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"SOME ASPECTS OF THE METABOLISM OF
SERRATIA MARCESCENS (BACILLUS PRODIGIOSUS) WITH
SPECIAL REFERENCE TO PIGMENTATION".

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Thesis presented for the Degree of Master of
Science in the University of Sydney.

By

ANTHONY W. LINNANE.
February, 1954.

(From the Department of Biochemistry, University of Sydney.)
The work presented in this thesis was carried out during the tenure of a demonstratorship in the Department of Biochemistry, University of Sydney, during the years 1952-53. Thanks are due to Professor J. L. Still and his staff for helpful advice and discussion.
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INTRODUCTION.

*Serratia marcescens* is the type species of the genus *Serratia* (Breed, 1948). This genus belongs to the order *Eubacteriales*, the family *Enterobacteriaceae* and the tribe *Serrateae*, thus it is closely related to the genera *Aerobacter* and *Escherichia*. Colourless strains of *Serratia* are very difficult to differentiate from some of these coliform organisms.

The *Serratia* are small gram negative aerobic rods, which usually produce a bright red or pink pigment on agar or gelatin media. Gelatin and blood are rapidly liquified, nitrate reduced and milk coagulated and peptonized. Typical species produce carbon dioxide and sometimes hydrogen from glucose and other sugars, as well as formic, acetic, succinic and lactic acids, ethanol, acetylmethylcarbinol and 2:3 butanediol. They are saprophytes living on decaying plant or even animal materials. There are five species in the genus; *S. marcescens*, *S. indica*, *S. plymuthicum*, *S. kilensis* and *S. piscatorum*. The two latter species are distinguishable from the others in that they pigment freely at 37° while the former three will pigment only below this temperature, about 27° being their optimum. *S. plymuthicum* can in turn be separated from *S. marcescens* and *S. indica* as it produces considerable hydrogen when grown on formate broth; the other two produce little or no hydrogen. Finally to distinguish
between S. marcescens and S. indica the organisms are grown on a medium composed of salts, glucose and urea as sole nitrogen source; S. indica grows profusely, while S. marcescens grows poorly or not at all. It will be appreciated that the difference is slight.

Serratia marcescens (E. prodigiosus), by virtue of its spectacular appearance, has been known since the dawn of history (Heffernan 1903, Scheurlen 1896). There is evidence of the early Egyptians having observed the blood red growth of the organism. The most characteristic feature of the metabolism of the bacterium is its synthesis of prodigiosin, a blood red tripyrryl methene, of known structure. The work presented in this thesis was undertaken in an attempt to gain some further insight into the biogenesis of prodigiosin.

Nearly all the work which has been carried out with this organism is referable in some way to pigmentation. The biochemistry of the bacterium is virtually an unexplored field. This review accordingly except for a short section on the known biochemistry of the organism is confined almost exclusively to information relevant to pigment synthesis by S. marcescens and it is divided into seven parts:

1. The inheritance of colour in S. marcescens.
2. Structure and properties of prodigiosin.
3. Possible multiplicity of pigments synthesised.
4. Physico-chemical requirements for growth and pigmentation.
5. Nutritional requirements for growth and pigmentation.
6. Some aspects of the biochemistry of S. marcescens.
7. Summary.
1. **THE INHERITANCE OF COLOUR IN SERRATIA MARCESCENS.**

Some organisms are remarkably constant in their pigmentation, notably the carotenoid producing *Mycobacterium phlei* (Ingraham & Steenbock 1935) in which no colour variants were noted over a three year period. Variants appear however to be the rule in *S. marcescens*, mutants, colourless and of different colours being regularly thrown. Pigmentation by the *Serratia* has been regarded by some as a sign of aged cells, (Moycho 1930, Amako 1930a, Crichton and Lazarus 1948), but this concept is incorrect as these authors failed to consider the nutritional and physico-chemical requirements for chromogenesis. Young 24 hour cultures will pigment intensely if the growth temperature, acidity and the carbon and nitrogen sources are correctly selected (Bunting 1940, Kuntze 1907, Labrum & Bunting 1953).

From the infancy of bacteriology up to the present time *S. marcescens* has been the subject of considerable study as an example of bacterial variation. As early as 1888, Wasserzug published a notable paper, reporting that, when a perfectly red colony was streaked on a gelatin medium not all cells gave red daughter colonies. It was possible by continued replating to obtain colourless colonies that did not commonly revert. Rettger & Sherrick (1911), found that different strains had quite different tendencies to mutate. This was confirmed by Eisenberg (1914) and he also reported no less than seven different colour types — dark-red, red, orange-red, rose, light-rose, white-pink and white. Five different colour types —
dark red, light red, bright pink, pale pink and white were recognized by Bunting (1946), who putting the problem on a quantitative basis found that the rate at which different variations occurred appeared to be relatively constant. The exceptions to this were rare, completely stable white or pale pink strains. Prior to Bunting's experiments Reed (1957), called attention to the fact that colour in the Serratia varied independently of capsulation and of colony structure. The usual chemical mutagens, viz desoxycholate, urethane, pyrogallic acid and nitrogen mustard (methyl bis ($\beta$ chloroethyl) amine hydrochloride), do not induce colour variation (Labrum & Bunting 1953).

It is clear from these reports that when studying the metabolism of S. marcescens care must be exercised to ensure that pure colour strains are used in the various experiments. Every 2-3 months the strain should be checked for its purity of colour and if variants are in evidence, a single colony of the same type as has been used in the worker's earlier investigations selected for the continuation of these studies.

2. STRUCTURE AND PROPERTIES OF PRODIGIOSIN.

Prodigiosin occurs intracellularly, being soluble only in organic solvents. How such a water-insoluble compound is arranged within the cell is a matter for conjecture. A study of its distribution would prove interesting in the light of recent work by Pardee, Schachman & Stanier (1952), who have shown
the photosynthetic bacteria to possess protein particles with which the water insoluble carotenoids and chlorophyll are associated.

The colour of the pigment is influenced by changes in pH; in acid solutions it is red while in alkaline solutions it is an orange colour. Within the living cell the colour of prodigiosin is always red.

Among the first to attempt to isolate prodigiosin in a pure state was Griffith (1892), who reported an empirical formula of $\text{C}_{38} \text{H}_{56} \text{N}_{05}$. This isolate was undoubtedly very impure, for the analysis was carried out on an evaporated alcohol-soluble extract of the organism. Various other workers attempted to isolate the pure pigment without success.

Wrede & Hettehe in 1929 successfully crystallised a derivative of the red dye. During the subsequent five years Wrede and co-workers determined its structure. Prodigiosin itself cannot be crystallised, being a stable red amorphous powder. It was isolated as a crystalline salt of various acids e.g. as a picrate, perchlorate, benzoate, etc. Its empirical formula was shown to be $\text{C}_{20} \text{H}_{25} \text{N}_{0} \text{O}_{4}$ (Wrede 1932). Sodium hydroxide fusion with the pure compound followed by distillation in nitrogen gave an Ehrlich-reacting substance of empirical formula $\text{C}_{10} \text{H}_{17} \text{N}_{4}$ (Wrede & Rothhaas 1933a). It was suggested that the isolated pyrrole was $\beta$-amyl methylpyrrole, $\text{C}_{5}^{\text{H}}_{\text{N}}$. Oxidation of the pigment with hydrogen peroxide in acetic acid led to the isolation of N-methyl methoxy maleinimide (Wrede & Rothhaas 1933a). It was concluded that this degradation product was derived from a methoxylated pyrrole,
viz β methoxy pyrrole. Oxidation with acidic or alkaline permanganate was shown to result in pyruvate formation (Wrede & Rothhaas 1933b). As at this stage only C₄H₅ N was to be accounted for, it was concluded that the pyruvate came from an unsubstituted pyrrole; hence the two following possibilities existed:

![Diagram A]

![Diagram B]

It was not possible by further experimentation to determine which of these two possibilities was the correct formula but the structures of rings 1 and 2 were confirmed (Wrede & Rothhaas 1933c, Wrede 1934). Of the proposed structures B would explain more satisfactorily that fourteen hydrogen atoms were required to hydrogenate prodigiosin and the observed colour changes in acid and alkaline solutions. Until Fischer & Gangl (1940) prepared the first synthetic tripyrryl methene a decision on the structure of prodigiosin had to be postponed. The synthetic tripyrryl methene, unlike prodigiosin, was highly substituted but a comparison of the spectral properties of the synthetic and naturally occurring tripyrryls led Hubbard & Rimington (1950) to conclude that formula B represented
prodigiosin. Both compounds exhibited colour changes in acid and alkaline solutions, their spectra being very similar under the same conditions. When partitioned between various solvent mixtures their distribution was the same.

