THE CAROTENOID PIGMENTS OF STAPHYLOCOCCUS PYOGENES

by

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A Thesis

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INTRODUCTION AND PURPOSE.

The majority of investigations on the carotenoid pigments of bacteria have dealt with the identification of the types of carotenoids present in bacteria. Since the publication of Sullivan's paper in 1905 little work has been done to show which factors affect the synthesis of carotenoids in bacteria. Most of the investigations which have been conducted along this line have dealt with the effect of various carbon sources, inorganic ions, pH and temperature on pigmentation. The majority of these observations have been visual comparisons. In preliminary investigations this method proved to be unreliable as a means of determining variations in pigment production. Thus, before any investigations could be attempted it became imperative that an accurate and reliable method be found whereby quantitative comparisons could be made.

It was intended in the beginning to do a complete survey of the physical and chemical factors influencing carotenoid production in Staphylococcus pyogenes. On further consideration, it was decided that such experiments would indeed yield a mass of information but would not give any insight as to the metabolic pathway of synthesis. Therefore investigations were designed so as to obtain a better understanding of pigment synthesis in Staphylococcus pyogenes.

HISTORICAL REVIEW.

Introduction:

Any attempt to evaluate the literature pertaining to the carotenoids of bacteria would prove futile since much of the work was done at a time when the chemical structure of the compound was unknown. Proper methods for the determination of variation in pigment production were lacking and therefore, investigators were unable to secure a true appreciation of the factors influencing pigment production. Since the discovery of the chemical structure many strides have been made in identification of the carotenoids present in bacteria but work on other aspects is still nil.

In order to give the reader a background as to the other pigments produced by bacteria, exclusive of the carotenoids, a section on the classification of bacterial pigments is included.

Classification of Bacterial Pigments:

Beijerinck (1891) suggested that pigment producers should be divided into three classes on the basis of their relationship to the cell protoplasm:

1. Chromophoric - pigment contained in the protoplasm of bacteria.
2. Parachromophoric - pigment located on cell wall or capsule.
3. Chomoparic - color external to cell.

Buchanan and Fulmer (1928) believed that the

emphasis placed by Beijerinck between the chromophoric and parachromophoric was unnecessary and that the essential differentiation should be made between the cellular and the extracellular pigments. They divided the cellular pigments into the following classes:

1. Pigment diffused into protoplasm.
2. A pigment may be a characteristic part of the cell inclusion.
3. Pigment may impregnate the cell wall.
4. The pigment may be present on or impregnate the sheath or capsule.

The extracellular pigments were classified by Buchanan and Fulmer (1928) according to the manner in which they developed:

1. Pigment produced inside the cell and excreted as such.
2. A leuco compound may be excreted by the cell and the color caused to develop through the activity of some external agent.
3. An enzyme excreted by the cell may bring about a color change in the medium.
4. Some other chemical excreted by the cell may be the cause of change of color in the environment.

Buchanan and Fulmer (1928) proposed several other methods for classification of the pigment produced by bacteria. One classification was based on the fact that certain pigments

of micro-organisms gave reactions similar to those of the higher plants and animals. They therefore classified bacterial pigments into four groups:

1. Carotenoids and related pigments.
2. Anthocyanins.
3. Melanins.
4. Miscellaneous.

Another classification was based upon the relative solubilities of the pigments.

Still another classification was based on the color produced by the bacteria.

At that time any of the above classifications could be used by investigators to designate the types of pigment present in bacteria.

White (1939) divided bacterial pigments into the following groups based on their chemical structure:

1. Pyrrole derivatives.
2. Phenazines.
3. Carotenoids.
4. A naphthoquinone.

Two pyrrole derivatives have been isolated from bacteria: bacteriochlorophyll and prodigiosin. Bacteriochlorophyll has been isolated from the photosynthesizing bacteria. The second pyrrole derivative, prodigiosin, is the red coloring matter produced by Serratia marcescens.

The phenazine derivatives are: pyocyanin, chlororaphin, and 1:2-dehydroxyphenazine-di-N-oxide. The first

phenazine derivative to be found in nature was pyocyanin, the dark-blue pigment, produced by Pseudomonas aeruginosa. The pigment, 1:2-dihydroxyphenazine-di-N-oxide has been isolated from Chromobacterium iodinum. Chlororaphin is a green pigment which has not received a great deal of attention.

There has been only one naphthoquinone isolated from bacteria. Anderson and Newman (1933) demonstrated the presence of phthiocol in the human tubercle bacillus. Ingraham and Steenbock (1935) and Turian (1951) have isolated the same pigment from Mycobacterium phlei.

The carotenoids isolated from bacteria will be discussed in the following section.

Carotenoids of Bacteria.

The presence of carotenoids in bacteria was demonstrated by Zopf in 1889, who designated this group of bacterial pigments lipochromes because of their solubility in the fat solvents. He demonstrated the presence of the carotenoids by means of the lipocyan reaction. This reaction is a color test for carotenoids and consists of adding concentrated sulfuric acid to an ethereal solution of the pigment and a positive test gives a deep indigo blue color. All the research on carotenoids up to 1928 depended on the use of the lipocyan reaction which did not distinguish the individual carotenoids. Thus, the earlier investigators were handicapped by the inability to separate the various

carotenoids. It was not until 1925 when Reader using Tswett (1906) adsorption column that investigators were able to separate the various pigments present in bacteria. Yet, since 1925 only a few of the large number of bacteria known to contain carotenoids have been subjected to pigment analysis.

Lankester (1873) gave the name bacteriopurpurin to the characteristic pigment of Bacterium rubescens one of the purple sulfur bacteria. Warming (1875) and Engelmann (1888) studied the spectrum of Lankester's Bacterium rubescens and Engelmann concluded he was dealing with a mixture of pigments. Butschlii (1890) noted that the red pigment could be extracted quickly with alcohol and the green color more slowly. Archichowsky (1904) named the red pigment bacterioerythrin. Molisch (1907) working with pure cultures of purple bacteria was able by means of absolute alcohol to extract a green pigment which he named bacteriochlorin and further treatment with chloroform yielded a garnet red solution. This latter pigment he termed true bacteriopurpurin. van Niel and Smith (1935) isolated a carotenoid pigment named spirilloxanthin from Rhodospirillium rubrum which had an identical absorption spectrum as Molisch's bacteriopurpurin. Karrer and Solmssen (1935-1936), Karrer, Solmssen and Konig (1938) investigated Rhodovibrio, Rhodobacillus, and Thiocystis and described five new pigments; rhodoviolascin, rhodopin, rhodopurpurin, flavorhodin and

rhodovibrin. Volk and Pennington (1950) investigated the pigments of Rhodomicrobium vannielii and isolated beta-carotene, rhodopsin and spirilloxanthin. Rhodopin was similar to that of Karrer and Solmssen (1935-1936) and spirilloxanthin was identical with that found by van Niel and Smith (1935) in Rhodospirillium rubrum.

Zopf (1899, a, b; 1891, 1892) described the carotenoids in eight species of bacteria. Palmer (1922) reviewing Zopf's work believed that only four species contained carotenoids; namely, Bacterium egregium, Bacterium chrysogloia, Staphylococcus pyogenes var. aureus, and Sphaerotilus roseus.

Bacterium ergregium formed yellow colonies on gelatin. Colonies gave lipocyan reaction. The alcoholic solution showed two absorption bands, one covering the F line, the other between F and G.

Bacterium chrysogloia and Staphylococcus pyogenes var. aureus showed the same properties as Bacterium ergregium.

Sphaerotilus roseus showed properties indicative of carotene.

Schrotter (1895) observed that the pigments of Sarcina aurantiaca and Staphylococcus pyogenes var. aureus showed the solubility properties and gave the lipocyan reaction (color reaction with sulfuric acid) of carotenoids.

Kligler (1914) investigated the pigments of Staphylococcus pyogenes var. aureus, Sarcina aurantiaca and

Sarcina lutea and found that the pigment from the three organisms was soluble in alcohol, ether and chloroform and gave the lipocyan reaction.

Reader (1925) extracted the carotenoid pigments from Sarcina aurantiaca and Streptothrix corallinus by grinding the bacteria with sodium sulfate and boiling with alcohol for five minutes. By employing Tswett (1906) adsorption column she was able to obtain carotene and lycopene from Sarcina aurantiaca and a hitherto unreported pigment which she named coralin, in Streptothrix corallinus.

Chargaff and Dieryck (1932) reported the presence of a xanthophyll and a hydrocarbon which they called sarcinene, in Sarcina lutea. Nakamura (1936) found a xanthophyll ester in the same organism. Takeda and Ohta (1941) isolated a new xanthophyll from Sarcina lutea which they called sarcinaxanthin. Sarcinaxanthin is probably the xanthophyll ester previously reported by Nakamura (1936).

Chargaff (1934) reported the presence of zeaxanthin in Staphylococcus pyogenes var. aureus. He also isolated beta-carotene and zeaxanthin from Sarcina aurantiaca.

Sobin and Stahly (1942) disagreed with Reader (1925) and Chargaff (1934) in regard to the pigment formed by Sarcina auratiaca. They discovered a single carotenol which was previously unreported. The same two authors disagreed with Nakamura (1936) and Chargaff and Dieryck (1932) with respect to the pigments of Sarcina lutea. They obtained two carotenoid

alcohols in contrast to the hydrocarbon, sarcinene reported by Chargaff and Dieryck. They also disagreed with Chargaff (1934) with respect to the pigment present in Staphylococcus pyogenes var. aureus. They reported the presence of delta-carotene and rubixanthin. Sobin and Stahly (1942) also reported the presence of unidentified carotenoids in the following organisms: Flavobacterium esteroacromaticum, Flavobacterium suaveolens, Flavobacterium fecale, Flavobacterium sulphureum, Flavobacterium arborescens, Sarcina flava, Micrococcus luteus, Micrococcus flavus, Erwinia lathyri, Erwinia ananas, Bacterium mycoides and Cellulomonas flavigena.

Sobin and Stahly (1942) in order to test the hypothesis that different strains of a species may produce different pigments, when grown on a standard medium, subjected twelve strains of Staphylococcus pyogenes var. aureus to pigment analysis. Two pigments, delta-carotene and rubixanthin, were common to all strains and the authors therefore concluded that the disagreement as to the pigments formed by a certain species of bacteria was not due to different strains of the same species. Starr and Saperstein (1952, 1953 b) observed the qualitative change in the carotenoid pigments of Corynebacterium poinsettiae when the concentration, in the basal medium, of the required growth factor, thiamine was altered. Under conditions of low thiamine concentration, two pigments were produced, spirilloxanthin and lycoxanthin. When a high concentration of thiamine was used, three pigments were

produced: lycoxanthin, a small amount of spirilloxanthin and cryptoxanthin. Baker (1952) observed a qualitative change in the carotenoid pigments of Staphylococcus pyogenes when grown on various culture media. We believe that the disagreement among various investigators as to the carotenoids produced by a species of bacteria is purely a question of the culture medium used and not one of source or strain.

Krainsky (1914) studying the red pigments of certain Actinomyces obtained a lipocyan reaction. He concluded that the pigments belonged to the carotenoids.

Gurd and Denis (1911) noted the presence of lipochromes in Mycobacterium leprae. The substance was soluble in ordinary fat solvents such as ether, alcohol, acetone and chloroform and gave with concentrated sulfuric acid the color change characteristic of the lipochromes. Grundmann and Takeda (1937) reported the presence of leprotin, a carotenoid hydrocarbon similar to beta-carotene, in the acid-fast bacteria isolated from a leprous lesion. The same pigment was later found by Takeda and Ohta (1939 b) in Mycobacterium phlei.

Chargaff (1930) reported the presence of beta-carotene in Mycobacterium phlei. Chargaff (1933) continuing his work on Mycobacterium phlei separated the pigments into carotenes, xanthophylls and xanthophyll esters and put each fraction through columns of aluminum oxide. Four bands were revealed when the hydrocarbons were examined, he concluded from spectrophotometric data that three of them were due to beta-carotene.

From the xanthophyll esters he obtained a pigment similar to lutein and two bands were not examined. Ingraham (1935), Ingraham and Steenbock (1935) absorbed a light petroleum solution of the mixed pigments of Mycobacterium phlei through a magnesium oxide column and then washed the column first with 20% ether in petroleum ether and then with chloroform. About a dozen bands were revealed by this procedure. Spectrometric analysis revealed the presence of alpha-carotene, beta-carotene, cryptoxanthin, lutein, zeaxanthin and azafrin. Takeda and Ohta (1939 a, 1940) reported that Mycobacterium phlei produced leprotin which has the spectrometric properties of beta-carotene and stated that this was the material reported by other workers as beta-carotene. They also reported the presence of azafrin and beta-carotene. Turian (1950 d) obtained an acidic carotenoid from the above organism which had properties similar to astaxanthin but with an absorption band, with a maximum, at a much lower wave length. He proposed the name chrysophlein for this new pigment. Two additional acid-fast bacteria were studied by Chargaff and Lederer (1935). Bacillus lombardo-pelligrini contained beta- and gamma-carotene and the Bacillus Grassberger was found to contain beta- and gamma-carotene and lycopene. Haas and Bushnell (1944) investigating Mycobacterium lacticola isolated beta-carotene, two pigments believed to be isomers of beta-carotene and a fourth pigment which possessed the chemical and spectrographic properties of astacin.

Reimann and Echler (1941) derived four varient types from the "white" form of Micrococcus tetragenous which had been isolated from a patient. The five types were named white, yellow, pink, pink-yellow and brown according to color of colonies. Pigments identified were: yellow, xanthophyll; white, no pigment; pink, rhodoxanthin; pink-yellow, alpha-carotene or rubixanthin; and brown, gamma-carotene or rubixanthin.

