of course this was true only if a particular level of growth was obtained. Such evidence supports the theory that pigmentation can be altered by some growth factors and is not simply a phenomenon which occurs when the bacterial population attains a particular level.

It is true that there was some discrepancy in the bacterial counts of culture 5 in that there were slightly greater numbers of cells in the autoclaved sugar medium than in the filtered sugar medium. However, this difference only serve further to support the argument that there are growth factors which directly affect pigmentation and have put a minor effect upon growth.

Effect of Amino Acids upon Pigmentation

No investigation of the effect of various metabolites upon pigmentation would be complete without some work being done on altering the amino acid composition of the media. Reid (26) reported that nitrogen as well as inorganic salts were necessary for pigmentation. Gladstone (13) developed a synthetic medium of 16 amino acids and found that 11 of these acids were necessary for the growth of 25 strains of staphylococci. This worker was interested simply in growth of the organism and did not mention the extent of pigmentation in these cultures.

For such a study, it would be necessary to use a chemically defined medium similar to Gladstone's in order that it could be determined if any particular amino acid had an effect upon pigmentation. When a synthetic medium is used, additional problems are presented which are not apparent when a peptone or even casamino acids are used for

the nitrogen source. The initial problem is to devise a medium in which the organism will grow. If this can be accomplished, the next difficulty to overcome is to obtain sufficient growth for pigment extraction.

The medium which was tried contained the following constituents:

Basal Medium

Water	1000 ml.
Mannite	2.0 gm.
Lactose	2.0 gm.
Dipotassium acid phosphate	5.0 gm.
Sodium chloride	0.1 gm.
Magnesium sulfate $7H_2O$	0.2 gm.
Ferrous sulfate $7H_2O$	0.1 gm.
Manganese sulfate H_xO	0.1 gm.
Thiamin	250.0 γ
Niacin	250.0 γ
Biotin	0.12 γ
Valine	175.0 mgm.
Leucine	150.0 mgm.
Glycine	40.0 mgm.
l Proline	60.0 mgm.
Methionine	60.0 mgm.
Phenylalanine	60.0 mgm.
Aspartic acid	150.0 mgm.
Arginine	40.0 mgm.
Histidine	40.0 mgm.
Cystine	10.0 mgm.
Tryptophane	10.0 mgm.

In order to determine if the above medium contained sufficient nutrients for growth of the three test organisms, the following procedure was tried. The medium was dispensed in three ml amounts and seeded from a 24 hour nutrient agar slant. Two transfers through the broth were necessary before growth was considered successful.

The results of these trials are observed in the first part of Table 15. Cultures 2 and 5 grew in the medium while 6 did not. The latter culture was reseeded into the above broth and shaken during incubation at 30 C and still no growth occurred. Attempts were also made to grow this organism at 37 C without success.

The following constituents were added to a previously tried medium in the combinations given below.

Vitamins (nonessential)	
Pyrodoxine	500.0 γ
Calcium pentothenate	250.0 γ
Riboflavin	250.0 γ
Para-amino benzoic acid	250.0 γ
Inositol	250.0 γ
Folic acid	2.0 γ
Amino acids (nonessential)	
Alanine	100.0 mgm.
Glutamic acid	75.0 mgm.
Tyrosine	27.0 mgm.
Lysine	75.0 mgm.
Purine bases	
Adenine	0.5 mgm.
Guanine	0.5 mgm.
Uracil	0.5 mgm.
Xanthine	0.5 mgm.

(1) Basal medium - calcium pentothenate.

(2) Basal medium - calcium pentothenate - purine bases.

(3) Basal medium - 5 nonessential B complex vitamins.

(4) Basal medium - 5 nonessential B complex vitamins - purine bases.

(5) Basal medium - 5 nonessential B complex vitamins - purine bases - folic acid

(6) Basal medium - 4 nonessential amino acids.

Calcium pentothenate, purine bases and folic acid were included in the various media as the work of Moller et al (20) had shown that about one-fourth of the races of staphylococci studied, required pantothenic acid, one-half needed purine bases and a few would not grow without folic acid.

The three cultures were seeded into these media in the same manner that had been used before. The results are shown in the remaining portion of Table 13. Cultures 2 and 5 grew in all the combinations of medium which were tried while culture 6 failed to grow. A final trial was made in which the nonessential amino acids, namely, alanine, glutamic acid, tyrosine and lysine were added individually; however still no growth was obtained. Due to these results the investigation of the extent of pigmentation in a purely synthetic medium was confined to cultures 2 and 5.