Prodigiosin has been considered as biologically unique, no other tripyrryl methene being known. A claim has recently been made by Dietzel (1948), that prodigiosin-like compounds are present in a variety of actinomycetes. This claim is based on spectral data and the isolation in crystalline form of a perchlorate derivative of a compound of elemental analysis similar to prodigiosin. This compound $C_25H_{35}ON_3 \cdot HClO_4$, is suggested as being a higher homologue of prodigiosin, $C_{20}H_{25}ON_3 \cdot HClO_4$. Spectral examination of other red actinomycetes led Dietzel to conclude that possibly prodigiosin or similar pigments may have a wide distribution among these organisms.

3. POSSIBLE MULTIPICLITY OF PIGMENTS SYNTHESISED.

When discussing the colouring matter synthesised by S. marcescens the various source books, e.g. Wilson & Miles (1947), Breed, Murray & Hitchens (1948), make no mention of pigments formed except the tripyrryl methene prodigiosin. Evidence is however accumulating that other pigments, (cf. part I), are produced by various strains of this organism.

Three independent groups of observers have noted that a yellow to greenish yellow pigment accumulates within the cells of certain strains of S. marcescens (Ehrismann & Noethling 1936,
Amako 1930a, Linnane & Still 1953). Amako suggested that the yellow pigment was identical with pyocyanin, a phenazine produced by *Pseudomonas aeruginosa*. The data presented by him do not permit such a conclusion to be drawn. A yellow mutant, thrown by a strain normally rapidly producing very considerable quantities of prodigiosin, was isolated by Linnane et al. (1953). This mutant if grown on a solid glycerol-

ammonium citrate-salts medium appeared quite red after 24 hours growth but if observed after 14–16 hours greenish yellow cells could be detected among the red cells. Using the same medium but in liquid form, the strain exhibited a lag in its prodigiosin formation, for after approximately 14–20 hours growth no red was visible but the cells were deep yellow. At the end of this period a red colour began to appear which finally after about 30 hours obscured the yellow pigment.

Variation of the absorption spectra of *n*-butanol extracts of cells of different ages was the subject of an investigation by Weiss (1949). He claimed to show definite spectral changes with the age of the cultures and this he attributed to changes in concentration of various intracellular pigments, which according to his work may be as many as five. Subsequent work of Hubbard & Rimington (1950) has shown that prodigiosin has two different spectral forms depending upon the pH. At pH's approximating 7 both forms can co-exist, while under acid conditions the characteristic red form of prodigiosin exclusively exists, whereas in alkaline solution the sole existing form is orange. Some of the absorption spectra presented by Weiss as representing different pigments may be due to insufficient consideration of the pH
of the solutions being examined, as prodigiosin in its various forms would give similar spectra to some of those obtained by him. However not all the data reported by Weiss can be explained in this manner.

Chromatogrammed extracts of a red strain of S. marcescens were shown to contain two pigments (Bunting 1940). Due consideration was paid to the pH of the extracts and it was noted that one pigment, presumably prodigiosin, was red in acid solution and orange in alkaline solution while the second was red in alkali and dark purple in acid. Bunting (1946) believes that five colony types can be recognised, dark red, red, bright pink, pale pink and white.

A red water-soluble pigment was claimed by Schreiner & Snow (1926) to be produced by a strain of S. marcescens at 37°. In classifying the various species of the genus Serratia, Breed & Breed (1927) state that S. marcescens produces no water-soluble pigments nor does it pigment at 37°. Related species can pigment at 37° and synthesise water-soluble pigments.

4. PHYSICO-CHEMICAL REQUIREMENTS FOR GROWTH AND PIGMENTATION.

(a) Temperature.

The optimum temperature for pigmentation by a microbe is frequently unrelated to that which is best for growth. S. marcescens grows over a wide range of temperatures from about 12° to 44°. As early as 1887 Schottelius reported that at
temperatures above 36° this organism did not synthesise prodigiosin, even though the growth of the organism was otherwise normal. If maintained at 37° for prolonged periods the ability to pigment at any temperature was lost. This observation has since been well substantiated and the optimum temperature for pigmentation suggested as being in the vicinity of 20°. Kasakow & Kotschergina (1933) claimed that the addition of sodium chloride, to a final concentration of 6-8%, to nutrient broth would enable synthesis of pigment by the bacterium to continue at temperatures in excess of 36°. This claim has received scant attention from nearly all subsequent workers. It has however been confirmed in this laboratory. An organism stated to be a strain of S.marcescens and studied by Schreiner & Snow (1926) was shown to pigment at 37°. The pigment formed however was not prodigiosin; for it was water-soluble.

Recently Linnane & Still (1953) demonstrated that the addition of certain long-chain unsaturated fatty acids stimulated S.marcescens to pigment appreciably even up to a temperature of 41°. This effect appears to be a metabolic one for the even numbered saturated acids from acetic through to stearic are without effect, as are the surface active Tweens which are polyoxyethylene sorbitan esters both of saturated and unsaturated fatty acids.

(b) Oxygen Tensions.

The organism as mentioned earlier is a facultative aerobe growing poorly under anaerobic conditions. The failure of some authors to obtain anaerobic growth may be attributed
to deficient media. Ritter (1900), showed that the nutritional demands for anaerobic growth are more exacting than those for aerobic growth. He found that a peptone - salts medium supported growth aerobically but not anaerobically. The inclusion of glucose was necessary for anaerobic growth.

Schottelius in 1887 demonstrated that highly aerobic conditions are required for pigmentation. Wasserzug soon after in 1888, observed that in liquid culture *S. marcescens* gave a smaller yield of pigment than when grown on solid gelatin media. This he realised was due to the low oxygen concentration in the first case as compared with the second. If cultivated in narrow test tubes it is noted that only about the top half inch of liquid is coloured, the rest of the growth being quite colourless. Often a lag period before the onset of pigmentation will be noted when the organism is grown on liquid media.

It has frequently been recorded that a shallow layer of paraffin oil placed over growth media results in a colourless growth. (Beguet 1928, Amako 1930). Goldsworthy & Still (1936) found that increasing the percentage of oxygen in the atmosphere was not beneficial to growth or pigmentation, an atmosphere of 50% oxygen permitted no increase in pigmentation or growth.

(c) **The Effect of Light.**

Ultraviolet light of various wave lengths, unless for short exposure periods, is lethal to *S. marcescens* as it is for probably all micro-organisms. For most organisms the wave length
2650Å is the most lethal, however 2801Å was found to be so for *S. marcescens* (Porter, 1947).

Novelle (1953) has examined the effect of ultraviolet light of wave length 2537Å on cells which have lost their power to pigment. When colourless cells grown on nutrient broth were exposed for 1-30 seconds some red pigmentation followed. Pigmentation was poor if cells were grown on nutrient broth plus chlorophyll; if however they were exposed to ultraviolet light, excellent prodigiosin production was observed on incubation for a further 24 hours. It is difficult to evaluate the significance of these results. They could perhaps be related to a photochemical decomposition of chlorophyll followed by utilisation of pyrrole decomposition products for prodigiosin synthesis.