Stone and Coulter (1931-32) reported the presence of lycopene in Corynebacterium diphtheriae. Ingraham and Baumann (1934) reported the presence of beta-carotene in Corynebacterium carotenum. Ingraham (1935) continued the work on Corynebacterium carotenum and isolated alpha-carotene, beta-carotene, cryptoxanthin, and zeaxanthin. She also studied the presence of carotenoids in a red diphtheroid and found lycopene, beta-carotene and zeaxanthin present. Starr and Saperstein (1952, 1953 b) found that when Corynebacterium poinsettiae was grown in a medium with a low thiamine concentration, two major pigments were produced; spirilloxanthin and lycoxanthin. High concentrations of thiamine yield three pigments: lycoxanthin, cryptoxanthin and a small amount of spirilloxanthin. Saperstein, Starr and Filfus (1954) observed that the yellow parent type of Corynebacterium michiganense produced cryptoxanthin and lycopene. A pink mutant formed lycopene and spirilloxanthin. A red-black mutant produced only lycopene. An orange mutant synthesized cryptoxanthin, beta-carotene and canthaxanthin.

Carotenoids were not detected in colorless mutants. They found that yellow strains of Corynebacterium michiganense were affected by thiamine in a manner similar to Corynebacterium poinsettiae. At relatively high concentrations of thiamine, cryptoxanthin and lycopene were synthesized. The formation of lycopene, relative to spirilloxanthin, in the pink mutant was favored by high thiamine concentration.

Gilman (1953) found that Chromobacterium chocalatum and Chromobacterium orangium produced two carotenoids one of which was lycopene, the other was not identified.

The Effect of Physical and Chemical Factors on Carotenoid Production by Bacteria.

Since the majority of investigators have been primarily interested in identifying the types of carotenoids present in bacteria, the studies on the physical and chemical factors influencing pigmentation have been almost completely neglected.

Carbon and Nitrogen Sources.

Sullivan (1905) observed that the yellow pigment produced by Micrococcus aurantiaca, Micrococcus citreus, Sarcina lutea and Micrococcus cereus flavus was formed very slowly in non-albiminous media. The pigment was quickly formed in peptone solution plus salts. In a 1 per cent solution of dialyzed peptone, Micrococcus citreus, Staphylococcus pyogenes citreus quickly developed color and Micrococcus cereus flavus, Sarcina lutea, and Micrococcus agilis did not grow at all.

Fujita and Yoshioka (1923) found that of the carbohydrates tested galactose and lactose gave the best pigment production in Staphylococcus pyogenes. Ingraham and Steenbock (1935) found that glycerol was the best carbon source for pigment production in bacteria. They observed that when Mycobacterium phlei was grown on a synthetic glucose-asparagine medium the pigment content of the cells was relatively low. The substitution of ammonium salts, urea, peptones and other sources of nitrogen for asparagine had no effect upon pigmentation when the reaction of the medium was controlled. In the studies of Ingraham and Steenbock (1935) attention should be called to the fact that the authors upon chromatographic studies observed the presence of a pigment resembling phthiocol. This pigment was largely responsible for the increased pigmentation in the presence of glycerol and glucose. The effect of this phthiocol-like pigment was not evaluated in their other determinations. Haas and Bushnell (1944) observed that Mycobacterium phlei, Mycobacterium leprae and Mycobacterium smegmatis were pigmented when grown on ordinary media, but failed to yield pigment when cultured with hydrocarbons. Mycobacterium lacticola was able to produce pigment in this medium. Sevag and Green (1944) grew 13 strains of Staphylococcus pyogenes var. aureus on beef-extract peptone agar and on the same extract agar plus 0.5 per cent of the following carbohydrates; glucose, d-galactose, d-mannitol, d-mannose, trehalose, cellobiose, d-fructose and pyruvate. Majority of strains showed increased pigmentation

on glucose, d-galactose, d-mannose, d-mannitol and trehalose. Development of pigment was determined after a period of 48 hours of growth at 37°C and then allowing the cultures to stand at room temperature for 24 hours. Kahler (1951) found that Staphylococcus pyogenes var. aureus gave greater pigmentation with filtered mannite and lactose than when these were autoclaved. Alanine, tyrosine, glutamic acid and lysine had no effect upon pigmentation but validity of these results is questionable due to the limited number of organisms that grew. Saperstein, Starr and Filfus (1954) investigated the effect of l-leucine on the pigmentation of Corynebacterium michiganense and found there was no increase with the addition of this amino acid to the medium.

Inorganic Ions.

Sullivan (1905) observed that when magnesium sulfate and dipotassium phosphate were added to a peptone solution there was an increase in production of pigment by Micrococcus aurantiaca, Micrococcus citreus, Sarcina lutea and Micrococcus cereus flavus. In a 1 per cent solution of dialyzed peptone, Micrococcus citreus, Staphylococcus pyogenes var. citreus quickly developed color. Micrococcus cereus flavus, Sarcina lutea and Micrococcus agilis did not grow at all. On adding 1 per cent sodium sulfate to the solution, Sarcina lutea gave a good yellow sediment, while Micrococcus cereus flavus was colorless and Micrococcus agilis did not develop at all. On adding 1/10 of 1 per cent K_2HPO_4 to the peptone-sodium sulfate

solution Micrococcus cereus flavus also developed it's characteristic pigment. $MgSO_4$ had the same effect as Na_2SO_4. The author concluded that little could be said regarding the salts necessary for the formation of the yellow pigment, since he found it impossible to free the peptone from impurities either by precipitation or by dialysis. He believed, however, that the presence of $MgSO_4$ and K_2HPO_4 facilitated the production of the pigment and gave it a more vivid color. Ingraham and Steenbock (1935) investigating the production of pigment by Mycobacterium phlei noted that increasing the concentration of ferric salts tended to prevent pigment formation. When glycerol was substituted for glucose pigmentation was greatly increased. The concentration of potassium was without effect on pigmentation in the presence of glycerol, but phosphates and ferric or cupric salts decreased the color of the cells. As previously mentioned the effect of phthiocol-like pigment was not evaluated in these determinations. Kharasch, Conway and Bloom (1936) observed that high concentrations of copper on beef infusion agar inhibited the growth of Staphylococcus pyogenes var. aureus. Similar media, however, to which liver extract was added resulted in a colorless growth. With low concentrations of copper on beef infusion agar a colorless growth was obtained. The addition of liver extract, however, to the medium containing low concentration of copper produced a good growth of pigmented Staphylococcus pyogenes var. aureus. The addition of liver extract to a medium containing low

concentrations of manganese was ineffective in restoring the color to the non-pigmented Staphylococcus. Turian (1950 a) found that the inhibition of pigment produced by diphenylamine on Mycobacterium phlei could be reversed by iron but not by manganese, cobalt, nickle or copper. Kahler (1951) in his studies on pigmentation of Staphylococcus pyogenes var. aureus reported that when manganese sulfate was added to a semi-synthetic media pigmentation was increased by one-third of that obtained with other salts (ferrous sulfate, magnesium and sodium chloride).

Temperature and Light.

Reader (1925) observed that Sarcina aurantiaca grew better at 20°C than at 37°C, and although the yield of carotenoids was higher at 20°C, the concentrations were identical at both temperatures. There was no difference in pigmentation of cultures grown either in the dark or diffuse daylight. She noted that when cultures were exposed to direct sunlight, growth was poor and no color developed. Even old cultures became bleached in direct sunlight. When bleached colonies were subcultured and removed from the strong light the new growth developed color as usual. Sesler, Stahly and Brode (1941) confirmed the results of Reader (1925) in regards to the effect of temperature on pigmentation. Kahler (1951) disagrees with the others in that he has shown 30°C to be the best temperature for pigmentation.

Hydrogen ion Concentration.

Fujita and Yoshioka (1923) observed that pigment production in Staphylococcus pyogenes var. aureus was more pronounced at pH 6 or slightly below that. Reader (1925) found that the pH limits for growth of Sarcina aurantiaca were 5.3 - 9.4 with maximum production occurring at pH 7.1. No other papers have been published in which a complete study has been made of the effect of pH on pigmentation.

Oxygen.

Lubinski (1894) was the first to point out that no pigment was produced under anaerobic conditions by Staphylococcus pyogenes.

Ingraham and Baumann (1934) reported that they found no anaerobic bacteria that produced carotenoids. Reid (1936-37) studied seventy-six cultures representing 24 genera of chromogenic bacteria and found none that would produce pigment in the absence of oxygen. Topley and Wilson (1946) state that oxygen is necessary for pigment production by Staphylococcus pyogenes and under anaerobic conditions the growth is colorless.

Growth Factors.

Lutz (1947) found that the degree of pigmentation in Bacillus lombardo-pellegrini and Bacillus boquet was directly proportional to the thiamine content of the medium. Braum (1949) found that the naturally occurring yellow type of Corynebacterium michiganense produced either a cream or a yellow growth,

depending upon whether the concentration of thiamine in the growth medium was low (0.1 ug/100 ml.) or high (1-100 ug/100 ml.), respectively. He noticed that a white mutant of this organism produced no pigment regardless of the thiamine concentration. In the related species, Corynebacterium poinsettiae, thiamine was observed to affect pigmentation (Staff and Pirone, 1942; Starr, 1949): later a more intensive examination of this effect was made by Starr and Saperstein (1953 b). Cells grown under conditions of low thiamine concentration, two major pigments were produced: spirilloxanthin and lycoxanthin. Cells formed under conditions of high thiamine concentrations, three major pigments were produced: lycoxanthin, cryptoxanthin and a small amount of spirilloxanthin. Starr and Saperstein (1953 b) and Saperstein, Starr and Filfus (1954) observed that carotenoid synthesis in the yellow strain of Corynebacterium michiganense was affected by thiamine in a manner similar to that previously described for Corynebacterium poinsettiae. Kahler (1951) found that thiamine was essential for maximum pigment production in Staphylococcus pyogenes var. aureus and that cocarboxylase could be substituted for thiamine.

Inhibition Studies.

Kharasch, Conway and Bloom (1936) demonstrated that the addition of diphenylamine in a concentration of 1-2000 or 1-6000 inhibited the production of pigment by Staphylococcus pyogenes var. aureus and Sarcina lutea. Turian (1950 c) confirmed these results by showing that diphenylamine in a

concentration of 1-35000 inhibited the production of carotenoids by Mycobacterium phlei.

Turian (1951) has also shown that phenol in a concentration of 1/2000-1/5000 inhibited pigment production in Mycobacterium phlei; resorcinol (1/1000-1/5000) is 2-3 times less active than phenol while alpha-naphthylamine, thiourea, KCN, salicylaldoxime are almost inactive. Dinitrophenol, on the other hand, stimulated the production of the carotenoids.

Ark (1951) investigating the pigment of Corynebacterium michiganense found that pigment production could be inhibited by the addition of the following compounds to the medium: acenaphthene, alpha-nitronaphthalene, 3-bromoacenaphthene, and ethyl-alpha-naphthoate.

Colonial Variation as Related to Carotenoid Production.

Mutation in bacteria is a well-recognized phenomenon. Of all the types of mutations studied, the one which has received the least attention is the changes in pigment brought about by mutation.

Neumann (1897) observed that when Staphylococcus pyogenes var. aureus was cultivated at room temperature, white, yellow, flesh-colored and orange colored colonies could be isolated and subcultured as stable strains. Noguchi (1911) observed that strains of Staphylococcus pyogenes var. aureus lost their pigment slowly on artificial media, and he described a slow transformation from aureus to citreus to cereus and finally

to albus, the complete aureus-albus transformation required more than a year. Bigger, Boland, and O'Meara (1927) found that by growing a normal Staphylococcus pyogenes var. aureus in broth, variant strains were produced and when such cultures were spread on agar plates and these incubated abnormal colonies were found. Such variants showed alteration in color (from aureus to albus), in texture (from "smooth" to "rough") and in cohesion (from "non-viscid" to"viscid"). On agar, variants were produced only in color and viscidity. Rough volonies were never found to appear in cultures of normal Staphylococci grown on agar. The variants under certain conditions reverted to the normal type. Pinner and Voldrich (1932) found that culturing Staphylococcus pyogenes var. aureus in phin broth, making daily transfers, and streaking the 24 hour growth on agar every second or third day, that occasional Staphylococcus pyogenes var. albus colonies would develop in the streak. This happened with every one of the nine Staphylococcus pyogenes var. aureus strains studied. During periods of from two to three months (daily transfers), there was no tendency for the Staphylococcus pyogenes var. albus colonies to increase in frequency or in number. More Staphylococcus pyogenes var. albus colonies appeared when Staphylococcus pyogenes var. aureus was grown in pleural fluid broth. The authors by seeding Staphylococcus pyogenes var. albus in undiluted anti-albus serum, were able to obtain the transformation of a Staphylococcus pyogenes var. albus strain to Staphylococcus pyogenes var. aureus.

The yellow colonies did not occur before the 20th day in anti-albus serum. The number of Staphylococcus pyogenes var. aureus colonies remained always small in proportion to that of the white colonies. They believed that the relation between Staphylococcus pyogenes var. aureus and Staphylococcus pyogenes var. albus strains is essentially similar to that between S and R strains of other organisms. The splitting-off of Staphylococcus pyogenes var. albus was enhanced by growing Staphylococcus pyogenes var. aureus in broth containing Staphylococcus pyogenes var. aureus agglutinins and by animal passage. Hoffstadt and Youmans (1932) brought about the dissociation of a virulent strain of Staphylococcus pyogenes var. aureus by: (1) daily transfers in lithium chloride medium and (2) animal passage. They found that the smooth orange Staphylococcus pyogenes var. aureus or S dissociated into two types, R and G. The G forms were isolated 14 times in stable cultures, three times in lithium chloride medium and 11 times by inoculation of animals. Four biochemical types were isolated: a yellow R that, unlike the yellow S, fermented lactose; a white R that fermented levulose, which was not fermented by either yellow form and G which fermented glycerin, which was not fermented by the other three. They found that the original strain S was the only virulent strain. Dissociated forms were not virulent and did not produce lesions of any kind. Swingle (1935) isolated several small colony variants of Staphylococcus pyogenes var. aureus from old broth or agar

cultures or from cultures in lithium chloride broth. These forms were characterised by loss of pigment. Reversion to the normal form occurred occasionally on agar and could be enhanced by aging in broth or by enrichment of the media. She believed that small colony forms occurred as the result of injury to an occasional cell which is retained through successive cell generations. Nutini, Kreke and Schroeder (1945) by means of spleen extracts were able to transform Staphylococcus pyogenes var. aureus to the albus form. The Staphylococcus pyogenes var. albus form was unstable and remained white only after frequent cultures and subcultures in media containing the extract. Nutini and Lynch (1946) using extracts of brain, spleen, heart and kidney were able to convert the yellow S form of Staphylococcus pyogenes var. aureus to the white R variant with altered biochemical characteristics conforming to those of the avirulent organism. The avirulence of the white R variant was established by tests, in mice. Byatt, Jann and Salle (1948) found it possible to stimulate or initiate chromogenesis in a non-chromogenic Staphylococcus by growing it in extracts of a pigmented strain of the same species. The new chromogenic strain retained the same biochemical properties of the parent culture. The intensity of pigment production was not as great in the new strain as in the strain from which the extract was prepared nor was it lasting. No explanation was given for the fact that some cells of the test organism was refractory to stimulation whereas others were not.