The medium which was used for pigmentation tests was the basal medium which contained the sugars, salts, essential B complex vitamins and the 11 required amino acids plus alanine, glutamic acid, tyrosine and lysine. The latter acids were added separately to lots of the above medium to determine the significance of each in pigment production. It was necessary to shake the incubating cultures for 48 hours rather than 24 hours in order to obtain sufficient cells for methanol extraction. Two hundred ml of the medium in flasks was seeded and treated in the usual manner.

In Table 14 are shown the data using culture 5. The amount of pigment produced varied from 4.10 to 4.77 x 10^{-10} mgm. The least amount of pigment was obtained when the medium consisted of the required amino acids plus glutamic acid (4.10 x 10^{-10} mgm), and the greatest amount when the medium contained the 11 required amino acids alone or with alanine (4.77 x 10^{-10} mgm). When lysine or tyrosine was used in the medium with the required amino acids, 4.41 and 4.28 x 10^{-10} mgm of pigment respectively were produced.

From the standpoint of the amount of pigment produced, the differences do not seem to be great enough to be significant. However when the data are examined in the light of the number of cells produced, some interesting results are apparent. The greatest number of cells, 790 x 10^6, were found when the least amount of pigment was produced; that is with the medium which contained the 11 required amino acids plus glutamic acid. The lowest bacterial count, 425 x 10^6, occurred when the amount of pigment was the greatest, that is with the required amino acids alone or with alanine. The other two media containing the required amino acids plus lysine or tyrosine showed bacterial populations of 510 x 10^6 organisms per ml of medium which were inversely proportional to the amount of pigment produced.

The results, when culture 2 was tried, are shown in Table 15. The data are erractic due to insufficient cells for extraction and to the variable ratio of the number of cells and pigment production. For example when the medium contained the 11 required amino acids plus alanine, insufficient cells were obtained. Pigment production and total cell variations were shown as follows: the 11 required amino acids alone gave 4.03×10^{-10} mgm of pigment and 368×10^{6} cells, with tyrosine - 2.72×10^{-10} mgm and 576×10^{6} cells, with glutamic acid - 2.96×10^{-10} mgm and 520×10^{6} cells, and with lysine - 3.48×10^{-10} mgm and 394×10^{6} cells.

Upon examination of the data obtained with culture 5, the initial conclusion that could be drawn is that pigmentation was not affected by the addition of any of the auxiliary amino acids which were tested. A level of pigmentation of approximatel 4.40×10^{-10} mgm was reached when any of the above media was tried. On the other hand the growth of the organism was definitely affected by the addition of the amino acids. Therefore under these test conditions pigmentation was not directly proportional to growth.

Such a high level of pigmentation in contrast with bacterial counts which were relatively low requires some explanation. One possible answer may be the length of time that the cells were grown, it being twice as long as when

the semisynthetic medium was under test. As mentioned before a 48 hour growing period was necessary to obtain sufficient cells for methanol extraction. Another possibility is that considerable error was introduced in the pigment production determinations when it was necessary to use values at the lower end of the curve denoting weight of pigment. The percent light transmission covered a range of 78.0 to 83.0. The fact that the most accurate readings on any type of a light transmission instrument are obtained between 25.0 and 75.0 and that below and above these values, the error increases in magnitude, causes some doubt as to the validity of these results. This is also true when the number of organisms was determined as the percent light transmission obtained with these cell suspensions was above 77.0 percent.

The inconsistent results obtained with culture 2 are also best explained by the inaccuracies which enter the calculations when percent light transmission values are high. With this organism much greater error would be expected, as in the determination of pigment weight, the spectophotometer readings were all above 90.0 percent although the bacterial counts were above 66.0 percent.

SUMMARY

The results obtained in this study have been discussed for the most part in that portion of the thesis where the data were given. Therefore, this section will be confined to some concluding or summarizing remarks of the observations which have been made in the study of S. aureus.