Kreitlow (1941), has made a number of observations on the effects of the different visible wave lengths on pigmentation. Cells exposed to red light showed increased pigmentation, while green light was without effect. When the organism was continually subjected to blue light, it grew but lost the power to pigment.

Bright red cultures exposed to sunlight were reported by Amako (1930a), to become dark violet and non viable.
(d) **Hydrogen Ion Concentration.**

Only a few investigations into the influence of pH have been carried out and these have but slight significance due to inaccuracies in the estimation of pH. The organism grows optimally at pH 6.0-7.0 but it will grow over a wide range of pH values, from about 4.5-9.0; (Porter 1947).

Most researchers, even the very early ones, are agreed that a weakly acid medium favours pigment production, (Bunting 1946, Dewey & Poe 1945, Wasserzug 1888 and Hefferan 1903). Carbohydrates are uniformly recommended for pigmentation and Kuntze (1907), suggested that the beneficial effects observed are due to acid production. Cultivating his organisms on a medium of ammonium succinate, glycerol and salts, he noted that the omission of glycerol led to poor pigmentation, the medium becoming alkaline; the regular addition of small amounts of acid in place of the glycerol, to maintain an acid pH resulted in good pigmentation. These findings were extended to include a study of a number of carbohydrates, in media maintained at weakly acid or alkaline pH; in all cases the acid media favoured prodigiosin synthesis.

(e) **Surface Tension and Osmotic Pressure.**

Beguet (1928), using sulphoricinoleate to lower the surface tension of gelatin media on which *S. marcescens* was growing, found that as the tension was lowered the colour intensified. The colour of the growth at a surface tension
of 45 dynes/cm. was carmine red, while at 30 dynes/cm. it was violet to dark red with diffusion of pigment into the gelatin mass. This is contrary to the findings of Bunting (1950), who observed that there was a tendency for the paler variants of S. marcescens to increase after logarithmic growth had ceased and that this effect was augmented by detergents such as sodium lauryl sulphate. The detergents used by Bunting showed no preferential toxic effects on the red cells and their effect was shown to be selective and not mutagenic. When cells were allowed to age in the presence of the surface active agents there was no evidence of the production of mutants. These findings were reiterated by Labrum & Bunting (1953).

At a temperature of 16°, according to Beguet, increasing concentrations of sodium chloride up to 6%, led to a gradual falling off in the intensity of the colour produced, from reddish violet to carmine red and finally pale pink. Kasakow et al. (1933), made no observations at temperatures below 38° but they demonstrated that the organism grown at that temperature on nutrient broth could be induced to pigment by the addition of high concentrations of sodium chloride. This effect was only observed with final salt concentrations of 5-8%. So little is known of the biosynthetic mechanisms involved in prodigiosin synthesis that it is impossible in the light of our present knowledge to visualise what specific effect osmotic pressure can be having on the metabolism of the organism.
5. **NUTRITIONAL REQUIREMENTS FOR GROWTH AND PIGMENTATION.**

The nutritional requirements of *S. marcescens* for growth are of the simplest. Ammonium ions serve as a source of nitrogen, three carbon atom compounds such as lactate or glycerol are adequate carbon sources and the inorganic requirements are sulphate, phosphate, magnesium and iron. If in addition to growth abundant pigmentation is desired, then the nutritional demands are more exacting. As Hefferan (1904), noted, there is little correlation between growth and pigmentation. The best media for growth and pigmentation will be discussed under two headings:

(a) Nitrogen and carbon sources.

(b) Inorganic requirements.

(a) **Nitrogen and Carbon Sources.**

There have been no extensive researches on the influence of nitrogen source on pigmentation. Most media upon which it is desired to cultivate *S. marcescens* for pigment production have ammonium ions as their nitrogen source, (Bunting 1946, Dewey *et al.* 1943, Kuntze 1907, Samkow 1904). Peptone has been employed for the dual purpose of growth and chromogenesis, (Goldsworthy & Still 1936, Bunting, Robinow & Bunting 1949). Single amino acids, (Amako 1950b) and pairs of amino acids, (Goldsworthy *et al.* 1936), have not been successfully used as sole nitrogen and carbon sources for growth. Weinberg (1951), found glutamic acid (0.25%) inhibited strains actively pigmenting on a glycerol-ammonium salts medium. A specific requirement of a
particular strain for \( \alpha \) amino nitrogen for the synthesis of prodigiosin was observed by Waring & Werkman (1942). Hubbard et al. (1950), have since shown, using isotopes, that the nitrogen \( \alpha \) and \( \alpha \) carbon atoms of glycine are directly concerned in prodigiosin synthesis. Asparagine proved to be a useful source of carbon and nitrogen for growth but not for pigmentation; (Sullivan 1905).

Generally it appears that if a high \( \frac{C}{N} \) ratio is provided by the medium the nitrogen source is not critical for growth or pigmentation. This is valid only when there is no physiological interaction between the growth substrates. An example of this is that a peptone-inorganic salts medium is satisfactory for growth and pigmentation; (Goldsworthy et al. 1936), as is a glycerol-citrate-salts-medium; (Bunting 1946) but a medium containing peptone-glycerol-phosphate results in little or no chromogenesis; (Bunting et al. 1949). The latter authors found that the omission of the phosphate from the peptone-glycerol-phosphate medium or else incorporating only low peptone concentrations, viz. 0.1%, enabled their strains to become highly coloured. Similarly Goldsworthy & Still (1933), noted that the addition of meat extracts to peptone media was inhibitory to pigmentation. The magnitude of this effect varied with the strain and the source of the extract.

The most comprehensive survey of the compounds which will enable \( S. marcescens \) to grow was made by den Dooren de Jong (1926). The survey included organic acids, alcohols, pentoses, hexoses, hexitols, amides, amines, amino acids and various miscellaneous compounds.
Attempts to utilise one and two carbon atom compounds as sole carbon sources for growth have been unsuccessful (Amako 1930b, Kuntze 1907, Sullivan 1905). The simplest sole carbon sources that can support growth and pigmentation contain three carbon atoms e.g. glycerol and lactate.

The organism grows well on most of the sugars but pigmentation is variable. Most authors are agreed that glucose as a carbon source is adequate for growth but undesirable for prodigiosin synthesis (Goldsworthy et al. 1936, Porter 1947, Sullivan 1905). This was attributed by Bunting et al. (1949) to the high acidity which developed but work in this laboratory suggests otherwise (Linnane et al. 1953). The latter workers found that the pH of media containing glycerol became just as low as similar media containing glucose. Wasserzug (1888) was of the opinion that sugars were essential for pigmentation. Kuntze (1907) disagreed, maintaining that, if the sugars were replaced by an organic acid together with regular additions of mineral acid to maintain an acid pH, then pigmentation would proceed optimally. Glycerol almost without exception is regarded as the best single carbon source (Sullivan 1905). Combinations of sugars and organic acids, such as citric and succinic, have proved the most successful (as media) for the dual purpose of growth and pigmentation (Bunting 1946, Sullivan 1905, Kuntze 1907, Dewey at al. 1943). Organic acids supported growth but the amount of colour produced by the cells was variable.
(b) **Inorganic Requirements.**

Much of the initial work on the inorganic requirements of *S. marcescens* for growth and pigmentation was performed without appreciation of the roles of sulphate, phosphate and metallic ions in bacterial nutrition.

Using chemically defined media devoid of organic sulphur and phosphorus Kuntze (1907), also Dewey et al. (1943), showed that as well as a requirement for growth additional sulphate and phosphate were required for pigmentation. There are various earlier reports of these two anions being dispensable for growth but these workers failed to appreciate the small concentrations required and the impurity of their chemicals. Some sulphur and phosphorus compounds must have been present for any growth to occur.