Reimann (1936 a, b) subjected a strain of Micrococcus tetragenus to ageing on agar or in broth. As a result 11 variant forms were encountered and studied, namely mucoid-yellow, yellow, mucoid white, white, small white, translucent, mucoid pink, pink, pink-yellow, brown and bacillary form. Bacteria from each form were characterized by rather distinctive morphology, immunology, and pigment production and by differences in regard to growth in various sugars. There was evidence of reversion from one form to another among all variants studied except the bacillary form. Reimann (1937 a, b) observed that Micrococcus tetragenus possessed at least five major variant forms, mucoid-yellow, mucoid-white, mucoid-pink, mucoid-pink-yellow and mucoid-brown, which were regarded as specific types. Each type was observed to have M, S, or R culture phases. He observed that one type may change into another spontaneously in vivo or in vitro. Reimann and Eckler (1941) identified the pigments in the respective type strains.

Bryan (1930, 1931) isolated "spontaneously" occurring white and pink strains of the normally yellow Corynebacterium michiganense. Ark (1951) using uranium salts was able to produce the following variants of a naturally smooth type of Corynebacterium michiganense: smooth, rough and intermediate white and smooth, rough, and intermediate dark and light pink. Saperstein, Starr and Filfus (1954) identified the pigments produced by these mutants.

Possible Function of Carotenoids in Bacteria.

The amount of work that has been carried out to determine the possible function of carotenoids in bacteria is exceedingly small. Baumann, Steenbock, Ingraham and Fred (1933) investigated the possibility of microorganisms converting carotene to vitamin A. They added colloidal carotene directly to the medium, which was then sterilized and incubated with the appropriate organisms. In some cases cultures were also made with pure carotene in the crystalline condition. The following media were used:

1. A crude corn mash.
2. A glucose peptone medium.
3. Yeast water prepared from autoclaved Brewer's yeast.
4. An inorganic medium.

Seven species of bacteria, 12 species of molds and 13 mixed cultures obtained from soil, manure, air, water, milk, sewage and peat were tested. Incubation varied from two days to three weeks.

In none of the organisms examined was the synthesis of vitamin A demonstrated.

A possible role in photosynthesis appears to have been ruled out in the case of purple bacteria including Sprillium rubrum (French, 1937).

Imshenetskii (1946) postulated that carotenoids may protect Sarcina lutea and Sarcina aurantiaca against the effects of ultra-violet light.

Darzin (1939) found that beta-carotene inhibited the in vitro growth of Mycobacterium phlei and Vasileva (1941) claimed that it stimulated the growth of the typhus bacteria. Darzin also noted an increase in the acid fastness of Mycobacterium tuberculosis var. hominis grown on carotene containing medium. Lutz (1947) using Mycobacterium phlei confirmed Darzin's observations.

Guirard, Snell and Williams (1946) have shown that catotenoids can replace sodium acetate in the nutrition of lactic acid bacteria.

We believe that the true function of carotenoids in bacteria has yet to be discovered. Some of the possible functions that have yet to be investigated are:

1. Do they protect sensitive enzymes?
2. Act as hydrogen acceptors?
3. Photochemically active in assimilation?
4. Transport of oxygen or oxidation reactions?
5. They have no function but are metabolic end products?

Biosynthesis of Carotenoid Pigments in Bacteria.

Turian (1950 b) investigating the effect of diphenylamine on pigmentation in Mycobacterium phlei proposed the following scheme, purely hypothetical, for the synthesis of carotenoids by this organism:

Non-colored precursors

↓ n times

Polyenes (C_{40}-)

Non-colored phytofluene ($C_{40}H_{64} \pm 2H$) → ox. Colored Leprotin ($C_{40}H_{54}$) → ox. Colored Keto-enol:

Carotenes	Chrysoflein
($C_{40}H_{56}$)	Astacine
Carotenols	($C_{40}H_{48}O_4$)

The essence of this scheme is that he believes that the more saturated polyenes are precursors of the less saturated carotenoids, which results from the step-wise removal of the hydrogen atoms from the former. Bonner et al. (1946) using seven ultra-violet mutants of Rhodotorula rubra and Porter and Lincoln (1950) carrying out inheritance studies involving crosses of commercial varieties of tomatoes are in agreement with Turian (1950 b). Saperstein, Starr and Filfus (1954) noted the absence of colorless polyenes in the Coryneform organism that they studied and therefore, they are of the opinion that alterations in polyene structure probably occurs prior to the formation of the complete C_{40} chain.

Many questions remain to be answered before the synthesis of carotenoids by bacteria can definitely be established. We believe that the following factors have to be investigated before any insight can be gained as to the synthesis of carotenoids by bacteria:

1. Is acetate the primary precursor involved in the

synthesis of carotenoids?

2. Are carotenoids synthesized *via* 5-carbon units?
3. If carotenoids are synthesized *via* 5-carbon units, are these units saturated or unsaturated?
4. Do four 5-carbon units combine "head to tail" to form a C_{20} compound which then dimerises "tail to tail" or do two 5-carbon units combine "tail to tail" and the molecule built up by "head to tail" condensation at either end of this ten carbon chain?
5. Does alternation in the polyene occur before or after formation of the complete C_{40} chain?

GENERAL PROCEDURE.

GENERAL PROCEDURE.

Introduction.

Previous investigators have measured pigment by visual comparison of the intensity of color of a culture grown upon a test medium with that produced upon a control medium. Reader (1925) and Reid (1936) classified differences in shade or tint of pigment by comparison with various color standards.

An attempt was made to use this comparative method but it was often found to be difficult to estimate relative intensities of pigment because of varying size of colonies. Since colonies may appear to have a greater intensity of color because of their larger size, this method of estimation of bacterial pigment proved unreliable.

Stahly, Sesler and Brode (1942) described a colorimetric method for measuring the comparative amounts of pigment produced by bacteria under various environmental conditions. This method also was found to be unsatisfactory reasons will be given for this conclusion in another section of the thesis. It therefore, became imperative that a method be devised which would yield more accurate and reliable results.

A method will be discussed whereby one may determine whether a given physical or chemical factor will increase or decrease pigmentation when compared with a control.

Test Organism.

The strain of *Staphylococcus pyogenes* # 4575 used in

all of the experimental work was obtained from the Clinical Laboratory, Department of Bacteriology and Immunology, McGill University. It was maintained on Chapman's medium (1946), transfers being made every two weeks. The formula for this medium may be found in Appendix A of this report.

This strain was adopted as the test organism because of its ability to produce abundant pigment on a wide assortment of culture media. Another reason being that on repeated subcultures over a period of six months, prior to its use, it did not lose its ability to produce pigment.

Method for Colorimetric Determination of Pigment.

Introduction.

Stahly, Sesler and Brode (1942) published a colorimetric method for measuring bacterial pigment. The method was as follows: agar slants of the test media are inoculated in a uniform manner and incubated at the temperature and for the time desired. At the end of the incubation period, the cells are scraped off gently into distilled water. The suspension is shaken and then filtered through several layers of cheese-cloth to obtain homogeneity. In the case of Staphylococcus pyogenes, the turbidity of the suspension was measured at 660 mμ. At this wavelength the light waves are not absorbed by the bacterial pigment. The turbidity was again read at 420 mμ which represented turbidity plus pigment, since pigment will absorb light at this wavelength. The true pigment absorption was obtained by substracting the absorption

at 660 mμ from 420 mμ. Since the turbidity may be taken as an index of the number of bacterial cells present, the average amount of pigment per unit of cells may be calculated and used for purposes of comparison. They recommended that the suspension be diluted to an optical density of 0.2000 or less. Results are calculated for convenience to a standard optical density of 0.1000 by the following proportion.

$$\frac{\text{Amount of pigment for 0.1000}}{0.1000} = \frac{\text{measured pigment}}{\text{measured turbidity}}$$

We had hopes of using this method since it was simple and rapid but found that it yielded erratic results and failed to give reproducible values. The fault with this method lies in the fact that the optical density obtained at 660 mμ. and the turbidity, independent of pigment, at 420 mμ is not equal. Species of bacteria which produced no pigment were subjected to the above method and results were obtained showing these species to absorb different amounts of light at the wavelengths tested. For example, optical densities were taken of a suspension of a Corynebacterium species, possessing no pigment, at 420 mμ, 520 mμ, 540 mμ, and 660 mμ. The following optical densities were obtained, 0.2596, 0.2157, 0.2007 and 0.1612, respectively. By the above method, it would appear that the species contained pigment.

With hopes of obtaining a solution to this problem we wrote to one of the authors, Grant L. Stahly. In his letter, he stated that one of his Master's candidates did

research using this method but her results were somewhat erratic. He also stated that it would appear that cells absorb different quantities of light depending on the wavelength passed by the filter.

In this section a method will be discussed which has been found adequate for our purposes.

Culture Medium Used for Experimental Studies.

In quantitative work involving the use of bacteria, all constitutents in the culture medium should be known. However, the nutritive requirements of many bacteria have not been thoroughly studied and therefore, investigators have been handicapped in finding a synthetic medium to be used for quantitative studies, such as pigment production. Fildes, Richardson, Knight and Gladstone (1936) and Fildes and Richardson (1937) developed a synthetic medium which will allow the growth of Staphylococcus pyogenes. This medium was found to be excellent for our purposes because it excluded chemically unknown substances. Another advantage to Fildes' medium is that the solution may be made up to 100 ml. by the addition of 26.5 ml. of distilled water. Since all the compounds to be tested were either in liquid form or aqueous solution it enabled us to maintain a constant volume throughout the experimental work.

The preparation of this medium is outlined in Appendix A. The original Fildes' medium contained glucose as a source of carbohydrate but in these experiments the

substance to be tested has been substituted for glucose.

In our studies 100 ml. of sterile Fildes' medium, including substrate to be tested, was dispensed in sterile 500 ml. Erlenmeyer flasks. All substrates to be tested were filtered through a Seitz filter prior to addition to the medium.

Inoculation and Incubation.

Preparation of Inoculum.

Prior to testing, the organism was subcultered from Chapman's medium onto five tubes of Watt and Werkman medium (1951) and incubated for 18 hours at 37°C. This medium was selected because it is a glucose-free medium and thus pigment production is scant. At the end of the incubation period, the growth was washed off the slants with distilled water. The growth from the five tubes was collected and washed three times with distilled water. After the final washing, the cells were resuspended in 10 ml. of distilled water and transferred to an absorption tube. This suspension was adjusted, without change in volume, to give a turbidity reading of ten per cent transmission of light with a 660 mu filter. An Evelyn photoelectric colorimeter* was used for this purpose. The colorimeter was adjusted by means of a blank containing 10 ml. of distilled water to give a reading of 100 per cent

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* Evelyn Photoelectric Colorimeter, Rubicon Company, 29 North Sixth Street, Philadelphia, Pa., U.S.A.

transmission of light.

To insure uniform inoculation, 0.5 ml. of this suspension was added to each flask of Fildes' medium.

Incubation.

After inoculation the flasks containing the substrate to be tested and controls were placed on a reciprocal shaker and shaken for a period of ninety hours at room temperature.

Preliminary investigations revealed that for the above incubation time a greater amount of pigment would be produced at room temperature than at 37°C.

At the end of the incubation period the growth from each flask was transferred to separate 200 ml. centrifuge tubes and centrifuged for a period of thirty minutes. The supernatant fluid was then discarded and the cells in each centrifuge tube were resuspended in 10 ml. of sterile distilled water and transferred by means of sterile Pasteur pipettes to test tubes (18 x 150 mm.). This washing procedure was repeated twice in the test tubes. After the final washing, the supernatant was discarded and the cells resuspended in 2 ml. of distilled water. Then the cells from each test tube were transferred by means of Pasteur pipettes to individual weighting bottles (10 ml.). The weighting bottles were then placed in a vacuum dessicator over calcium chloride and the dessicator evacuated and placed in the 37°C. incubator. The weighting bottles were weighed at intervals

until constant weights were obtained ($\pm$ 0.0002 gm).

Extraction and Colorimetric Determination of Pigment.

Extraction of Pigment.

After constant weights were obtained the cells in each weighting bottle were resuspended in approximately 4 ml. of distilled water. The cells were then transferred by Pasteur pipettes to individual test tubes (18 x 150 mm.). The weighting bottles were again washed with 4 ml. of distilled water and again dried to constant weight by the method discussed in the foregoing section.

The test tubes were centrifuged for thirty minutes and after centrifugation the supernatant was discarded from each tube and replaced with 5 ml. of methanol. The tubes were then placed in a 70°C water bath for one hour. At this temperature the pigment was extracted from the cells (Sobin and Stahly, 1942). At the end of one hour of extraction, the test tubes were centrifuged for thirty minutes and the supernatant collected individually into separate test tubes. The cells were re-extracted with 5 ml. of methanol for one hour and the tubes then centrifuged for thirty minutes. The supernatant obtained was added to that previously collected in the first extraction. It was found that two extractions were sufficient to extract the pigment from the cells because a third extraction with 6 ml. of methanol gave an optical density reading of zero or 100 per cent transmission of light.