The method which was devised for determining the amount of pigment has proved satisfactory for the majority of the tests made. Close agreement has been obtained with the method of Stahly et al (32) as shown in Table 16. With a small amount of pigment (A) 0.0190 units were obtained with Stahly's method and 1.91×10^{-10} mgm of pigment with the method used in these tests. With greater amounts of pigment agreement was also close, (B) and (C) showing 0.0248 and 0.0266 units respectively of pigment with Stahly's method and 3.88 and 4.32×10^{-10} mgm respectively of pigment with the extracted pigment method. Enough samples were not tested to determine the superiority of one method over the other but the agreement of both show that the method used in this study was scientifically sound.

Attempts to attribute pigmentation to particular chemical compounds have been made by many workers with a variety of organisms. Some have gone as far as to state that organisms can be made to produce pigment simply by the addition of a particular salt, sugar, vitamin or amino acid or

can completely suppress pigmentation by withholding any such compound from the medium. Sullivan (1905) reported that the organic salts - malate, tartrate and oxalate allow growth but do not allow pigmentation while lactate, citrate or succinate give good pigmentation. Several species of micrococci were used in this study. From the observations made in this study this was not the case with staphylococci. With this organism the degree of pigmentation can be altered by the addition of various chemical compounds but it cannot be induced or completely suppressed in the presence or absence of such components. Perhaps the methods of measuring the extent of pigmentation have been responsible for some of the results which have been obtained in the past.

In some instances changes in the amount of pigmentation were closely allied with the bacterial population. A good yield of pigment was obtained when the bacterial counts were relatively high and low pigment formation resulted when relatively low bacterial counts occurred. This was well exemplified when the vitamin supplement was substituted for the yeast extract in the liquid modification of Chapman's medium and when decreasing amounts of thiamin and niacin were used in the semisynthetic medium. In both cases altering the amount of vitamin resulted in changes in the bacterial populations which in turn caused a proportional change in the amount of pigment produced.

Although a direct correlation was also very apparent with all three test organisms when the amount of pigment was compared in the presence and absence of the phosphate salt. The data obtained with the salts and sugars for the most part presented a different picture. Variations in the amount of pigment produced were not accompanied by similar changes in the bacterial populations. The studies made with the various salts demonstrated that the addition of manganese sulfate increased pigmentation by one third of that obtained with the other salts (ferrous sulfate, magnesium and sodium chloride) that were tried.

Likewise the comparison of the various sugars using culture 5 showed that differences in the amount of pigment were not correlated directly with cell numbers. There was less pigment when dextrose served as the carbohydrate source than with any of the other sugars but bacterial populations were about the same throughout.

The comparison of the filtered and autoclaved mannite and lactose solutions presented a similar picture in that, greater pigmentation was obtained with the filtered carbohydrates than was obtained with the autoclaved carbohydrates although the number of organisms per ml of medium remained approximately the same.

The results obtained when the amino acid content of the medium was altered would indicate that alanine, tyrosine, glutamic acid and lysine had no effect upon pigmentation although the validity of these results may be open to question due to the limited number of organisms which grew in a purely synthetic medium.

To summarize, it may be stated that specific salts and sugars had a direct effect upon pigmentation and very little or no effect upon cell proliferation after the growth requirements of the organism were apparently completely satisfied.

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Table 1. A Comparison of Pigment Production by S. aureus on Solid Media at Two Incubation Temperatures with Petri Plates Sealed and Unsealed

Media		Incubating Temperatures	
		21 C	37 C
Dextrose Agar	Sealed	N P	N P
	Unsealed	F P	F P
Proteose Peptone #3 Agar	Sealed	N P	N P
	Unsealed	F P	G P
Proteose Lactose Agar	Sealed	N P	N P
	Unsealed	G P	F P
Chapmans #110 Agar	Sealed	N P	N P
	Unsealed	G P	G P

N P --- No visible Pigment
F P --- Fair Pigmentation
G P --- Good Pigmentation

Table 2. A Comparison of Pigmentation by S. aureus 5 at three Incubating Temperatures Using 10 Days Growth

Incubating Temperatures	Pigmentation Observed in Cells	Pigmentation by Methanol Extraction
20 C	F P	Orange
30 C	G P	Very Dark Orange
37 C	G P	Dark Orange

F P --- Fair Pigmentation
G P --- Good Pigmentation

Table 3. The Effect of Omitting Various Constituents of Chapmans # 110 Medium on Growth and Pigmentation of S. aureus 5