Most researchers are agreed that magnesium had an essential role in pigmentation, for its presence is unconditionally required (Bunting 1940, Samkow 1904). This is also true of iron but agreement has not been universal. Iron is required only in very low concentrations, of the order of 0.050 mg/l for growth and 0.2 mg/l for growth and pigmentation. It was not until Waring et al. (1942) developed a satisfactory method of removing the last traces of iron from media that this could be established.

A claim that zinc increases prodigiosin synthesis has been made (Bortels 1927). This has not been confirmed by other researchers.

Several investigators have observed that calcium in low concentrations is beneficial, while higher concentrations,
6. SOME ASPECTS OF THE BIOCHEMISTRY OF SERRATIA MARCESCENS.

A most thorough investigation of the products resulting from the fermentation of glucose by *S. marcescens* has been made by Neish, Blackwood, Robertson & Ledingham (1947, 1948). Two temperatures, 30° and 35°, were selected for study, also the fermentation was carried out under aerobic and anaerobic conditions. Three strains were examined and, although the percentages of the various compounds formed were not the same under all conditions, the same compounds were found. The most striking differences in the amounts found were with formate, acetoin and acetate, the concentration of the former two varying with the oxygen tension, while the accumulation of the latter was considerably influenced by the growth temperature. The products detected by Neish and co-workers were: acetoin, 2,3 butanediol, glycerol, ethanol, lactate, formate, acetate, succinate, carbon dioxide and sometimes traces of hydrogen. Nothing is known of the enzymes or mechanisms involved in carbohydrate dissimilation by *S. marcescens*.

The enzymes for the oxidation of fatty acids were shown by Silliker & Rittenberg (1951a), to be constitutive for a number of bacteria but adaptive for *S. marcescens*. A second paper (Silliker et al., 1951b), dealt with the nature of the adaptation and possible intermediates involved in the oxidation.

The assimilation of ammonia and the fixation of carbon.
dioxide has been investigated, (McLean & Fisher 1947, McLean & Purdie 1952). Ammonia uptake was accompanied by increased oxygen consumption, 2.2 oxygen atoms being taken up for each nitrogen atom assimilated. While fixation of carbon dioxide on a glycerol-ammonium salts medium was considerable, it fell to 6% of the maximum upon the addition of asparagine, arginine, uracil, adenine and guanine. Each compound separately replaced a definite proportion of the total carbon dioxide fixation.

Miscellaneous information has been collected by Porter (1947) on the biochemistry of the organism. The occurrence of a number of the enzymes being recorded, e.g. catalase, peroxidases, aspartase and a lecithinase have all been recognised in *S. marcescens*.

The data available on the biochemistry of the bacterium is seen to be indeed meagre and any contributions to this field would be of value.

7. **SUMMARY.**

The exact chemical structure of one pigment from *S. marcescens* has been determined. There is evidence that others occur. These have not been isolated and characterised.

A considerable amount of work has been performed on the physiology of pigment formation by the bacterium. The effect of pH, oxygen tension, temperature, light, surface tension, osmotic pressure and various media on chromogenesis has been studied in some detail. In the light of modern concepts much of this work
is however of little value.

Compared with some other aerobes very little is known of the biochemistry and intermediary metabolism of this organism. The fermentation products of glucose have been determined, some enzymes present recognised, assimilation and carbon dioxide fixation have been investigated and lastly some study of the oxidation of fatty acids has been initiated.

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The foregoing survey of the literature reveals that contributions to the biochemistry of _S. marcescens_ and any additional information regarding pigmentation would be of considerable value. Aspects of both of these fields were investigated. The experimental work carried out has been divided into three parts:

Pigment production by _Serratia marcescens_ at elevated temperatures and the effect of supplements of unsaturated fatty acids on pigmentation at 27° and 37°.

Some aspects of the metabolism of glycerol and related compounds by _Serratia marcescens_.

Some observations on the number of pigments synthesised by _Serratia marcescens_.

(Handwritten notes: Need to work on Ch. 4 and Part II; it needs additional work to Part II.)
CHAPTER II.

PIGMENT PRODUCTION BY SERRATIA MARCESCENS AT ELEVATED TEMPERATURES AND THE EFFECT OF SUPPLEMENTS OF UNSATURATED FATTY ACIDS ON PIGMENTATION AT 27° AND 37°.

INTRODUCTION.

Although the growth of, and pigmentation by, S.marcescens are independent the nutritional requirements for both are very simple and similar. Thus the use of different media appears to offer little hope of gaining much insight into the biogenesis of prodigiosin. Another approach to the problem must be made. The use of isotopes and biochemical mutants are the two main methods of approach left open to the biochemist seeking information as to the chain of reactions involved in the biosynthesis of a compound.

Using isotopes, Hubbard et al. (1950) have shown that the nitrogen atom and the α carbon atom of glycine and both carbon atoms of acetic acid are directly implicated in prodigiosin synthesis. Apart from this nothing is known of the biosynthesis of prodigiosin; indeed this is virtually all that is known of pyrrole synthesis of any kind (Shemin & Wittenberg, 1951). The simplest approach to the problem at hand would have been to follow similar lines of experimentation but this was not possible as isotopes were unavailable.

Deficient mutants have been extensively used in bacterial problems, being employed in the study of the biosynthesis of essential nutrients. This technique utilises the genetical blocking of reaction chains involved in the synthesis of some essential nutrients. Investigations on the genetic control of biochemical reactions in Neurospora crassa (Beadle, 1945)
led to the concept that a one to one relation exists between biochemical reaction and gene. Thus alteration of a single gene will block a single reaction. It is possible to obtain mutants all requiring the same end product for growth, in which different consecutive steps in the synthesis of the end product have been blocked. This makes possible a step-wise study of the biosynthesis of essential growth requirements. The genetical approach to our problem does not at first seem possible as prodigiosin is not essential to the economy of \textit{S. marcescens}.

It is not possible to obtain mutants requiring an unessential compound such as prodigiosin for growth. However, according to Lederberg (1951), the failure of \textit{S. marcescens} to pigment at 37\textdegree{} is a genetic phenomenon. Theoretically it should then be possible to induce pigmentation at 37\textdegree{} in a strain of the organism capable of pigmenting below 37\textdegree{}, provided the precursor whose synthesis is blocked or retarded is added to the growth medium. The problem is not a simple one, for it is regarded as characteristic of \textit{S. marcescens} (Breed, 1948), that it does not pigment at 37\textdegree{}. Any compounds selected for addition to media would have to be of a different nature from those present in the usual standard bacteriological media. This became obvious when a basal medium composed of glycerol, ammonium citrate and salts enriched separately and together with yeast extract, tryptic digest of casein, ox heart extracts and peptone supported pigmentation at 27\textdegree{} but not at 37\textdegree{}.

An opportunity to study the effect of the addition of different types of compounds to media presented itself in the form of a mutant. This mutant normally pigmented red if
incubated for 20 hours or more at 27°, but observation at the
end of 16 hours showed that it was an intermediate yellow colour. Y
Closer examination revealed that the yellow colour always
preceded the formation of any red pigment and that it also was
not produced at 37°. This suggested that it might be possible
to induce prodigiosin synthesis at 37° by the addition of a
preparation of the yellow pigment to growth media; and indeed
it was found that the yellow pigment did appear to induce some
red pigment synthesis. Subsequently it was shown that long-
chain unsaturated fatty acids contaminating the crude yellow
pigment had an even greater effect on prodigiosin synthesis at
37° than the yellow pigment. This section of the thesis deals
mainly with the effect of unsaturated fatty acids on pigmentation
both at 27° and 37°.
EXPERIMENTAL.

Organisms.