Colorimetric Determination.

The individual 10 ml. extracts were then centrifuged for thirty minutes to remove any possible debris carried over in the supernatant from previous centrifugations. These methanol extracts were then placed in individual absorption tubes and, in cases where some methanol may have been lost during extraction, further methanol was added to give a final volume of 10 ml.

A final check was done on the extracts to be sure that no debris or cells were present. This was done by ascertaining the optical densities of the extracts with a 660 mμ filter. If the optical densities of the extracts were found to be greater than zero, it indicated that these substances were present and therefore a further centrifugation would have to be done. At 660 mμ the pigment in the extracts will not absorb light and therefore any deflection in the optical densities would be due to debris or cells.

Optical densities were now obtained for the pigment present in the extracts by using a 420 mμ filter for at this wavelength pigment will absorb light. Before and after the observations were made upon each sample, the galvanometer was adjusted until it provided 100 per cent transmission of light or optical density of zero with a control tube of methanol. The readings thus obtained gave the optical densities of the extracted pigment from known dry weights of cells.

An Evelyn photoelectric colorimeter was used for all

colorimetric determination. Standardization of absorption tubes and operation of the colorimeter are discussed in Appendix B.

Calculations for Obtaining Optical Density for a Standard Dry Weight of Cells.

Having obtained the optical densities of the pigment extracted from known dry weights of cells, the following proportion was used to obtain the optical density for 100 mgm. of dry weight of cells.

$$\frac{\text{Optical density of extracted pigment}}{\text{Dry weight of extracted cells}} = \frac{\text{Optical density for 100 mgm. of cells}}{\text{100 mgm. of cells}}$$

$$\text{Optical density for 100 mgm. of cells} = \frac{\text{Optical density of extracted pigment (x) 100 mgm. of cells}}{\text{Dry weight of extracted cells}}$$

It was necessary to calculate the results to a standard dry weight of cells in order to compare a series of measurements.

EXPERIMENTAL INVESTIGATIONS.

EXPERIMENTAL INVESTIGATIONS.

THE EFFECT OF TEMPERATURE AND AERATION ON PIGMENT PRODUCTION.

Baker (1952) showed that aeration, by means of a shaking machine, will increase pigment production by *Staphylococcus pyogenes* but it was not determined whether this effect was greater at room temperature or 37°C. The following experiment was carried out to substantiate the previous findings and also to determine the effect of temperature on pigment production. Ten ml. of a 2 M solution of glycerol* was added to ninety ml. of Fildes' medium to give a final concentration in the medium of 0.2 M glycerol. This medium was inoculated with 0.5 ml. of a suspension of *Staphylococcus pyogenes* prepared in the way discussed in the section on General Procedure. Three flasks were incubated at room temperature, three flasks at 37°C, three flasks were agitated at 37°C and three flasks were agitated at room temperature. All flasks were incubated for a period of ninety hours. The method used for the extraction and colorimetric determinations of the pigment is as described in the previous section. The results are tabulated in Table 1 and it may be concluded that shaking at room temperature yielded the greatest amount of pigment. The reason for the low amount of pigment when

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* Glycerine, C. P., Eimer and Amend, New York, N.Y.

TABLE 1.

THE EFFECT OF TEMPERATURE AND AERATION ON PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES.

Temperature	Without Aeration.			Aeration.		
	Dry wt. mgm.	O/D of extracted pigment.	Calc. O/D for extracted pigment.from 100 mgm. dry wt.	Dry wt. mgm.	O/D of extracted pigment	Calc. O/D for extracted pigment from 100 mgm. dry wt.
Room Temperature	31.6	0.0680	0.215	35.4	0.0862	0.244
	30.2	0.0642	0.212	35.2	0.0862	0.244
	31.4	0.0680	0.215	35.1	0.0862	0.244
37°C.	33.2	0.0642	0.193	38.6	0.0875	0.227
	33.6	0.0642	0.191	39.0	0.0875	0.224
	34.1	0.0655	0.191	38.8	0.0862	0.222

the organisms were grown without agitation was due to the limited amount of oxygen available to the organisms. The organisms in this case settling to the bottom of the flasks and covered by approximately one inch of medium which would have a tendency to limit the amount of oxygen available to the organisms. The organisms when shaken have a greater access to oxygen and to the available nutrients in the medium and therefore, are able to produce more pigment.

Due to these results, it was decided that incubation at room temperature on the shaking machine would be used through out our experiments.

THE EFFECT OF ENZYME INHIBITORS ON PIGMENT PRODUCTION BY *STAPHYLOCOCCUS PYOGENES*.

Introduction.

These experiments were conducted to determine whether enzyme inhibitors such as sodium fluoride, sodium arsenite, sodium fluoroacetate and sodium malonate would inhibit or enhance pigment production by *Staphylococcus pyogenes*. It should be stated here that these studies were done with proliferating organisms and not with cell-free extracts or resting cell suspensions. Due to this fact, it would be quite impossible to state that any of the inhibitors used in these experiments inhibited any specific enzyme. Investigators in the field of enzyme studies have been mainly interested in isolated enzyme systems and have not dealt with organisms in the process of growth. In the following experiments the organisms were grown in the presence of the enzyme inhibitor for a period of ninety hours and during proliferation may have the ability to overcome the effect of the inhibitor. In some cases the inhibitor may prevent growth from being initiated. In other cases the organisms may be capable of utilizing the inhibitor and therefore, preventing it from functioning as such. For example, sodium malonate which is known to inhibit the conversion of succinic acid to fumaric acid in the citric acid cycle may be broken down by *Pseudomonas aeruginosa* to acetate

(Gray, 1952) and therefore, would be unable to act as an inhibitor.

Although there are many limitations to this type of study, it was felt that it would be interesting to know whether enzyme inhibitors increased or decreased pigment production in Staphylococcus pyogenes.

In these experiments Staphylococcus pyogenes was incubated at room temperature on the shaking machine for a period of ninety hours and the extraction and colorimetric determinations of the pigment was in accordance with the methods given in the section on General Procedure.

The Effect of Sodium Fluoride on Pigment Production by Staphylococcus Pyogenes.

In large concentrations inorganic and organic fluoride compounds inhibit growth. Little is known about the effect of fluorides on bacteria. Davis and Dubos (1947) showed that fluoride inhibited the action of a lipase on "tween 80" in concentrations that did not affect the growth of Mycobacterium tuberculosis H37RV. Fritzgerald and Bernheim (1948) observed that at pH 6.7 and 7.8 the oxidation of glucose, fructose, and trehalose by Mycobacterium tuberculosis was increased by fluoride. At pH 6.0 fluoride inhibited the oxidation of these carbohydrates by this organism.

The following experiment was carried out to determine the effect of sodium fluoride on pigment production by Staphylococcus pyogenes. The following substances were

added to separate flasks of Fildes' medium as the final concentration.

1. 0.2 M glycerol (Control).
2. 0.2 M glycerol plus 0.02 M sodium fluoride.
3. 0.2 M glycerol plus 0.04 M sodium fluoride.
4. 0.2 M glycerol plus 0.08 M sodium fluoride.

Each test was set up in duplicate. Separate tests were carried out at pH 6.8 and pH 6.0.

The control flasks were prepared by adding ten ml. of a filtered two molar solution of glycerol to ninety ml. of Fildes' medium to give a final concentration in the medium of 0.2 M glycerol.

The sodium fluoride* was prepared as a 0.8 M solution. 0.2 M and 0.4 M solutions were prepared by dilution of the 0.8 M solution. Ten ml. of each molarity was added to ninety ml. of Fildes' medium containing 0.2 M glycerol to give the desired molar concentrations. All flasks had a final volume of 100 ml.

The results presented in Table II demonstrate that sodium fluoride at pH 6.8 will result in an increase in pigment production by Staphylococcus pyogenes. With increasing concentrations of sodium fluoride there will be a subsequent decrease in growth but the amount of pigment produced per 100 mgms. dry weight will remain somewhat higher than that of

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*Sodium fluoride, reagent grade, Merck Co. Ltd., Montreal, Quebec.

TABLE II.

THE EFFECT OF SODIUM FLUORIDE AT pH 6.8 AND pH 6.0 ON PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES.

Test Substance In Fildes' Medium	pH	Dry wt. mgm.	O/D of extracted pigment.	Calc.O/D for extracted pigment from 100mgm.dry wt.	Average O/D.
0.2 M Glycerol	6.8	35.8 34.1	0.0901 0.0809	0.251 0.237	0.2445
0.2 M Glycerol + 0.02 M NaF	6.8	34.6 37.1	0.1565 0.1707	0.452 0.460	0.456
0.2 M Glycerol + 0.04 M NaF	6.8	21.7 21.4	0.0706 0.0706	0.325 0.329	0.3275
0.2 M Glycerol 0.08 M NaF	6.8	11.7 10.1	0.0315 0.0269	0.269 0.266	0.2675
0.2 M Glycerol	6.0	49.1 43.9	0.1024 0.0888	0.208 0.203	0.2055
0.2 M Glycerol + 0.02 M NaF	6.0	NO GROWTH NO GROWTH			

the control. At pH 6.0 sodium fluoride will inhibit the growth of Staphylococcus pyogenes.

Fritzgerald and Bernheim (1948) observed that the oxidation of glucose, fructose and trehalose by Mycobacterium tuberculosis was increased by sodium fluoride at pH 6.8. This effect of sodium fluoride on oxidation may also apply to Staphylococcus pyogenes and therefore, with increased oxidation of glycerol there was a corresponding increase in pigment production. Another explanation may be that at pH 6.8 a fluoride-insenstive pathway exist such as the hexose-monophosphate oxidative system which would function when the glycolytic pathway has been blocked by sodium fluoride.

The Effect of Sodium Malonate on Pigment Production by Staphylococcus pyogenes.

The following experiment was carried out to determine the effect of sodium malonate on pigment production. The following substances were added to separate flasks of Fildes' medium as the final concentration.

1. Distilled water to make up volume (Control).
2. Distilled water plus 0.04 sodium malonate.
3. 0.2 M glycerol (Control).
4. 0.2 M glycerol plus 0.02 M sodium malonate.
5. 0.2 M glycerol plus 0.04 M sodium malonate.

Each test was done in duplicate at pH 6.8.

The preparation of the 0.2 M glycerol solution has

has been described previously.

Sodium malonate* was prepared as a 0.4 M solution, ten ml. and five ml. of this solution were added to 90 and 95 ml. respectively, of Fildes' medium to give a final concentration of 0.04 M and 0.02 M sodium malonate in the medium.

Gray (1952) has demonstrated that dried cell preparations of Pseudomonas aeruginosa will decarboxylate malonic acid under aerobic and anaerobic conditions with the accumulation of acetic acid. In later experiments it will be shown that Staphylococcus pyogenes can utilize sodium acetate for growth and pigment production. In one part of this experiment sodium malonate was added to Fildes' medium containing no source of carbohydrate to determine whether Staphylococcus pyogenes could utilize this compound as a source of carbon. If there was an increase in growth and pigment production as compared to the control, this would suggest that Staphylococcus pyogenes possibly contained the decarboxylase for malonic acid.

The results presented in Table III demonstrate that the amount of pigment produced per 100 mgm. dry weight of Staphylococcus pyogenes will be increased by sodium malonate. It would also appear from the results that sodium malonate may act as a carbon source because the compound when added

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*Sodium malonate, Eastman Kodak Co., Rochester, N.Y.

TABLE III.

THE EFFECT OF SODIUM MALONATE ON PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES.

Test Substance in Fildes' Medium	Dry wt. mgm.	O/D of extracted pigment.	Calc.O/D for extracted pigment from 100mgm.dry wt.	Average O/D.
Distilled Water	12.1 12.3	0.0155 0.0155	0.128 0.126	0.127
Distilled Water + 0.04 M Sodium Malonate	15.4 14.7	0.0315 0.0304	0.204 0.207	0.2055
0.2 M Glycerol	35.0 34.8	0.0848 0.0848	0.240 0.244	0.242
0.2 M Glycerol + 0.02 M Sodium Malonate	37.1 38.3	0.1051 0.1079	0.280 0.282	0.281
0.2 M Glycerol + 0.04 M Sodium Malonate	36.8 37.7	0.1135 0.1163	0.308 0.308	0.308

to Fildes' medium containing no carbohydrate resulted in an increase in growth as well as pigment production. It may be that the above increases were a result of Staphylococcus pyogenes being able to decarboxylate malonic acid to acetic acid. The acetic acid produced by decarboxylation could be used as a carbon source and also as a precursor of pigment.

The Effect of Sodium Fluoroacetate and Sodium Arsenite on Pigment Production by Staphylococcus pyogenes.

Having found that sodium fluoride and sodium malonate enhanced pigment production in Staphylococcus pyogenes it was decided to try two other enzyme inhibitors, namely, sodium fluoroacetate and sodium arsenite. Chenoweth (1949) reported that the fluoroacetates are quite non-toxic to isolated enzymes; they are, however, very toxic to dogs, rabbits and guinea pigs, rats and monkeys. The present view is that this compound functions as an inhibitor by blocking the pyruvate oxidase system at the citrate stage (Peters, 1953). Arsenites function by inhibiting the respiration of intact cells in the presence of glucose and pyruvate (Quastel, 1953).

The following experiment was carried out to determine the effect of sodium fluoroacetate and sodium arsenite on pigment production by Staphylococcus pyogenes.

1. 0.2 M glycerol (Control).
2. 0.2 M glycerol plus 0.02 M sodium fluoroacetate.
3. 0.2 M glycerol plus 0.04 M sodium fluoroacetate.
4. 0.2 M glycerol plus 0.00025 M sodium arsenite.

5. 0.2 M glycerol plus 0.0005 M sodium arsenite.

The above are the final concentrations of the test substances in Fildes' medium. Each test was carried out in duplicate at pH 6.8.