Omitted Constituent	No. of Organisms /ml Media x 10^9	No. of Organisms Extracted x 10^9	Wt. of Pigment/ Cell x 10^{-10}
Complete Medium	---	314	4.70
Without Mannite	1.8	314	3.56
Without Lactose	2.6	314	2.54
Without Sodium Chloride	2.1	314	5.37
Without Yeast Extract	1.1	314	0.95
Without Tryptone	2.2	307	1.79
Without Gelatin	N.G.	---	---

Table 4. The Effect of Omitting Various B Complex Vitamins on Pigmentation When Substituted for Yeast Extract in Chapmans #110 Medium

Omitted Constituents	No. of Organisms /ml of Medium($\times 10^9$)	No. of Organisms Extracted($\times 10^9$)	Wt. of Pigmen Cell ($\times 10^{-10}$
Complete Medium	4.9	350	3.81
Without B Complex	1.9	350	0.48
Without Thiamin	2.7	350	0.51
Without Pyridoxine	5.1	350	4.14
Without Riboflavin	5.2	357	4.06
Without Biotin	5.3	350	4.31
Without Niacin	4.3	350	3.71
Without P.A.B.A.	4.3	350	3.77
Without Ca Pento thenate	5.0	350	4.08
Without Inositol	3.8	350	3.68

Table 5. The Effect of Omitting Various B Complex Vitamins from a Semi-synthetic Medium on Pigmentation by S. aureus.

Omitted Constituents	No. of Organisms /ml of Medium(x 10^9)	No. of Organisms Extracted(x 10^9)	Wt. of Pigmen Cell (x 10^{-10}
Complete Medium	4.0	281	4.09
Without Thiamin	N.G.	---	---
Without Niacin	N.G.	---	---
Without Biotin	0.7	350	0.57
Without Pyridoxine	3.5	326	3.55
Without Riboflavin	2.7	350	3.40
Without P.A.B.A.	4.0	350	3.54
Without Ca Pento-thenate	4.0	350	3.42
Without Inositol	4.7	350	4.20

Table 6. The Effect of Various Concentrations of Thiamin on Pigmentation of Three Cultures of S. aureus

Organism	of Thiamin / 100 ml of Medium			
	25.0	12.5	5.0	2.5
2				
Wt. of Pigment x 10^{-10}	2.32	2.20	1.52	0.77
No. of Organisms x 10^{9}/ ml of Medium	1.7	1.2	0.52	0.35
5				
Wt. of Pigment x 10^{-10}	4.42	4.42	2.40	0.78
No. of Organisms x 10^{9}/ ml of Medium	3.5	3.3	2.7	2.0
6				
Wt. of Pigment x 10^{-10}	3.62	2.85	0.78	0.88
No. of Organisms x 10^{9}/ ml of Medium	2.9	2.0	0.68	0.60

Table 7. The Effect of Various Concentrations of Niacin on Pigmentation of Three Cultures of S. aureus

Organism	of Niacin / 100 ml of Medium			
	25.0	12.5	5.0	2.5
2				
Wt. of Pigment x 10^{-10}	3.30	3.11	1.26	---
No. of Organisms x 10^{9}/ ml of Medium	2.3	2.2	0.66	N.G.
5				
Wt. of Pigment x 10^{-10}	3.32	2.28	1.70	0.90
No. of Organisms x 10^{9} / ml of Medium	3.4	3.7	2.8	1.9
6				
Wt. of Pigment x 10^{-10}	4.32	4.00	3.39	0.98
No. of Organisms x 10^{9} / ml of Medium	4.9	4.1	1.3	0.90

Table 8. A Comparison of the Amount Pigment Producted When Cocarboxylase Is Substituted for Thiamin in a Semisynthetic Medium Using Three Cultures of S. aureus

	Wt. of Pigment ($\times 10^{-10}$) / Cell	
Organism	Cocarboyxlase	Thiamin
2	2.58	2.33
5	3.88	3.88
6	4.68	4.27

Table 9. The Effect of Sodium Chloride, Ferrous Sulfate, Manganese Sulfate and Magnesium Sulfate on Pigment Production and Growth in Three Cultures of *S. aureus*.