The stock cultures were maintained on a solid medium suggested by Bunting (1940). All the strains of S. marcescens used in this work were isolated locally. The red and white races were isolated from single pigmented or colourless colonies. The colourless strains were very stable, only occasional coloured variants being observed over a two year period. Periodically the coloured strains were plated out and a single pigmented colony selected from each strain for its maintenance. This was necessary as from time to time the pigmenting organisms throw a considerable number of colourless variants. Except where otherwise stated a strain denoted "C" (red), which shows prolific growth and pigment production at 27°C, was used.

Estimation of Growth and Pigmentation.

As only gross differences were being sought, for the purpose of these experiments it was sufficient to estimate both the growth and pigmentation visually; in both cases, − = none, tr. = trace, + to ++++ gradations to maximum.

Method of Cultivation.

The organisms were grown in liquid medium in two ways, either in Roux bottles or else in 6" x ½" test tubes. Where Roux bottles were used the conditions were highly aerobic as the liquid layer was very shallow, and when the organisms were producing a maximum of pigment the growth had the appearance of a thin layer of blood. Conditions of restricted oxygen tension exist where the organisms are grown in test tubes, for the tubes
contain about 7.0 ml of medium which is approximately 3" in depth. Even at 27º the amount of pigment produced during growth in these tubes is small compared with the more aerobic Roux bottle growth.

Since the growth and pigmentation were assayed visually, as a standard procedure when an assay was being carried out at 37º a control bottle or tube was also incubated at 27º and the amount of growth and pigmentation at 37º was estimated relative to this.

Yeast Extract.

Dried bakers' yeast was steamed for 20 minutes with twice its volume of water and then filtered. The filtrate was autoclaved and used as the extract.

Tryptic Digest of Casein.

This was a 3% casein hydrolysate prepared as described by Cole (1942).

Nutrient Broth.

Two different broths were prepared. One contained a local commercially available meat concentrate marketed by the Riverstone Meat Company, Sydney and the other an extract of fresh ox heart obtained by steaming the meat for 30 minutes with an equal volume of water. The composition of the broths thus became:

Meat Concentrate Medium.
Peptone, 10 g.
Riverstone meat extract, 5 g.
Water to 1L. pH adjusted to 7.2.

Fresh Beef Heart Medium.
Peptone, 10 g.
Sodium chloride, 5 g.
Aqueous extract of fresh ox heart 500 ml.
Water to 1L. pH adjusted to 7.2.
Bunting's Medium.

Glycerol, 10 g.
Citric acid, 5 g.
Dipotassium hydrogen phosphate, 7.6 g.
Sodium chloride, 0.5 g.
Magnesium sulphate heptahydrate, 0.5 g.
Ferric ammonium citrate, 0.05 g.
Water to 1 L.
N NH₄OH used to adjust the final pH to 6.8.

If a solid medium was desired, agar (1.5%) was added.

Fatty Acids.

Samples of oleic, palmitic and stearic acids were about 97% pure. Other acids used were purchased from British Drug Houses Ltd., London, and were nearly all of a technical grade.
RESULTS AND DISCUSSION.

Exploratory Experiments at 27° and 37°.

These experiments were designed to show that the usual standard bacteriological media support pigmentation by *S. marcescens* at 27° but not at 37°.

Goldsworthy et al. (1938), have shown that, while growth is normal on a variety of nutrient broths, not all support pigmentation even at a temperature of 27°. This earlier work was confirmed; pigmentation occurred with the fresh beef heart medium at 27° but not at 37°, while the organism remained colourless irrespective of the temperature with the meat concentrate medium.

Weinberg (1951) has reported that a 1.0% casein hydrolysate inhibits pigmentation at 27°, but that more dilute preparations do not. With the present strain a 3.0% casein hydrolysate did not support pigmentation but a 0.3% hydrolysate supplemented with Bunting's medium did permit prodigiosin synthesis to occur but only at 27°.

An aqueous extract of yeast enriched with glucose (0.5%) also allowed pigment synthesis only at 27°.

Red strains regularly throw white variants and although Bunting's medium tends to stabilize the red strains at 27° colourless growth results from cultivation at 37°. Supplements of tryptic digest of casein, nutrient broth and yeast extract to Bunting's medium tended to increase the growth of the organism as compared with the unsupplemented medium but pigmentation still occurred only at the lower temperature.
The results of this series of experiments conform with the experiences of other workers. Growth and pigmentation are clearly independent. The necessity for thoughtful selection of media when it is desired to obtain coloured growth is indicated.

The Induction of Pigment Synthesis at 37°.

As mentioned earlier this study was initiated by the discovery of a mutant strain of *S. marcescens* which became red only after passing through an intermediate yellow stage. This strain in the rest of the thesis will be referred to as "P" (yellow). It was considered likely that this yellow pigment was an intermediate in prodigiosin synthesis whenever the yellow was observed, subsequently the organism turned red. Since the strain was colourless at 37° the inability of *S. marcescens* to pigment at this temperature might be due to the blocking of the reaction chain involved in red pigment synthesis prior to the yellow stage. The isolation of the yellow compound(s) was undertaken in order to add it to media in the hope of inducing pigment synthesis by *S. marcescens* at 37°.

Large quantities of the yellow mutant were accumulated and the cells exhaustively extracted with ethanol. The yellow extracts were then partially purified by partition between a member of different solvents and finally by adsorption chromatography. The procedures followed in the purification are described in detail in Chapter IV of this thesis. It was soon evident that a complex mixture of coloured compounds was being investigated. The yellow compounds were all soluble in various organic solvents, some dissolved in dilute NaOH, all were insoluble in water and mineral acid solutions. A number of different
preparations of the yellow compounds of varying purity were suspended in dilute NaOH and added to Bunting's medium. When strain "C" (red) was grown on this at 37°C, the results were promising as all fractions showed some pigment-promoting property but not as much as was desired. The comparatively poor response was attributed to a number of different factors. There may have been slow assimilation of the yellow compounds. Due to their limited solubility in alkaline aqueous solutions they may not have been readily available to the cell at its site of pigment synthesis. There may have been an inhibition of prodigiosin synthesis as the added pigments were not pure, containing by virtue of the method of preparation various lipides. Kodicek (1949) has shown certain lipides to be inhibitory to growth and conceivably they could also inhibit pigment synthesis. Controls were therefore set up to test the effect of various fats on the pigmentation of strain "C" (red) at 27°C and 37°C (cf. Table I).

The results were most surprising. Intense pigmentation took place under the influence of the added fats at both temperatures. The organism growing at 27°C on Bunting's medium alone was not as highly coloured as some of the growths at 37°C. It is to be appreciated that the conditions of the assay are not sufficiently aerobic for optimal pigmentation at 27°C as the assay is performed in a depth of medium contained in test tubes. It is not possible to determine whether the colour observed at 37°C in the presence of the added yellow compounds results from these compounds or from contaminating fat. The purification of the yellow compounds proved to be more difficult than was anticipated.
TABLE I.
The Effect of Complex Lipides and a Crude Mixture of Pigments Isolated from a Yellow Mutant on Pigmentation at 27° and 37°.

<table>
<thead>
<tr>
<th>INCUBATION PERIOD</th>
<th>20 Hours</th>
<th>Pigmentation</th>
<th>48 Hours</th>
<th>Pigmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADDITIONS</td>
<td>Growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27°</td>
<td>Nil</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>NaOH</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Lard</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Peanut Oil</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>37°</td>
<td>Nil</td>
<td>+++</td>
<td>—</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>NaOH</td>
<td>+++</td>
<td>—</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Lard</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Peanut Oil</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>Castor Oil</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Crude mixture of yellow compounds isolated from &quot;P&quot; (yellow).</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

Incubation temperature as indicated. The assay was performed in test tubes (see experimental section). The tubes contain 7.0 ml of Bunting's medium and several drops of a thick suspension of strain "C" (red) so that initially the solution is slightly turbid with little or no detectable colour. The lipide and pigment additions were 0.4 ml of 1.0% suspensions in NaOH (1%). NaOH added was 0.4 ml of a 1% solution. Where additions were made the initial pH approximated 7.3, otherwise it was 6.8.

and only very small amounts were available (about 0.5g of a crude mixture of pigments from 1kg. wet weight of cells). These factors and the insolubility of the pigments in water, led to the abandonment of this approach to pigmentation at 37° in favour of a more detailed study of the observed fat effect.
Specificity of the Fat Effect.