The 0.2 M glycerol solution has been described previously.

Sodium fluoroacetate* was prepared as a 0.4 M solution, ten ml. and five ml. respectively, of this solution were added to 90 and 95 ml. of Fildes' medium to give a final concentration of 0.04 M, and 0.02 M sodium fluoroacetate in the medium.

0.5 ml. and 0.25 ml., respectively, of the 0.1 N solution of sodium arsenite** were added to 99.5 ml. and 99.75 ml. of Fildes' medium to give a final concentration of 0.0005 M and 0.00025 M sodium arsenite in the medium.

Results presented in Table IV demonstrate that the above enzyme inhibitors are extremely toxic for Staphylococcus pyogenes. An attempt to evaluate the results would prove futile due to the limited amount of growth obtained in the presence of the inhibitors. However, it was interesting to note that although the inhibitors caused a marked decrease in growth they did not completely suppress pigment production. It would therefore appear that in the presence of a carbohydrate the complete suppression of pigment production by enzyme

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* Sodium fluoroacetate, Bios Laboratories, New York, N.Y.

**Sodium arsenite, N/10, Anachemia Ltd., Montreal, Canada.

TABLE IV.

THE EFFECT OF SODIUM ARSENITE AND SODIUM FLUOROACETATE ON PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES.

Test Substance in Fildes' Medium	Dry wt. mgm.	O/D of extracted pigment.	Calc.O/D for extracted pigment from 100 mgm. dry wt.	Average O/D.
0.2 M Glycerol	35.1 35.8	0.0862 0.0888	0.244 0.248	0.246
0.2 M Glycerol + 0.00025 M Sodium Arsenite	6.6 6.2	0.0132 0.0121	0.200 0.195	0.1975
0.2 M Glycerol + 0.0005 M Sodium Arsenite	No growth No growth			
0.2 M Glycerol + 0.02 M Sodium Fluoroacetate	5.4 7.5	0.0088 0.0121	0.163 0.161	0.162
0.2 M Glycerol + 0.04 M Sodium Fluoroacetate	4.4 3.4	0.0066 0.0055	0.150 0.161	0.1555

inhibitors was impossible.

Summary.

In the foregoing section the effect of various enzyme inhibitors on pigment production by Staphylococcus pyogenes have been studied. Sodium fluoride and sodium malonate were found to increase pigmentation in Staphylococcus pyogenes. Possible explanations for the increases are given in the appropriate sections. Sodium fluoroacetate and sodium arsenite were found to be toxic for the organism but it was interesting to note that as long as there was growth pigment was present. The use of enzyme inhibitors with proliferating cells had its limitations in that it is not possible to determine which enzyme system is affected by the inhibitor. However, the foregoing experiments revealed that proliferating cells in some cases possessed the ability to overcome the effect of the inhibitors and are able to carry out their normal function such as pigment production.

THE PRODUCTION OF PIGMENT BY *STAPHYLOCOCCUS PYOGENES* WITHOUT A SOURCE OF CARBOHYDRATE.

Investigators have been mainly interested in the influence of carbohydrates on pigment production by bacteria but none have investigated the possibility of an organism producing pigment in the absence of a source of carbohydrate. In order to study this problem, two conditions must be satisfied: first, a method must be found which will allow the measurement of small amounts of pigment and, second, a synthetic medium must be used to eliminate the possibility of a carbohydrate being present in trace amounts. The method described in the General Procedure for the estimation of pigment will detect small amounts of pigment. Fildes' medium being a synthetic medium consisting of amino acids and vitamins would satisfy the second condition in that trace amounts of carbohydrates would be absent. Having satisfied the above two conditions the following experiment was carried out to determine whether *Staphylococcus pyogenes* could produce its pigment in the absence of a source of carbohydrate. Flasks containing Fildes' medium without a source of carbohydrate were incubated for ninety hours at room temperature on a shaking machine. Flasks containing Fildes' medium and 0.2 M glycerol were incubated at the same time to serve as comparisons.

The results presented in Table V show that *Staphylococcus pyogenes* will produce pigment in the absence of a source of carbohydrate, but the amount will be less than its

TABLE V.

THE PRODUCTION OF PIGMENT BY STAPHYLOCOCCUS PYOGENES WITH AND WITHOUT A SOURCE OF CARBOHYDRATE.

Fildes' Medium	Dry wt. mgm.	O/D of extracted pigment.	Calc.O/D for extracted pigment from 100mgm.dry wt.	Average O/D.
0.2 M Glycerol	35.2	0.0862	0.244	0.244
	35.1	0.0862	0.244	
No Carbohydrate	12.1	0.0155	0.128	0.127
	12.3	0.0155	0.126	
	12.2	0.0155	0.127	

presence allows. A possible explanation for the production of pigment in the absence of a source of carbohydrate would be that some of the amino acids were utilized as a source of carbon and energy. If this were not so, no growth would have appeared in the medium. The compounds which served as the source of carbon could also be used for the production of the pigment by the bacteria.

THE EFFECT OF SODIUM ACETATE ON PIGMENT PRODUCTION BY *STAPHYLOCOCCUS PYOGENES*.

Since *Staphylococcus pyogenes* will produce its pigment in the presence of a carbohydrate, it must be capable of utilizing one of the products of carbohydrate metabolism for this function. Investigations have yet to be carried out to determine whether some end product of carbohydrate metabolism could serve as a precursor of pigment. It was felt that in studies of this type it would be advisable to start with a relatively simple compound that is known to occur in carbohydrate metabolism, namely, acetate. If, for example, pyruvic acid was tested for its ability to produce pigment it would not be known whether *Staphylococcus pyogenes* oxidized this compound to acetate and utilized it for pigment production. Before acetate could be tested for its ability to produce pigment it became imperative to know whether this compound occurred as an end-product in carbohydrate metabolism of *Staphylococcus pyogenes*. Kligler, Grossowicz and Bergner (1943) studying the role of niacin and thiamine in the metabolism of glucose by *Staphylococcus pyogenes* observed that in the presence of these vitamins the end-products consisted of about 40 per cent acetate, 20 per cent lactic acid and only trace amounts of pyruvic acid. Chen and Tang (1940) reported the direct oxidation of pyruvic acid to acetic acid by *Staphylococcus pyogenes*. Sevag and Swart (1947) observed that cells derived from a glucose-containing medium principally oxidized pyruvic

acid, yielding, on the average, two to four moles of acetate for one mole of lactate produced. From the above it may be concluded that acetate will be one of the end-products of carbohydrate metabolism by Staphylococcus pyogenes.

The following experiment was carried out to determine whether acetate would promote pigment production by Staphylococcus pyogenes and the required substance was added to separate flasks of Fildes' medium to the indicated final concentration.

1. Distilled water to make up volume (Control).
2. 0.05 M sodium acetate.
3. 0.1 M sodium acetate.
4. 0.2 M sodium acetate.
5. 0.3 M sodium acetate.
6. 0.4 M sodium acetate.

Each test was done in duplicate at pH 6.8.

The above concentrations of sodium acetate* were prepared as ten-fold concentrations. Ten ml. of each ten-fold concentration was added to ninety ml. of Fildes' medium to give the desired molarity of sodium acetate in the medium.

For the purpose of comparison, another experiment was carried out in which glycerol was added to separate flasks of Fildes' medium to similar final concentrations.

1. Distilled water (Control).
2. 0.05 M glycerol.

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* Sodium acetate, C.P., Eimer and Amend, New York, N.Y.

3. 0.1 M glycerol.

4. 0.2 M glycerol.

5. 0.3 M glycerol.

6. 0.4 M glycerol.

Each test was done in duplicate at pH 6.8.

The above concentrations of glycerol were prepared as ten-fold concentrations. Ten ml. of each ten-fold concentration was added to ninety ml. of Fildes' medium to give the desired molarity of glycerol in the medium.

The results presented Table VI demonstrate that sodium acetate was utilized by Staphylococcus pyogenes for growth and pigment production. Although, less growth was obtained with acetate than with glycerol (Table VII), the amount of pigment produced per 100 mgm. dry weight of cells was considerably higher in the presence of acetate. Sodium acetate at a concentration greater than 0.2 M inhibited growth to such an extent that the yield of growth was less than that of the control but the amount of pigment was relatively higher. From the results of the two experiments it would appear that the controlling factor in pigment production is the amount of acetate available to the organism and therefore, acetate serves as the primary precursor of pigment. If a compound other than acetate were utilized for pigment production, the amount of pigment produced with glycerol would have been higher than that produced by acetate.

TABLE VI.

THE EFFECT OF SODIUM ACETATE ON PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES.

Test Substance in Fildes' Medium	Dry wt. mgm.	O/D of extracted pigment.	Calc.O/D for extracted pigment from 100 mgm.dry wt.	Average O/D.
Distilled Water	12.2 12.3	0.0155 0.0155	0.127 0.126	0.1265
0.05 M Sodium Acetate	20.5 20.2	0.1079 0.1051	0.526 0.520	0.523
0.1 M Sodium Acetate	20.5 19.5	0.1192 0.1135	0.581 0.582	0.5815
0.2 M Sodium Acetate	17.5 17.4	0.1079 0.1079	0.617 0.620	0.6185
0.3 M Sodium Acetate	8.8 8.6	0.0482 0.0470	0.547 0.547	0.547
0.4 M Sodium Acetate	5.0 5.3	0.0246 0.0257	0.492 0.485	0.4885

TABLE VII.

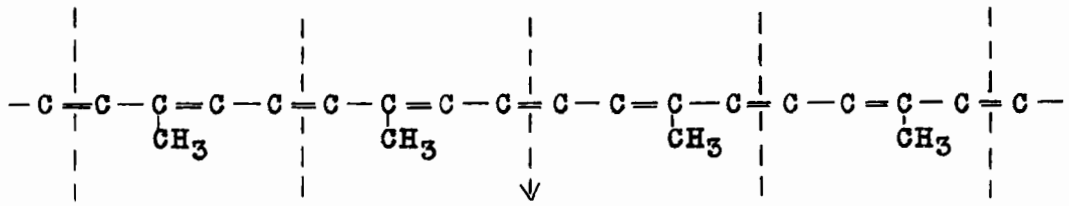
THE EFFECT OF GLYCEROL ON PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES.

Test Substance in Fildes' Medium	Dry wt. mgm.	O/D of extracted pigment.	Calc.O/D for extracted pigment from 100mgm.dry wt.	Average O/D.
Distilled Water	12.2 12.3	0.0155 0.0155	0.127 0.126	0.1265
0.05 M Glycerol	25.4 24.9	0.0386 0.0386	0.152 0.155	0.1535
0.1 M Glycerol	31.2 31.1	0.0642 0.0642	0.206 0.206	0.206
0.2 M Glycerol	35.4 35.2	0.0862 0.0862	0.244 0.244	0.244
0.3 M Glycerol	40.2 41.0	0.1192 0.1192	0.296 0.291	0.2935
0.4 M Glycerol	46.3 45.0	0.1503 0.1487	0.324 0.331	0.3275

THE EFFECT OF FIVE CARBON COMPOUNDS ON PIGMENT PRODUCTION BY *STAPHYLOCOCCUS PYOGENES*.

Introduction.

Having found that acetate could serve as the primary precursor of pigment, the next step would be to find the possible intermediary compound necessary for pigment synthesis. On examination of the structural formula of a carotenoid it will be noted that the pigment could possibly be formed by a condensation of isoprene units.



Center of molecule

Although it would appear that an unsaturated five carbon compound was the repeating unit, the possibility of the compound being saturated must not be overlooked.

The experiments in this section will deal with the effect of various five carbon compounds such as iso-valeric acid, iso-valeraldehyde and beta-methylcrotonic acid on pigment production by *Staphylococcus pyogenes*. Iso-valeric acid and iso-valeraldehyde are saturated compounds while beta-methylcrotonic acid is unsaturated.

In the experiments to follow each compound was tested for its ability to act as a carbon source by comparing the amount of growth obtained in Fildes' medium without a source

of carbon with that obtained when the compound was present. This was a necessary step because if the compound acted as a carbon source it would mean that the compound was metabolized and any increase in pigment production would possibly be due to the end products of this particular substance. The effect of the test substance on pigment production was determined with sodium acetate as a source of carbon. The amount of pigment produced with sodium acetate and test substance was compared to the amount produced with sodium acetate alone. If there was an increase in pigment production due to the test substance with no accompanying increase in growth as compared to the control this would indicate that the increase in pigment was due to condensations of the five carbon compound.

The Effect of Iso-Valeric Acid on Pigment Production by Staphylococcus pyogenes.

The following experiment was carried out to determine the effect of iso-valeric acid on pigment production by Staphylococcus pyogenes. The following substances were added to separate flasks of Fildes' medium as the final concentration.

1. Distilled water to make up volume (Control).
2. Distilled water plus 0.005 M iso-valeric acid.
3. 0.05 M sodium acetate (Control).
4. 0.05 M sodium acetate plus 0.001 M iso-valeric acid.
5. 0.05 M sodium acetate plus 0.005 M iso-valeric acid.
6. 0.05 M sodium acetate plus 0.01 M iso-valeric acid.

Each test was done in duplicate at pH 6.8.

Sodium acetate was prepared as a 0.5 M solution and ten ml. of this solution was added to ninety ml. of Fildes' medium to give a final concentration in the medium of 0.05 M sodium acetate.

Iso-valeric acid* was prepared as a 0.1 M solution and 10 ml., 5 ml. and 1 ml. of this solution was added, respectively, to 90 ml., 95 ml. and 99 ml. of Fildes' medium to give a final concentration of 0.01 M, 0.005 M and 0.001 M iso-valeric acid in the medium.

The results presented in Table VIII demonstrate that iso-valeric acid was not utilized as a source of carbon since it did not increase growth when added to Fildes' medium containing no source of carbon other than the test substance. This experiment also showed that the saturated compound iso-valeric acid will increase pigment production by Staphylococcus pyogenes without an accompanying increase in growth. This would indicate that the organism is capable of utilizing this compound directly as a building unit for pigment and therefore it could be the intermediary compound in pigment synthesis.