	Culture 2		Culture 5		Culture 6	
Chemical Constituents	Wt. of Pig. x 10^{-10}	No. of Organisms x 10^{9}	Wt. of Pig. x 10^{-10}	No. of Organisms x 10^{9}	Wt. of Pig. x 10^{-10}	No. of Organisms
Four Salts Added	3.93	1.8	4.06	4.0	4.16	2.0×10^{9}
No Salts Added	1.29	1.5	1.52	4.6	2.86	1.0×10^{9}
Sodium Chloride Added	1.26	2.2	2.42	4.4	2.27	1.0×10^{9}
Ferrous Sulfate Added	1.44	2.2	2.48	4.0	2.27	900×10^{6}
Manganese Sulfate Added	2.32	2.2	3.93	4.4	2.58	1.4×10^{9}
Magnesium Sulfate Added	1.12	1.7	2.26	4.6	2.45	900×10^{6}

Table 10. A Comparison of the Number of Organisms, Weight of Pigment and pH Obtained with and without di-potassium Acid Phosphate Using Three Cultures of S. aureus

Organism		No. of Organisms x 10^9/ml of Medium	Wt. of Pigment x 10^{-10}/Cell	pH of Supernatant Fluid After Growth
2	With K_2HPO_4	1.8	2.33	5.90
	Without K_2HPO_4	0.58	1.72	4.70
5	With K_2HPO_4	3.7	3.88	6.30
	Without K_2HPO_4	0.56	1.90	4.75
6	With K_2HPO_4	2.95	4.27	6.18
	Without K_2HPO_4	0.70	1.36	4.97

Table 11. The Effect of Various Carbohydrates in a Semi-synthetic Medium on Pigmentation by *S. aureus*

Carbohydrate	No. of Organisms ($\times 10^{9}$)/ml of Medium	Wt. of Pigment ($\times 10^{-10}$)/Cell
Lactose & Mannite	4.0	4.09
Lactose	4.6	3.43
Mannite	4.6	3.50
Dextrose	4.1	2.79
Sucrose	4.4	3.60

Table 12. A Comparison of Pigment Production by Three Cultures of S. aureus When Grown on Media Containing Filtered or Autoclaved Lactose and Mannite.

Organism	No. of Organisms ($\times 10^9$)/ ml Medium		Wt. of Pigment ($\times 10^{-10}$)/ Cell	
	Filtered Sugars	Autoclaved Sugars	Filtered Sugars	Autoclaved Sugars
2	2.2	2.2	3.42	2.04
5	4.1	4.7	5.72	4.68
6	2.9	2.8	6.24	4.39

Table 13. The Growth Response of Three Test Organisms to Various Synthetic Media

	Growth of Organism		
Media	Culture 2	Culture 5	Culture 6
B.M.	+	+	-
B.M. + C.P.	+	+	-
B.M. + C.P. + P.B.	+	+	-
B.M. + Non-essential Vitamins	+	+	-
B.M. + Non-essential Vit. + P.B.	+	+	-
B.M. + Non-essential Vit. + P.B. + Folic Acid	+	+	-
B.M. + Four Amino Acids	+	+	-

B.M. = basal medium
C.P. = calcium pentothenate
P.B. = purine bases

Table 14. The Effect of Various Amino Acids on Pigmentation of S. aureus 5.

Amino Acids	No. of Organisms ($\times 10^{6}$)/ ml Medium	Wt. of Pigment ($\times 10^{-10}$)/ Cell
11 Required Amino Acids	425	4.77
11 Required Amino Acids + Tyrosine	510	4.28
11 Required Amino Acids + Alanine	425	4.77
11 Required Amino Acids + Glutamic Ac.	790	4.10
11 Required Amino Acids + Lysine	510	4.41

Table 15. The Effect of Various Amino Acids of Pigmentation of S. aureus 2.

Amino Acids Added	No. of Organisms ($\times 10^{6}$)/ ml Medium	Wt. of Pigment ($\times 10^{-10}$)/ Cell
11 Required Amino Acids	368	4.06
11 Required Amino Acids + Tyrosine	576	2.72
11 Required Amino Acids + Alanine	*---	---
11 Required Amino Acids + Glutamic Ac	520	2.96
11 Required Amino Acids + Lysine	394	3.48

* Insufficient Cells for Extraction

Table 16. A Comparison of Stahly's Method with the Extracted Pigment Method for Determining Amount of Pigment Produced.

Medium	Stahly's Method	Extracted Pigment Method ($\times 10^{-10}$)
Semisynthetic Medium A	0.0190	1.91
Semisynthetic Medium B	0.0248	3.88
Semisynthetic Medium C	0.0266	4.32