A number of different complex lipides are active in inducing pigmentation at 37°; of these lard appears to be the most effective. As the lipides assayed differed considerably in their activity and in their fatty acid composition (Table 2), it was thought that the different component acids might account for the observed difference in activity. Accordingly a number of fatty acids were investigated for pigment promotion (cf. Table 3).

TABLE 2.
Percentage of Fatty Acids of Lard, Peanut Oil and Castor Oil from Maynard (1947) and Hilditch (1940).

<table>
<thead>
<tr>
<th>FATTY ACIDS PRESENT</th>
<th>Lard</th>
<th>Castor Oil</th>
<th>Peanut Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Palmitic</td>
<td>25</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>Stearic</td>
<td>13</td>
<td>0.3</td>
<td>4</td>
</tr>
<tr>
<td>Arachidic</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dihydroxystearic</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Oleic</td>
<td>54</td>
<td>7</td>
<td>61</td>
</tr>
<tr>
<td>Linoleic</td>
<td>7</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>Ricinoleic</td>
<td>-</td>
<td>88</td>
<td>-</td>
</tr>
</tbody>
</table>

Various small amounts of other acids are also present.

The even numbered saturated fatty acids from acetic through to stearic were found to be without influence on pigmentation as were azelaic propionic and ω hydroxy butyric acids. Valeric acid was inhibitory to growth. In striking contrast to these, the long chain unsaturated acids erucic, oleic,
linoleic and ricinoleic enabled the organism to pigment at 37°. These four unsaturated acids all appeared to be equivalent, at

TABLE 3.

Unsaturated Fatty Acids Inducing Pigmentation at 37°.

<table>
<thead>
<tr>
<th>INCUBATION PERIOD</th>
<th>20 Hours</th>
<th>48 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>Pigmentation</td>
</tr>
<tr>
<td>NaOH 27°</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>NaOH</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Valeric acid</td>
<td>tr.</td>
<td></td>
</tr>
<tr>
<td>Acetic, propionic,</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>butyric, caproic,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>caprylic, lauric,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>myristic, palmitic and steaeric acids.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crotonic &amp; β hydroxy butyric acids</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Azelaic acid</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Δ9° Undecenoic acid</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Ricinoleic acid</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>Erucic acid</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Incubation temperature 37° except where indicated. All additions were 0.4ml of 0.5% suspensions in NaOH(1%). Other details as given in legend for Table I.

least at the concentration assayed. It followed that the difference observed between the complex lipides was probably not a reflection of their composition so much as to their age as none of them were fresh and varying amounts of free fatty acids would have been
present. There existed however a certain specificity in the requirements for unsaturated fatty acids, as the presence of added crotonic and undecenoic acids did not effect the course of pigmentation by the organism at 37°. There were no other unsaturated fatty acids available to us to assay.

The four acids active in promoting pigmentation at 37° were all assayed at the final concentration of approximately 300μg/ml. Except for the oleic acid (see experimental section) the active acids were very impure and contamination could have conceivably explained their activity. Accordingly the four acids were assayed at final concentrations of 10-600 μg/ml. All showed little activity below 80 μg/ml; optimum pigmentation occurred at concentrations of 200-400 μg/ml. This made the likelihood of a contaminating chemical being the active agent improbable, unless it also contaminated the highly purified oleic acid.

The four acids erucic, oleic, linoleic and ricinoleic are equivalent in promoting pigmentation by strain "C" (red) of S. marcescens. Other red strains were next examined as to the effects of the fatty acids on their pigmentation and Table 4 shows that the four acids are approximately equivalent for all the red strains. All of the strains do not however pigment at 37° to the same extent as strain "C" (red) in the presence of these acids. The concentration of the acids required for optimal pigmentation was again about 200 μg/ml.

Researches by Williams, Broquist & Snell (1947), on Lactobacillus bulgaricus, which requires oleic acid for growth,
have shown that the acid is utilised only over a narrow pH range. At pH 5.0 - 6.0 and above 7.0 the growth response was negligible, while at pH 6.5 growth was optimal. This work suggested that the high concentrations of unsaturated fatty acids required by _S. marcescens_ for pigmentation at 37° might be a function of the initial pH of the growth medium and that at different pH values smaller concentrations of these acids might suffice. To investigate this possibility samples of

**TABLE 4.**

Pigmentation by a Number of Strains of _S. marcescens_ at 37°.

<table>
<thead>
<tr>
<th>ADDITIONS</th>
<th>NaOH</th>
<th>Oleate</th>
<th>Linoleate</th>
<th>Ricinoleate</th>
<th>Erucate</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRAIN</td>
<td>Pigmentation at the End of 22 Hours.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;C&quot; (red)</td>
<td>—</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>&quot;N&quot; (red)</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>&quot;D&quot; (red)</td>
<td>—</td>
<td>+++</td>
<td>++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>&quot;E&quot; (red)</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>&quot;J&quot; (red)</td>
<td>—</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>&quot;P&quot; (red)</td>
<td>—</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>&quot;B&quot; (red)</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>&quot;H&quot; (red)</td>
<td>—</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>&quot;L&quot; (red)</td>
<td>—</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>&quot;S&quot; (red)</td>
<td>—</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

Incubation temperature 37°. The growth at the end of 22 hours approximated +++ in all cases. All additions were 0.4ml of 0.5% suspensions in NaOH (1%). Other details as given in the legend for Table I.

Bunting's basal medium were adjusted to pH 5.0, 6.0, 6.5, 7.0,
7.5, 8.0 and an aqueous suspension of oleic acid was added to all in final concentrations ranging from 50 μg to 200 μg/ml. Pigmentation by S. marcescens at 37° was optimal at pH 7.5 and at pH values below 7.0 there was little pigmentation. The critical nature of the initial pH is thus evident. The concentration of unsaturated acid required was not influenced by the pH, 200 μg/ml of oleate still being required at pH 7.5 for optimal pigmentation. The procedure adopted in the initial assay, (cf. Table I), of making any additions in NaOH (1%) was henceforth continued as a routine. Thus the initial pH of all assay tubes was approximately pH 7.3.

**Possible Mechanism of Fat Effect.**

An explanation of the observed effect of unsaturated fatty acids on pigmentation must account for the inactivity of the saturated fatty acids. A survey of factors involved in the metabolism of fatty acids suggested a number of possible mechanisms for the observed fat effect. A summary of relevant data from the survey and the results of the investigation of various possibilities are included here.

(a) **Synthesis and oxidation of fatty acids.**

Comparatively little is known of the metabolism of the fatty acids mainly because of the difficulties inherent in their detection and estimation.

The most vigorously investigated aspect of the metabolism of the fatty acids has been the mechanism of their oxidation. This oxidation is now realised to be very complex. Recently
Green & Mii (1953) have formulated the steps involved in the oxidation of the saturated fatty acids. The initial reactions led to the formation from free fatty acids, of acyl coenzyme A derivatives which are then oxidised to \(\beta\) keto acyl derivatives. This oxidation is followed by coenzyme A-dependent cleavage of the \(\beta\) keto acyl compound to acetyl coenzyme A and an acyl coenzyme A with two carbon atoms less than the parent \(\beta\) keto acid. This sequence of reactions is repeated until the saturated fatty acid has been completely converted to acetyl coenzyme A, which is then oxidised per medium of the citric acid cycle.