The Effect of Iso-Valeraldehyde on Pigment Production by Staphylococcus Pyogenes.

Since iso-valeric acid was utilized as a building unit for pigment synthesis, the following experiment was carried

* Iso-valeric acid, Eastman-Kodak Co., Rochester, N.Y.

TABLE VIII.

THE EFFECT OF ISO-VALERIC ACID ON PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES.

Test Substance in Fildes' Medium	Dry wt. mgm.	O/D of extracted pigment.	Calc.O/D for extracted pigment from 100mgm.dry wt.	Average O/D.
Distilled Water	12.8	0.0166	0.130	0.1295
	12.9	0.0166	0.129	
Distilled Water + 0.005 M Iso-Valeric Acid	12.8	0.0315	0.246	0.248
	12.6	0.0315	0.250	
0.05 M Sodium Acetate	20.5	0.1079	0.526	0.5265
	20.2	0.1065	0.527	
0.05 M Sodium Acetate + 0.001 M Iso-Valeric Acid	20.6	0.1838	0.893	0.895
	20.3	0.1821	0.897	
0.05 M Sodium Acetate + 0.005 M Iso-Valeric Acid	20.2	0.1871	0.926	0.922
	20.2	0.1855	0.918	
0.05 M Sodium Acetate + 0.01 M Iso-Valeric Acid	17.3	0.1543	0.887	0.887
	17.8	0.1580	0.887	

out to determine whether iso-valeraldehyde would exert a similar effect. The following substances were added to separate flasks of Fildes' medium as the final concentration.

1. Distilled water to make up volume (Control).
2. Distilled water plus 0.005 M iso-valeraldehyde.
3. 0.05 M sodium acetate (Control).
4. 0.05 M sodium acetate plus 0.001 M iso-valeraldehyde.
5. 0.05 M sodium acetate plus 0.005 M iso-valeraldehyde.
6. 0.05 M sodium acetate plus 0.01 M iso-valeraldehyde.

Each test was done in duplicate at pH 6.8.

The preparation of the sodium acetate solution has been described in the previous experiment.

Iso-valeraldehyde* was prepared as a 0.1 M solution and 10 ml., 5 ml. and 1 ml. of this solution was added, respectively, to 90 ml., 95 ml. and 99 ml. of Fildes' medium to give a final concentration of 0.01 M, 0.005 M and 0.001 M iso-valeraldehyde in the medium.

The results in Table IX demonstrate that iso-valeraldehyde had no effect on pigment production by Staphylococcus pyogenes. The compound caused a slight inhibition of growth at concentrations of 0.001 M and 0.005 M and inhibited growth completely at a concentration of 0.01 M. From the results it may be concluded that Staphylococcus pyogenes is unable to utilize this compound for pigment

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* Iso-valeraldehyde, Eastman-Kodak Co., Rochester, N.Y.

TABLE IX.

THE EFFECT OF ISO-VALERALDEHYDE ON PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES.

Test Substance in Fildes' Medium	Dry wt. mgm.	O/D of extracted pigment.	Calc.O/D for extracted pigment from 100mgm.dry wt.	Average O/D.
Distilled Water	12.4	0.0155	0.125	0.1255
	12.3	0.0155	0.126	
Distilled Water + 0.005 M Iso-Valeraldehyde	8.0	0.0099	0.124	0.1215
	7.4	0.0088	0.119	
0.05 M Sodium Acetate	20.4	0.1079	0.529	0.528
	20.2	0.1065	0.527	
0.05 M Sodium Acetate + 0.001 M Iso-Valeraldehyde	18.4	0.0942	0.512	0.512
	18.4	0.0942	0.512	
0.05 M Sodium Acetate + 0.005 M Iso-Valeraldehyde	18.5	0.0969	0.524	0.523
	18.8	0.0982	0.522	
0.05 M Sodium Acetate + 0.01 M Iso-Valeraldehyde	No growth			

production because of its aldehyde group.

The Effect of Beta-Methylcrotonic Acid on Pigment Production by Staphylococcus pyogenes.

The following experiment was carried out to determine whether beta-methylcrotonic acid, an unsaturated five carbon compound, was capable of being used directly as a building unit for pigment. The following substances were added to separate flasks of Fildes' medium to the indicated final concentration.

1. Distilled water to make up volume (Control).
2. Distilled water plus 0.0005 M beta-methylcrotonic acid.
3. 0.05 M sodium acetate (Control).
4. 0.05 M sodium acetate plus 0.005 M beta-methylcrotonic acid.
5. 0.05 M sodium acetate plus 0.001 M beta-methylcrotonic acid.
6. 0.05 M sodium acetate plus 0.005 M beta-methylcrotonic acid.

Each test was done in duplicate at pH 6.8.

The preparation of the 0.05 M sodium acetate solution has been described in a previous experiment in this section.

Beta-methylcrotonic acid* was prepared as a 0.05 M solution and 10 ml., 2 ml. and 1 ml. of this solution was added, respectively, to 90 ml., 98 ml. and 99 ml. of Fildes' medium to give a final concentration of 0.005 M, 0.001 M and 0.0005 M

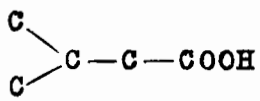
* Beta-methylcrotonic acid, Courtesy of Dr. Leger, Monsanto Canada Ltd, Montreal, Canada.

beta-methylcrotonic acid in the medium.

The results presented in Table X demonstrate that with increasing concentrations of beta-methylcrotonic acid there is a decrease in growth with an accompanying decrease in the amount of pigment produced per 100 mgm. dry weight. These results reveal that beta-methylcrotonic acid exerts an effect opposite to that of iso-valeric acid as regards pigment production.

Summary.

In the foregoing section, three five carbon compounds, namely, iso-valeric acid, iso-valeraldehyde and beta-methylcrotonic acid, were tested as possible intermediary compounds in carotenoid synthesis. Of the three compounds tested only iso-valeric acid was active as a possible intermediary compound in pigment production. Beta-methylcrotonic acid had an inhibitor effect on growth and pigment production while iso-valeraldehyde was inactive at low concentrations and at a concentration of 0.01 M was extremely toxic for the organism. The results of the foregoing experiments indicate that the possible intermediary compound in carotenoid synthesis by Staphylococcus pyogenes is a five carbon compound having the following structure and



the pigment could possibly be produced by condensation of eight

TABLE X.

THE EFFECT OF BETA-METHYLCROTONIC ACID ON PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES.

Test Substance in Fildes' Medium	Dry wt. mgm.	O/D of extracted pigment.	Calc.O/D for extracted pigment from 100 mgm. dry wt.	Average O/D.
Distilled Water	12.3	0.0155	0.126	0.1255
	12.4	0.0155	0.126	
Distilled Water + 0.0005 M Beta-Methylcrotonic Acid	10.7	0.0132	0.123	0.125
	10.4	0.0132	0.127	
0.05 M Sodium Acetate	20.5	0.1079	0.526	0.5275
	20.4	0.1079	0.529	
0.05 M Sodium Acetate + 0.0005 M Beta-Methylcrotonic Acid	21.0	0.1121	0.529	0.5265
	20.6	0.1079	0.524	
0.05 M Sodium Acetate + 0.001 M Beta-Methylcrotonic Acid	18.3	0.0915	0.500	0.503
	18.1	0.0915	0.506	
0.05 M Sodium Acetate + 0.005 M Beta-Methylcrotonic Acid	11.3	0.0555	0.491	0.4915
	11.8	0.0580	0.492	

five carbon units. From the results of the last two sections it would appear that Staphylococcus pyogenes produces its pigment by utilizing acetate to form iso-valeric acid which is then used as the building unit for the synthesis of pigment.

THE EFFECT OF VITAMIN A ON PIGMENT PRODUCTION BY *STAPHYLOCOCCUS PYOGENES*.

The similarity in chemical structure between carotene and vitamin A suggested the possibility that this vitamin could perhaps exert an influence on pigment production by *Staphylococcus pyogenes*.

The vitamin A* used in this experiment was obtained from Hoffman-LaRoche, Basle, Switzerland and was found suitable for this experiment since it was dissolved in an emulsifying agent thereby facilitating its dispersion in Fildes' medium. The solution of vitamin A contained 150,000 International Units of vitamin A per ml. or approximately 170 mgm. per ml. The emulsifying agent without vitamin A served as the control. These substances were added to separate flasks of Fildes' medium as follows:

1. 0.5 ml. of vitamin A solution was added to 99.5 ml. of Fildes' medium to give a final concentration of 750 I.U. per ml. or 0.85 mgm. per ml.
2. 0.5 ml. of the emulsifying agent was added to 99.5 ml. of Fildes' medium.
3. 1.0 ml. of vitamin A solution was added to 99 ml. of Fildes' medium to give a final concentration of 1500 I.U. per ml. or 1.7 mgm. per ml.
4. 1.0 ml. of the emylsifying agent was added to

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* Arovit-Tropfenlosung, Hoffman-LaRoche, Basle, Switzerland.

99 ml. of Fildes' medium.

5. 1.5 ml. of Vitamin A solution was added to 98.5 ml. of Fildes' medium to give a final concentration of 2250 I.U. per ml. or 2.55 mgm. per ml.
6. 1.5 ml. of the emulsifying agent was added to 98.5 ml. of Fildes' medium.

Each test was done in duplicate at pH 6.8.

The results presented in Table XI demonstrate that with increasing amounts of the emulsifying agent there was a decrease in growth but the amount of pigment produced per 100 mgm. dry weight remained relatively the same. Increasing amounts of vitamin A plus the emulsifying agent resulted in an increase in growth with an accompanying increase in the amount of pigment produced per 100 mgm. dry weight. These results would indicate that vitamin A was able to suppress the inhibitor effect of the emulsifying agent on Staphylococcus pyogenes and also that it stimulated both growth and pigment production. Since vitamins are catalysis it would appear that vitamin A is catalyzing some step in the metabolic pathway resulting in an increase in growth and pigment production. However, the possibility of two molecules of vitamin A condensing to form a carotenoid, that is, beta-carotene should not be overlooked as another explanation for the increase in pigment production by Staphylococcus pyogenes.

TABLE XI.

THE EFFECT OF VITAMIN A ON PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES.

Test Substance in Fildes' Medium	Dry wt. mgm.	O/D of extracted pigment.	Calc.O/D for extracted pigment from 100 mgm.dry wt.	Average O/D.
0.5 ml. Emulsifying Agent	25.8	0.0796	0.309	0.308
	25.1	0.0770	0.307	
0.5 ml. Emulsifying Agent + 750 I.U. per ml. Vitamin A	27.0	0.0809	0.299	0.299
	27.0	0.0809	0.299	
1.0 ml. Emulsifying Agent	14.1	0.0434	0.308	0.3065
	14.6	0.0446	0.305	
1.0 ml. Emulsifying Agent + 1500 I.U. per ml. Vitamin A	28.1	0.1135	0.404	0.403
	28.9	0.1149	0.402	
1.5 ml. Emulsifying Agent	12.0	0.0362	0.302	0.303
	11.9	0.0362	0.304	
1.5 ml. Emulsifying Agent + 2250 I.U. per ml. Vitamin A.	39.6	0.2076	0.524	0.522
	38.3	0.1190	0.520	

PIGMENT PRODUCTION BY *STAPHYLOCOCCUS PYOGENES* UNDER ANAEROBIC CONDITIONS.

Topley and Wilson (1946) state that oxygen is necessary for the development of pigment by *Staphylococcus pyogenes* and under anaerobic conditions the growth is colorless. In preliminary investigations we observed a slight trace of pigment when large amounts of growth were collected from nutrient agar plates which had been incubated under anaerobic conditions. Although it could be argued that the trace of pigment was a result of a small amount of oxygen being present in the Fildes-McIntosh jar, we concluded that it was due to incomplete oxidation of the available carbohydrate in the medium. The reason for this conclusion is that the amount of energy obtained from the anaerobic oxidation of a carbohydrate is approximately 4-5 per cent of that resulting from aerobic oxidation and therefore, more of a given substrate must be present in order for the organism to gain access to the same amount of energy that is normally supplied in aerobic oxidation. We thought it possible that if *Staphylococcus pyogenes* were grown on a medium containing high concentrations of a carbon source it would produce its pigment anaerobically.

The following experiments were carried out to determine whether *Staphylococcus pyogenes* when grown on nutrient agar containing an excess of a carbon source would produce pigment under anaerobic conditions. Two series of two per cent nutrient agar plates (Difco) were prepared, one series

contained 4, 8 and 12 per cent glycerol and the other contained similar concentrations of sodium acetate. Duplicate series were inoculated with washed suspensions of Staphylococcus pyogenes and one set was incubated aerobically and the other anaerobically at 37°C for a period of five days. Plates to be incubated anaerobically were placed in a Fildes-McIntosh jar with a tube of glucose containing methylene blue to serve as an indicator for anaerobiosis. The use of the Fildes-McIntosh jar is discussed in Appendix C. Since in these experiments we were primarily interested in determing whether or not Staphylococcus pyogenes would produce its pigment with increased concentrations of substrate, the results reported are of a purely qualitative nature and no attempt was made to estimate the relative amounts of pigment.

The results presented in Table XII demonstrate that Staphylococcus pyogenes will produce pigment anaerobically provided the carbon source is present in high concentrations. This would indicate that the production of pigment is not directly dependent on the presence of oxygen but rather on the amount of energy made available by carbohydrate oxidation.

TABLE XII.

PIGMENT PRODUCTION BY STAPHYLOCOCCUS PYOGENES UNDER ANAEROBIC CONDITIONS.

Test Substance in Nutrient Agar	Aerobic	Anaerobic
4 per cent Glycerol	+	+
8 per cent Glycerol	+	+
12 per cent Glycerol	+	+
4 per cent Sodium Acetate	+	+
8 per cent Sodium Acetate	No Growth	+
12 per cent Sodium Acetate	No Growth	No Growth

+ = Pigment

GENERAL DISCUSSION.