It may be inferred from a report by Kennedy & Lehninger (1950) that the unsaturated fatty acids follow a similar pathway. Oleic acid oxidation by rat mitochondria was found to require the same co-factors as did various saturated acids.

Evidence presented by Stansley & Beinert (1953) suggests that the biosynthesis of saturated fatty acids is essentially the reverse of their method of oxidation as described by Green & Mii. Opinions are divided as to the pathway of biosynthesis of the unsaturated fatty acids. Artom (1953), adheres to the older idea that dehydrogenation of saturated fatty acids account for their synthesis, while Block (1948), on the basis of isotope studies, believes a separate unknown process is involved in the biosynthesis of the unsaturated acids.

The distinction between the unsaturated and saturated fatty acids on the basis of their pathways of oxidation and synthesis is seen from the foregoing information to be slight.
It was considered that this date did not suggest any investigation, undertaken by us, on this aspect, would yield results relevant to pigment synthesis by \textit{S. marcescens} at 37°.

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Nutritional studies, particularly \underline{microbial work}, have clearly shown that the saturated and unsaturated fatty acids have different \underline{biological functions}. Various unsaturated acids are essential growth factors for some animals and bacteria but as yet no such requirement for the saturated acids has been found (Burr & Burr, 1929, 1930. Williams, Broquist & Snell, 1947).

(b) \underline{Pyridoxine and polyunsaturated fatty acids.}

The di- and tri-unsaturated acids linoleic and linolenic are essential growth factors for the rat, being required respectively for the synthesis of the tetraenoic and hexaenoic fatty acids (Widmer & Holman, 1950). More recently Witten & Holman (1952) have demonstrated that pyridoxine is involved in the synthesis of tetra- and hexa-enoic acids. These researchers also showed that oleic did not contribute to the formation of these polyunsaturated acids.

\underline{As oleic acid was active in promoting pigmentation by \textit{S. marcescens} at 37° and boiled yeast extract, which would be rich in pyridoxine, failed to promote such pigmentation, it was concluded that polyunsaturated fatty acids were not involved in pigmentation.}
(c) **The effect of biotin.**

Williams & Fieger (1946) discovered that the biotin requirements of various lactobacilli could be replaced by \( \Delta^9 \) octadecenoic acid (oleic acid). Various saturated fatty acids assayed could not duplicate this effect. Subsequently Cheng, Greenberg, Deuel & Melnick (1951) reported that at least five octadecenoic acids could emulate the oleate effect. It was also noted that the ability of these various acids to replace biotin decreased as the double bond became more remote from the 9-10 position, until finally no growth resulted when \( \Delta^{7-8} \) octadecenoic acid was substituted for biotin. The comparatively non-specific nature of this nutritive requirement for unsaturated acids is similar to the demand for unsaturated fatty acids by *S. marcescens* for pigmentation at 37°. The influence of biotin on such pigmentation was therefore investigated \( (\text{cf Table 5}) \) and found to be without effect. Similarly pimelic acid, a seven carbon dicarboxylic acid shown by Mueller (1951) to be a precursor of biotin was also without effect as were the dicarboxylic acids, adipic and sebacic.

(d) **The effect of lipoic acid.**

Oleic acid, independently of biotin, was shown by Guirard, Snell & Williams (1946) to replace the growth requirements for acetate of some lactobacilli. Later the new growth factor lipoic acid, present in acid hydrolysates of yeast, was found to substitute for both the oleic and acetic acids (Reed, De Busk, Johnston & Getzendaner 1951; Reed, De Busk, Gunsalus & Hornberger 1953).
It was inferred from these reports that lipoic acid might be involved in pigment synthesis by *S. marcescens* at 37°. Table 5 shows that an acid hydrolysate of yeast (prepared as described by Reed et al., 1951) and therefore presumably lipoic acid, has no affect on pigmentation.

**TABLE 5.**

The Effect of Biotin, its Precursors and Lipoic Acid on Pigmentation.

<table>
<thead>
<tr>
<th>INCUBATION PERIOD</th>
<th>20 Hours</th>
<th>48 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>Pigmentation</td>
</tr>
<tr>
<td><strong>ADDITIONS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nil</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>NaOH</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>Biotin (1µg)</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>Pimelic acid</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>Adipic acid</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>Sebacic acid</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>Acid hydrolysate</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>of yeast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Incubation temperature 37°.

NaOH addition was 0.4ml of a 1.0% solution.

Acid hydrolysed yeast addition was 0.4ml of a neutralised acid hydrolysate of yeast (5%). All of the added acids were 0.4ml of 0.5% suspensions in NaOH (1%).

Other details as given in legend of Table I.
(e) The effect of surface active agents.

In all of the nutritional studies on bacteria involving unsaturated fatty acids, reported in the literature, the concentrations of acid required were very small, viz about 1 - 10\(\mu\)g/ml. In this investigation very high concentrations of oleic acid, even up to 200\(\mu\)g/ml, are required to induce pigmentation by \textit{S. marcescens} at 37\(^\circ\). Such high concentrations of these acids required for pigmentation suggest that their function may not be metabolic. It can of course be argued that the apparent requirement for such high concentrations of unsaturated acids is a reflection of the inability of the organism to assimilate them at low concentrations or else that considerable oxidation of the acids occurs with little concomitant assimilation. An experimental approach to the problem of assimilation was not feasible.

Let us then consider the hypothesis, that the unsaturated acids are not directly metabolically involved in pigment synthesis. Williams & Fieger (1947) have considered such a possibility in relation to the involvement of the unsaturated acids in biotin synthesis. They suggest that an important biological function of biotin is its surface activity and that unsaturated fatty acids by virtue of their surface activity replace the biotin requirements of bacteria. By making use of the highly surface active Tweens (fatty acid esters of polyoxyethylene derivatives of sorbitan) they investigated this concept and found high concentrations of the Tweens could replace the biotin requirements of some bacteria. The effects of Tween 20 and 80, containing lauric and oleic acids respectively, on pigmentation
are shown in Table 6; both of the Tweens are seen to be inactive in pigment promotion. High concentrations of Tween 20 completely inhibited the growth of the organism while lower concentrations permitted growth but no pigmentation. As Tween 80 does not influence pigmentation it can be concluded that either the bacterium is incapable of hydrolysing this fatty acid ester or it is impermeable to it. Our results are thus

**TABLE 6.**

The Effect of the Tweens on Pigmentation.

<table>
<thead>
<tr>
<th>INCUBATION PERIOD</th>
<th>20 Hours</th>
<th>48 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADDITIONS</strong></td>
<td>Growth</td>
<td>Pigmentation</td>
</tr>
<tr>
<td>Tween 20 (20mg)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tween 20 (14,10,6, 2mg)</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>Tween 20 (0.5,0.1mg)</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>Tween 80 (10mg)</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>NaOH (1%)</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>Oleic acid (2mg)</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Incubation temperature 37°. Figures in brackets are the amounts of the various compounds used per assay and added in a volume of 0.4ml of NaOH (1%). Other details as given in the legend of Table 1.

Similar to those of Williams, Broquist & Snell (1947), who, contrary to the findings of Williams & Fieger (1947), found that the Tweens would not replace the growth requirement of *L. bulgaricus* for oleic acid. The former authors did however indicate that the efficiency of the utilisation of oleic acid was increased in the presence of one of the Tweens. This was not found to be the case
for S. marcescens of Table 7, which even in the presence of the Tweens still required high concentrations of oleic acid for pigmentation.

**TABLE 7.**

The Effect of Tween 20 in Combination with Oleic Acid on Pigmentation at 37°.