Attempts to measure pigment by visual comparison of a mass of cells or individual colonies proved unreliable because of the difficulty in determining whether the increase in color was a result of more pigment per cell or due to an increase in growth thereby resulting in a greater intensity of color. The method of Stahly, Sesler and Brode (1942) for the colorimetric measurement of pigment proved unsatisfactory because it yielded erratic results and failed to give reproducible values. In the above method the authors failed to realize that bacterial cells absorb different quantities of light depending on the wavelength passed by filter. A method is discussed in the section on General Procedure which was found to yield more accurate and reliable results than the previously mentioned methods. This method was capable of determining whether a given physical or chemical factor would increase or decrease pigmentation when compared to a control.

The liquid medium of Fildes, Richardson, Knight and Gladstone (1936) and Fildes and Richardson (1937) was found suitable for use since it is a synthetic medium and therefore, all constituents of the medium are chemically defined. Another factor favoring the use of this medium was that the test substance could be added in lieu of glucose enabling us to determine the effect of the test substance on pigmentation in the absence of another source of carbon.

Aeration, by means of a shaking machine, at room

temperature was found to be the most satisfactory method for the production of pigment by Staphylococcus pyogenes. The reason being that when the organisms are shaken they have a greater access to oxygen and to the available mutrients in the medium.

Staphylococcus pyogenes will produce its pigment in the absence of a source of carbohydrate but the amount will be less than its presence allows. A possible explanation for the production of pigment in the absence of a source of carbohydrate would be that some of the amino acids present in the medium were utilized as a source of carbon and energy. If this were not so, no growth would have appeared in the medium. The compounds which served as the source of carbon could also be used for the production of the pigment by the bacteria. Staphylococcus pyogenes may be capable of metabolizing some of the amino acids to acetic acid which is then utilized for pigment production. Acetic acid has been shown to be produced from glycine by Escherichia coli (Stephenson and Gale, 1937), from alanine by Mycobacterium tuberculosis (Campbell, 1925), and from glutamic acid by Hemophilus parainfluenza (Klein, 1940). Staphylococcus pyogenes may also be able to utilize the oxidation deamination product of valine, iso-valeric acid, is a building unit for pigment.

Experiments were conducted to determine whether enzyme inhibitors such as sodium fluoride, sodium arsenite, sodium fluoroacetate and sodium malonate would inhibit or enhance pigment production by Staphylococcus pyogenes. These studies

were done with proliferating organisms and not with cell-free extracts or resting cell suspensions. Sodium fluoride at pH 6.8 resulted in an increase in pigment production by Staphylococcus pyogenes. With increasing concentrations of sodium fluoride there was a subsequent decrease in growth but the amount of pigment produced per 100 mgm. dry weight of growth remained somewhat higher than that of the control. At pH 6.0 sodium fluoride inhibited the growth of Staphylococcus pyogenes. Fritzgerald and Bernheim (1948) observed that the oxidation of glucose, fructose and trehalose by Mycobacterium tuberculosis was increased by sodium fluoride at pH 6.8. This effect of sodium fluoride on oxidation may also apply to Staphylococcus pyogenes and therefore, with increased oxidation of glycerol there was a corresponding increase in pigment production. Another explanation may be that pH 6.8 a fluoride-insensitive pathway exist which would function when the glycolytic pathway has been blocked by sodium fluoride. The results obtained with sodium fluoride at pH 6.0 were similar to those obtained by Fritzgerald and Bernheim (1948) with Mycobacterium tuberculosis. The amount of pigment produced per 100 mgm. dry weight of growth was increased by sodium malonate. The results also indicated that this compound acted as a carbon source because when it was added to Fildes' medium containing no carbohydrate there was an increase in growth as well as pigment production. Gray (1952) demonstrated that dried cell preparations of Pseudomonas aeruginosa decarboxylated

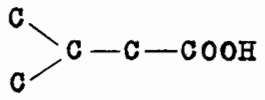
malonic acid to acetic acid. It may be that the increases in growth and pigment production were a result of Staphylococcus pyogenes being able to decarboxylate malonic acid to acetic acid and carbon dioxide. The acetic acid produced by decarboxylation could be used as a carbon source and also as a precursor of pigment. Sodium arsenite and sodium fluoroacetate were found to be extremely toxic for the organism. Although the inhibitors caused a marked decrease in growth they did not completely suppress pigment production, thus showing that in the presence of a carbohydrate the complete suppression of pigment production by enzyme inhibitors is impossible. It would therefore appear that the amount of pigment produced by an organism is proportional to the degree of oxidation of the substrate.

Since Staphylococcus pyogenes will produce its pigment in the presence of a carbohydrate, it must be capable of utilizing one of the end-products of carbohydrate metabolism for this function. Investigations were carried out to determine whether sodium acetate would have any influence on pigment production. Results indicated that sodium acetate was utilized by Staphylococcus pyogenes for growth and pigment production. Although, less growth was obtained with sodium acetate than with glycerol, the amount of pigment produced per 100 mgm. dry weight of growth was considerably higher in the presence of sodium acetate. Sodium acetate at a concentration greater than 0.2 M inhibited growth to such an extent that the yield of growth was

less than that of the control but the amount of pigment was relatively higher. It would appear from the results of the experiment that the controlling factor in pigment production was the amount of acetate available to the organism and therefore, acetate could serve as the primary precursor of pigment. If a compound other than acetate were utilized for pigment production, the amount of pigment produced with glycerol would have been higher than that produced by acetate.

In the laboratory it is often noticed that Staphylococcus pyogenes when first isolated will produce abundant pigment when grown on, for example, nutrient agar but upon subsequent subculture it will lose this ability to produce pigment. In the majority of cases there is a gradual decrease in pigment until finally the growth is colorless. Goldschmidt and Powelson (1953) observed that tryptone yeast-extract medium contained a dialyzable substance which interfered in some way with the ability of the cells of Staphylococcus pyogenes to oxidize acetate. In a synthetic medium, glucose produced a similar effect. Could it be that the media employed in the laboratory contain a similar substance? This could possibly explain why Staphylococcus pyogenes on first isolation produces abundant pigment but on further subculture the organism is able to overcome this inhibitor effect by degrees until finally acetate is completely oxidized and therefore, no pigment would be produced.

Having found that acetate could serve as the primary precursor of pigment, experiments were carried out to find the

possible intermediary compound necessary for pigment synthesis. Examination of the structural formula of a carotenoid revealed that the pigment could possibly be formed by a condensation of isoprene units. Experiments were undertaken to determine the effect of various five carbon compounds such as iso-valeric acid, iso-valeraldehyde and beta-methylcrotonic acid on pigment production by Staphylococcus pyogenes. Of the three compounds tested only iso-valeric acid was active as a possible intermediary compound in pigment synthesis. Beta-methylcrotonic acid had an inhibitor effect on growth and pigment production while iso-valeraldehyde was inactive at low concentrations and at a concentration of 0.01 M was extremely toxic for the organism. The results of the experiments indicate that the possible intermediary compound in carotenoid synthesis by Staphylococcus pyogenes is a saturated five carbon compound having the following structure  and the pigment could possibly be produced by condensations of eight five carbon units. The fact that the organism utilized the saturated compound would indicate that the organism contains a dehydrogenase capable of removing the hydrogen atoms either prior to or after the formation of the pigment. This step is necessary since the carotenoid is an unsaturated compound. It would appear that Staphylococcus pyogenes produces its pigment by utilizing acetate to form iso-valeric acid which is then used as the building unit for the synthesis of the pigment. The question

which remains to be answered is whether four five carbon units combine "head to tail" to form a twenty carbon compound which then dimerises "tail to tail" or whether two five carbon units combine "tail to tail" and the molecule built up by "tail to tail" condensation at either end of this ten carbon chain to form the pigment.

The similarity in chemical structure between carotene and vitamin A suggested the possibility that this vitamin could perhaps exert an influence on pigment production. Experiments with vitamin A revealed that this vitamin stimulated both growth and pigment production by Staphylococcus pyogenes. Since vitamins are catalysis it would appear that vitamin A was catalyzing some step in the metabolic pathway resulting in the increases in growth and pigment production. However, the possibility of two molecules of vitamin A condensing to form a carotenoid, that is, beta-carotene should not be overlooked as another possibility for the increase in pigment production.

Topley and Wilson (1946) state that oxygen is necessary for the development of pigment by Staphylococcus pyogenes and under anaerobic conditions the growth is colorless. From the results of preliminary experiments it was concluded that the lack of pigment anaerobically was due to the incomplete oxidation of the available carbohydrate in the medium. We found it possible to produce pigment anaerobically by growing the organism on a medium containing high concentrations of a carbon source. This would indicate that the production of

pigment is not directly dependent on the presence of oxygen but rather on the amount of energy made available by carbohydrate oxidation.

In conclusion we would like to quote Palmer (1922): "The whole subject of carotenoids and related pigments is a fascinating one and offers as many unsolved problems as any other phase of experimental biology and biochemistry ..... The extension of the frontiers of our knowledge regarding these pigments which are so abundantly distributed in so many plants and animals is certain to prove a profitable as well as an interesting undertaking."

GENERAL SUMMARY OF RESULTS.

1. A method has been discussed which was found capable of determining whether a given physical or chemical factor would increase or decrease pigmentation when compared to a control.
2. Fildes' synthetic medium was found suitable for use as a test medium.
3. Aeration, by means of a shaking machine, at room temperature was found to be the most satisfactory method for the production of pigment by Staphylococcus pyogenes.
4. Staphylococcus pyogenes will produce its pigment in the absence of a source of carbohydrate but the amount will be less than its presence allows.
5. Sodium fluoride at pH 6.8 resulted in an increase in pigment production by Staphylococcus pyogenes and at pH 6.0 it inhibited the growth of the organism.
6. Pigment production by Staphylococcus pyogenes was increased by sodium malonate.
7. Sodium arsenite and sodium fluoroacetate were found to be extremely toxic for the organism. Although the inhibitors caused a marked decrease in growth, they did not completely suppress pigment production.
8. Sodium acetate was utilized by Staphylococcus pyogenes for growth and pigment production. Although, less growth was obtained with sodium acetate than with glycerol, the amount of pigment produced per 100 mgm. dry weight of growth was

considerably higher in the presence of sodium acetate. Results indicate that sodium acetate could possibly serve as the primary precursor of pigment.

9. Experiments revealed that the saturated five carbon compound iso-valeric acid will increase pigment production by Staphylococcus pyogenes without an accompanying increase in growth. This would indicate that the organism was capable of utilizing this compound directly as a building unit for pigment.
10. Iso-valeraldehyde was found to have no effect on pigment production by Staphylococcus pyogenes.
11. Results revealed that beta-methylcrotonic acid exerted an effect opposite to that of iso-valeric as regards pigment production.
12. Vitamin A was found to stimulate both growth and pigment production by Staphylococcus pyogenes.
13. It was found possible to produce pigment anaerobically by growing the organism on a medium containing high concentrations of a carbon source.

CLAIM TO ORIGINALITY.

The results presented in this report constitute an original contribution to the understanding of pigment production by Staphylococcus pyogenes in that the following facts have been established:

1. A method has been developed which is suited for determining whether a given physical or chemical factor would increase or decrease pigmentation when compared to a control.
2. Staphylococcus pyogenes will produce its pigment in the absence of a source of carbohydrate but the amount will be less than its presence allows.
3. Sodium fluoride at pH 6.8 resulted in an increase in pigment production by Staphylococcus pyogenes and at pH 6.0 it inhibited growth.
4. Pigment production was increased by sodium malonate.
5. Sodium fluoroacetate and sodium arsenite were found to be extremely toxic for the organism. Although the inhibitors caused a marked decrease in growth, they did not completely suppress pigment production.
6. Sodium acetate was utilized for growth and pigment production. Although, less growth was obtained with sodium acetate than with glycerol, the amount of pigment produced per 100 mgm. dry weight of growth was considerably higher in the presence of sodium acetate. Results indicate that sodium acetate could possibly

serve as the primary precursor of pigment.

7. Experiments revealed that the saturated five carbon compound iso-valeric acid will increase pigment production without an accompanying increase in growth. This would indicate that the organism was capable of utilizing this compound directly as a building unit for pigment.
8. Iso-valeraldehyde was found to have no effect on pigment production.
9. Results revealed that beta-methylcrotonic acid exerted an effect opposite to that of iso-valeric acid as regards pigment production.
10. Vitamin A was found to stimulate both growth and pigment production.
11. It was found possible to produce pigment anaerobically by growing the organism on a medium containing high concentrations of a carbon source.

BIBLIOGRAPHY.

BIBLIOGRAPHY.

Anderson, R. J. and Newman, M. S. J.Biol.Chem., 101:773, 1933.

Archichowski, V. Bull.Jardin Imp. de St. Petersbourg 3: Part 4, 1904.

Ark, P. A. J.Bact., 61: 293, 1951.

Baker, H. A. M.Sc. Thesis, Department of Bacteriology and Immunology, McGill University, Montreal, Canada, 1952.

Baumann, C. A., Steenbock, H., Ingraham, M.A. and Fred, E.B. J.Biol.Chem., 103: 339, 1933.

Beijerinch, M.W. Bot.Zeitg., 49: 705, 725, 741, 757, 773, 1891.

Bigger, J.W., Boland, C.R. and O'Meara, R.A. J.Path. and Bact., 30: 261, 1927.

Bonner, J., Sandoval, A., Tang, Y.W. and Zechmeister, L. Arch. Biochem., 10: 193, 1946.

Braum, C.A. Phytopathology, 39: 171, 1949.

Bryan, M.K. Phytopathology, 20: 141, 1930.

Bryan, M.K. Phytopathology, 21: 559, 1931.

Buchanan, R.E. and Fulmer, E.I. Physiology and Biochemistry of Bacteria, The William and Wilkins Co., 1928, Baltimore.

Butschii, O. Uber den Bau der Bakterien u. verwandten Organismen. Winter. Cited: Buchanan and Fulmer, 1928.