<table>
<thead>
<tr>
<th>INCUBATION PERIOD</th>
<th>20 Hours</th>
<th>48 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth</td>
<td>Pigmentation</td>
</tr>
<tr>
<td>Tween 20 (2mg) &amp; oleic acid (2mg)</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Tween 20 (2mg) &amp; oleic acid (0·5mg)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Tween 20 (2mg) &amp; oleic acid (0·1mg)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Oleic acid (2mg)</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Oleic acid (0·5mg)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Oleic acid (0·1mg)</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

Incubation temperature 37°. Other details as given in the legends of Tables I and 5.

These experiments thus show the over-all effect of the Tweens to be an adverse one, growth being somewhat depressed and the course of pigmentation uninfluenced. The unsaturated fatty acids would therefore appear not to influence pigmentation by virtue of their surface activity.
(f) **Temperature and degree of unsaturation.**

As a general rule it may be stated that the higher the temperature at which micro-organisms are grown the lower the iodine number of the constituent fats (Kleinzeiler 1948, Hilditch 1953). It follows that the necessity for *S. marcescens* to be supplied with unsaturated fatty acids at 37° for pigmentation to occur might be a reflection of the low activity of the constituent fatty acid dehydrogenases at that temperature.

A preliminary study of the activity of these dehydrogenases in washed suspensions of *S. marcescens* was initiated. In this instance the organism was grown at 27° and 37° in Roux bottles on Bunting's liquid medium supplemented with 100 µg/ml of stearic acid. The usual Thunberg technique was used to estimate the activity of the enzymes. Table 8 shows a representative set of results. In the absence of any inhibitor there was no difference between Thunberg tubes containing substrate or otherwise. It is shown in the table and it is also characteristic of red strains of *S. marcescens* that they have very high metabolic activity even in the absence of any added substrate. White strains of *S. marcescens* or white cells derived from a red strain grown at 37° are characterized by comparatively low metabolic activity.

Making use of malonate to inhibit the basal respiration of the organism the presence of a stearic acid dehydrogenase is established. It is not possible to state with certainty on the basis of the results as given in Table 8 that an oleic acid dehydrogenase is present in the bacterium.

Unsuccessful attempts were made by a number of different
methods to reduce the high basal respiration of the red cells. Exhaustive washing of the cells with large volumes of water,

**TABLE 8.**

Presence of Stearic Acid Dehydrogenase in *S. marcescens* Grown at 27° and 37°.

<table>
<thead>
<tr>
<th>ADDITIONS</th>
<th>DECOLORISATION TIME (MINS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells Grown at 27°</td>
</tr>
<tr>
<td>Nil</td>
<td>7</td>
</tr>
<tr>
<td>Stearate</td>
<td>7</td>
</tr>
<tr>
<td>Oleate</td>
<td>7</td>
</tr>
<tr>
<td>Malonate</td>
<td>11</td>
</tr>
<tr>
<td>Stearate &amp; malonate</td>
<td>6.5</td>
</tr>
<tr>
<td>Oleate &amp; malonate</td>
<td>9</td>
</tr>
</tbody>
</table>

The activity of the stearic acid dehydrogenase was measured in anaerobic Thunberg experiments. Each Thunberg tube contained 1.0ml of a suspension of washed cells in water equivalent to 5 mg. dry weight, 1.0 ml of 0.05 M phosphate buffer pH 8.0, 0.1ml of methylene blue (0.1%). The oleate and stearate added were 0.2ml of saturated solutions of the sodium salts and the malonate was 0.3ml of a 0.1 M solution. Final volume per tube was 3.0ml. Usual Thunberg technique, 30°.

Aeration of washed suspensions, and incubation at 37° for 60 minutes of washed cell suspensions, all proved to be without affect on the blank. The use of a number of inhibitors, as shown in Table 9, did not improve the differentiation between control and experiment. It appeared that broken cell preparations would have to be used in a study of this kind in order to deal with the high blanks but no way of rupturing the cells could be found. All the known techniques using various abrasives met with
no success, presumably due to the small size of the cells. The project was therefore temporarily abandoned. It had been

TABLE 9.
The Effect of Various Inhibitors on Stearic Acid Dehydrogenase.

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>DECOLORISATION TIME (MINS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INHIBITOR</td>
<td>NIL</td>
</tr>
<tr>
<td>Sodium iodoacetate (0.002M)</td>
<td>25</td>
</tr>
<tr>
<td>Sodium fluoride (0.01M)</td>
<td>25</td>
</tr>
<tr>
<td>Potassium cyanide (0.001M)</td>
<td>20</td>
</tr>
<tr>
<td>2:4 Dinitrophenol (0.0005M)</td>
<td>11</td>
</tr>
<tr>
<td>Sodium malonate (0.01M)</td>
<td>17</td>
</tr>
<tr>
<td>Potassium cyanide (0.001M) &amp;</td>
<td>23</td>
</tr>
<tr>
<td>sodium malonate (0.01M)</td>
<td></td>
</tr>
<tr>
<td>Potassium cyanide (0.001M) &amp;</td>
<td>25</td>
</tr>
<tr>
<td>sodium fluoride (0.01M)</td>
<td></td>
</tr>
</tbody>
</table>

The cells used in the experiment were grown at 27°. Usual Thunberg technique, 30°. Each tube contained 1.0ml of 0.05 M phosphate buffer pH 8.0, 1.0ml of a suspension of washed cells in water, equivalent to 4mg. dry weight, 0.1ml of methylene blue (0.1%). Final concentrations of the inhibitors are indicated. The stearate added was 0.2ml of a saturated solution of the sodium salt. Final volume per tube was 3.0ml.

established however that a stearic acid dehydrogenase was present in the cells. The point of desaturation of the stearic acid chain was not determined and it could have been taking place at any position along the carbon chain. The most likely point of desaturation would be the 2–3 position which would be the site of initial oxidation in conventional β oxidation. Although fatty acid dehydrogenases which oxidise stearic acid to oleic acid are known (Fanti & Lincoln, 1949), this reaction is unlikely
as stearate does not influence pigmentation. Any definite conclusion on the dehydrogenation of the fatty acids must await further experimentation.

(g) Influence of oleate on enzyme formation.

A strain of Escherichia coli requiring biotin for growth was investigated by Lichstein & Boyd (1951). They found oleic acid independently of biotin, which it could replace, influenced the synthesis of formic dehydrogenase and formic hydrogenlyase. These enzymes were present in high concentrations in oleate-grown cells, while biotin-grown organisms contained little or none of them.

The two formate-decomposing enzymes were sought in S. marcescens grown at 27° and 37° in the presence and absence of added oleate.

The two enzymes decompose formic acid thus:

\[ \text{HCOOH} \rightarrow \text{CO}_2 + \text{H}_2 \] — formic hydrogenlyase.

\[ \text{HCOOH} + \text{A} \rightarrow \text{CO}_2 + \text{H}_2\text{A} \] — formic dehydrogenase. A is oxygen or some other acceptor.

Formic hydrogenlyase is formed only by micro-organisms when they are grown under conditions of restricted oxygen tension. The activity of this enzyme is estimated by measuring any hydrogen evolution, from formic acid, by conventional manometric techniques. This enzyme was not found in S. marcescens under any of the experimental conditions created by us, which included growing the organism at 27° and 37° both aerobically and anaerobically on Bunting's medium supplemented with oleate and formate.

Formic dehydrogenase was estimated by measuring the oxygen uptake of a washed suspension of the organism in the
presence of formate. A representative series of results is shown in Figure I. The cells were grown aerobically on Bunting's medium and where oleate was added to the medium its final concentration was adjusted to 200 \( \mu g/ml \). It can be seen that, irrespective of the growth temperature and the presence of oleate, formic dehydrogenase is present in the organism. Although the cells grown in the presence of oleate do oxidise formate at a slightly faster rate, this difference in the enzyme activity of the various cells was not considered significant.