Byatt, P.H., Jann, G.J. and Salle, A.J. J.Bact., 55: 787, 1948.

Campbell, L.K. Am.Rev.Tuberc., 11: 458, 1925.

Chapman, G.H. J.Bact., 51: 409, 1946.

Chargaff, E. Zentr.Bakt., I: 119, 121, 1930.

Chargaff, E. and Dieryck, J. Naturwissenschaften, 20: 872, 1932.

Chargaff, E. Compt. rend. acad.Sci., 197: 946, 1933.

Chargaff, E. Ann.Inst. Pasteur, 52: 415, 1934.

Chargaff, E. and Lederer, E. Ann.Inst.Pasteur, 54: 383, 1935.

Chen, H. K. and Tang, P.S. J.Cell. and Comp.Physiol., 16: 293, 1940.

Chenoweth, M.B. J.Pharmacol., 1: 383, 1949.

Darzin, E. Ann.Inst.Pasteur, 63: 455, 1939.

Davis, B.D. and Dubos, R.J. J.Bact., 55: 11, 1947.

Englemann, T.W. Bot.Zeitz., 46: 661, 677, 693, 709, 1888.

Fildes, P. and McIntosh, J.Brit.J.Exper.Path., 2: 153, 1921.

Fildes, P., Richardson, G.M., Knight, B.C.J.G. and Gladstone, G.P. Brit.J.Exper.Path., 17: 481, 1936.

Fildes, P. and Richardson, G.M. Brit.J.Exper.Path., 18: 292, 1937.

French, C.S. J.Gen.Physiol., 23: 469, 483, 1937.

Fritzgerald, R.J. and Bernheim, F. J.Bact., 55: 677, 1948.

Fujita, A. and Yishioka, J. Japanese Medical World, 3: 47, 1923.

Gilman, J.P. J.Bact., 65: 48, 1953.

Goldschmidt, M.C. and Powelson, D.M. Arch.Biochem. and Biophys., 46: 154, 1953.

Gray, C.T. J.Bact., 63: 813, 1952.

Grundmann, C. and Takeda, Y. Naturwissenschaften, 25: 27, 1939.

Guirard, B.M., Snell, E.E. and Williams, R.J. Arch.Biochem., 9: 361, 1946.

Gurd, F.B. and Denis, W. J.Exper.Med., 14: 606, 1911.

Haas, H.F. and Bushnell, L.D. J.Bact., 48, 219, 1944.

Hoffstadt, R.E. and Youmans, G.P. J.Infect.Dis., 51: 216, 1932.

Imsenecki, A.I. Comptes Rendus (Doklady) de L'Academie des Sciences de l'Urss, 53: 467, 1946.

Ingraham, M.A. and Baumann, C.A. J.Bact., 28: 31, 1934.

Ingraham, M.A. J.Bact., 29: 74, 1935.

Ingraham, M.A. and Steenbock, H. Biochem.J., 29: 2553, 1935.

Kahler, D.J. Dissertation, Michigan State College, 1951.

Karrer, P. and Solmssen, V. Helv.Chim.Acta., 18: 1306, 1935.

Karrer, P. and Solmssen, V. Helv. Chim.Acta., 19: 3, 1936.

Karrer, P., Solmssen, V. and Konig, H. Helv.Chim.Acta., 21: 454, 1938.

Kharasch, M.S., Conway, E.A. and Bloom, W. J.Bact., 32: 533, 1936.

Klein, J.R. J.Biol.Chem., 134: 43, 1940.

Kligler, I.S. Biochem.Bull., 3: 358, 1914.

Kligler, I.S., Grossowicz, N. and Bergner, S. J.Bact., 46: 399, 1943.

Krainsky, A. Centralbl. f. Bakteriol (etc), Abt.2, 41: 649, 1914.

Lankester, E.R. Quart.J.Micro.Sci., 13: 408, 1873.

Lubinski, W. Zbl.Bakt., 16: 769, 1894.

Lutz, A. Ann.Inst.Pasteur, 73: 1089, 1947.

Molish, H. Die Purpurbakterien 1907. Cited: Buchanan and Fulmer 1928.

Nakamura, T. Bull.Chem.Soc. Japan, 11: 176, 1936.

Neuman, R. Arch.f.Hyg., 30: 1, 1897.

Noguchi, Y. Arch. f.klin.Chir., 96: 696, 1911.

Nutini, L.G., Kreke, C.W. and Schroeder, M.P. J.Bact., 50: 177, 1946.

Nutini, L.G. and Lynch, E.M. J.Exper.Med., 84: 247, 1946.

Palmer, L.S. Carotenoids and Related Pigments, American Chemical Society Monograph No.9, Chemical Catalog Co., New York, 1922.

Peters, R. Brit.Med.Bull., 9: 116, 1953.

Pinner, M. and Voldrich, M. J.Infect.Dis., 50: 185, 1932.

Porter, J.W. and Lincoln, R.E. Arch.Biochem., 27: 390, 1950.

Quastel, J.H. Brit.Med.Bull., 9: 142, 1953.

Reader, V. Biochem.J., 19: 1039, 1925.

Reid, R.D. Zentralbatt fur Bakteriologie Parasitenknude und Infektionskrankheiten, Abt. 2, 95: 379, 1936-37.

Reimann, H.A. J.Bact., 31: 385, 1936, a.

Reimann, H.A. J.Bact., 31: 407, 1936, b.

Reimann, H.A. J.Bact., 33: 499, 1937, a.

Reimann, H.A. J.Bact., 33: 513, 1937, b.

Reimann, H.A. and Echler, C. J.Bact., 42: 435, 1941.

Saperstien, S., Star, M.P. and Filfus, J.A. J.Gen.Microbiol., 10: 85, 1954.

Schrotter-Kristelli, H.R. Bot.Centr., 61: 33, 1895.

Sesler, C.L., Stahly, G.L. and Brode, W.R. J.Bact., 41: 46, 1941.

Sevag, M.G. and Green, M.N. J.Bact., 48: 496, 1944.

Sevag, M.G. and Swart, E.A. Arch.Biochem., 13: 401, 1947.

Sobin, B. and Stahly, G.T. J.Bact., 44: 265, 1942.

Stahly, G.L., Sesler, C.L. and Brode, W.R. J.Bact., 43: 149, 1942.

Stahly, G.L. Personnal Communication, 1952.

Starr, M.P. and Pirone, P.P. Phytopathology, 32: 1076, 1942.

Starr, M.P. J. Bact., 57: 253, 1949.

Starr, M.P. and Saperstein, S. Bact.Proceedings, page 162, 1952.

Starr, M.P. and Saperstein, S. Sixth International Congress of Microbiology Abstracts of Communication, 1: 169, 1953 a.

Starr, M.P. and Saperstein, S. Arch. Biochem. and Biophys., 43: 157, 1953 b.

Stephenson, M. and Gale, E.F. Biochem. J. 31: 1316, 1937.

Stone, F.M. and Coulter, C.B. J.Gen.Physiol., 15: 629, 1931-32.

Sullivan, M.E. J.Med.Sci., 14: 109, 1905-06.

Swingle, E. J.Bact., 29: 467, 1935.

Takeda, Y. and Ohta, T. Z.physiol.Chem., 258: 6, 1939, a.

Takeda, Y. and Ohta, T. Z.physiol.Chem., 262: 168, 1939 b.

Takeda, Y. and Ohta, T. Z.physiol.Chem., 265: 233, 1940.

Takeda, Y. and Ohta, T. Z.physiol.Chem., 268: 1, 1941.

Topley, W.W.C. and Wilson, G.S. Principles of Bacteriology and Immunity, 3rd Edition, Vol.1. Revised by Wilson, G.S. and Miles, A.A. Edward Arnold Co., London, England, 1946.

Tswett, M. Ber.deut.bot.Ges., 24: 316, 1906.

Turian, G. Helv.Chim.Acta., 33: 12, 1950, a.

Turian, G. Helv.Chim.Acta., 33: 17, 1950, b.

Turian, G. Helv.Chim.Acta., 33: 19, 1950, c.

Turian, G. Helv.Chim.Acta., 33: 1303, 1950, d.

Turian, G. Helv.Chim.Acta., 34: 27, 1951.

van Niel, C.B. and Smith, J.H.C. Arch.Microbiol., 6: 219, 1935.

Vasileva, V.A. Z.Mikrobiol.Epidemol.Immunitatsforch, U.R.S.S., 27: 1, 1941.

Volk, W.A. and Pennington, D. J.Bact., 59: 169, 1950.

Warming, E. Videnskabelige Meddelelser far den Naturhistoriske Forening i Kjobenhavn 1875. Cited: Buchanan and Fulmer 1928.

Watt, D. and Werkman, C.H. Arch. Biochem. and Biophys., 31: 383, 1951.

White, D.E. Aust.Chem.Inst.J.and Proc., 6: 313, 1939.

Zopf, W. Bot.Zeitz., 49: 53, 69, 85, 1889, a.

Zopf, W. Z.wiss.Micro., 6: 172, 1889, b.

Zopf, W. Ber.deut.bot. Ges., 9: 22, 1891.

Zopf, W. Beitr. Physiol.Morphol. Niederer Organismen 2: 3, 1892. Cited: Buchanan and Fulmer, 1928.

APPENDICES.

APPENDIX A.

FORMULAE AND METHODS OF PREPARATION OF MEDIA.

1. Chapman's Medium:

Mannitol	1 per cent
Gelatin	3 per cent
Tryptone	1 per cent
K_2HPO_4	0.5 per cent
Lactose	2.5 per cent
NaCl	7.5 per cent
Agar	1.5 per cent

Autoclaved twenty minutes at fifteen pounds pressure.

2. Fildes' Medium:

Part 1.

Distilled water	550 ml.
dl-Alanine	0.12 gm.
dl-Valine	0.15 gm.
l-Leucine	0.17 gm.
Glycine	0.05 gm.
l-Proline	0.07 gm.
l-Hydroxyproline	0.08 gm.
l-Asparatic acid	0.18 gm.
d-Glutamic acid	0.09 gm.
dl-Phenylalanine	0.08 gm.
l-Tyrosine	0.05 gm

d-Arginine.HCl	0.05 gm.
l-Histidine.HCl	0.05 gm.
l-Lysine.HCl	0.09 gm

The amino acids were dissolved, pH adjusted to 6.8 with M/10 phosphate buffer and volume adjusted to 600 ml. Autoclaved for twenty minutes at fifteen pounds pressure.

Part 2.

The following were added to 60 ml of Part 1 to give a final volume of 100 ml.

$MgSO_4 . 7H_2O$, M/60, autoclaved,	1 ml.
$NaNO_3$, M/5, autoclaved,	1 ml.
$Fe(NH_4)_2SO_4$, M/500 in M/50 HCl, autoclaved	2.5 ml.
Thiamine.HCl, M/2000, filtered. Diluted 1/100.	2 ml.
Nicotinamine, M/200, autoclaved. Diluted 1/10.	2 ml.
dl-Methionine, M/100, autoclaved.	2 ml.
l-Tryptophane, M/1000, autoclaved.	1 ml.
l-Cystine, M/100 in M/50 HCl, filtered.	2 ml.
Distilled water plus compound to be tested.	26.5 ml.

Concentration of phosphate buffer was now approximately M/16. pH of final solution was 6.8. The above solutions were stored in refrigerator at 5°C.

3. Watt and Werkman's Medium:

Peptone	1 per cent
Yeast Extract	0.5 per cent
NaCl	0.5 per cent

Beef Extract 0.5 per cent

Agar 2 per cent

Autoclaved twenty minutes at fifteen pounds pressure.

APPENDIX B.

OPERATION OF EVELYN PHOTOELECTRIC COLORIMETER.

The correct filter was placed in the light beam of the instrument. Next an Evelyn tube containing the blank was placed in the tube holder. The galvanometer lamp was turned on by means of the galvanometer lamp switch, and the galvanometer adjusted to zero by moving the coarse zero adjustor on top of the galvanometer. The colorimeter lamp was then turned on and the galvanometer adjusted to 100 per cent transmission of light or optical density of zero by means of the coarse and fine adjustments on the instrument. The tubes containing the samples were now placed in the instrument and the per cent transmission read directly from galvanometer scale. Per cent transmission converted to optical density by means of the following formula optical density = 2-Log of G, "G" being galvanometer reading.

STANDARDIZATION OF ABSORPTION TUBES.

The absorption tubes used in these experiments were selected 7" x 7/8" round bottom test tubes which had been checked for uniformity of physical dimensions within plus or minus 1 per cent by the manufacturer (Rubicon Co., Philadelphia, Pa.).

Before using these tubes for any accurate work it was necessary to test their uniformity under actual operating

conditions as follows. All tubes were cleaned thoroughly with cleaning solution and 15 ml. of distilled water was placed in each and the lower end of all the tubes were polished with a clean cloth. Using the 10 cc. aperature and a 660 mu filter, the galvanometer was adjusted to exactly 50 with the holder empty. The tubes were then inserted one at a time, rotating each one slowly through one full revolution to determine the position of greatest stability of reading and a diamond pencil mark was made at the upper end of the tube to coincide with the vertical white line on front of the bakelite sleeve (this line was used to locate the tube in the same relative position every time it was placed in the apparatus). All tubes which differed by more than 0.25 divisions from the mean was discarded, as well as any tubes which fluctuated more than 0.5 divisions when rotated through 360 degrees. Tubes which conformed to the above specifications were used in our experimental work.

APPENDIX C.

OPERATION OF THE FILDES-MCINTOSH JAR.

The cultures were placed in a steel jar. The metal lid was put in place and sealed with a beeswax-vaseline mixture. The lid was equipped with two valves, two electric terminals and on the under surface there was an electric light bulb surrounded by a wire net holding asbestos impregnated with palladium black. A suction pump was used to exhaust most of the air in the jar and it was replaced with hydrogen gas. The electric terminals were connected and the electric bulb provided heat to activate the catalytic activity of the palladium black tobring about the union of hydrogen and the remaining oxygen in the jar.