A STUDY OF SOME PORPHYRIN COMPOUNDS IN MICROORGANISMS.

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by

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ABSTRACT

The bacterium Serratia marcescens, is notable for its bright red pigment, the tripyrrolic compound prodigiosin. Some strains mutate in such a way that the biosynthetic pathway to prodigiosin is interrupted, and they remain white. By supplying an intermediate beyond the blocked step it may be possible to restore pigmentation.

A white mutant strain of S. marcescens was grown in the presence of some pyrrolic compounds and some known precursors of pyrrole rings. One of the latter, δ-aminolaevulinic acid, was not a prodigiosin precursor but was converted by the bacterium into a dark red-brown pigment, which was excreted into the culture medium. The pigment was isolated and identified as a mixture of porphyrins, together with a brown compound which was non pyrrolic in nature.

The porphyrins were identified separately, and some aspects of their synthesis were investigated.

ABBREVIATIONS

ALA	δ-aminolaevulinic acid.
ATP	Adenosine triphosphate.
DPN	Diphosphopyridine nucleotide. (nicotinamide adenine dinucleotide).
PALPO	Pyridoxal phosphate.
TCA cycle	Tricarboxylic acid cycle.
PBG	Porphobilinogen.
COPRO'gen	Coproporphyrinogen.
URO'gen	Uroporphyrinogen.

CHAPTER 1. A REVIEW OF THE LITERATURE

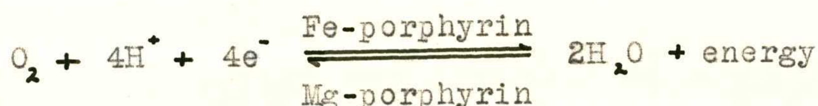
SECTION 1. PORPHYRINS IN NATURE

Porphyrins are intimately involved in the fundamental processes of life. No free porphyrins are of great physiological significance but in combination with a metal their function is of primary importance. Of all possible metal complexes, only Fe- and Mg-porphyrins are biologically active --- iron in haem enzymes and magnesium in chlorophyll.

Photosynthesis is a process whereby light energy of the sun is stored primarily in carbohydrates, secondarily in proteins and fats. Chlorophyll catalyses the removal of oxygen from water with the release of electrons and protons. There is thus the provision of food and the release of oxygen for aerobic cellular respiration.

The general importance of haems was realised through the studies by Warburg on the respiratory ferment and through Keilin's rediscovery (1933) of the cytochromes first described by McMunn, although the more specific oxygen carrying capacity of haemoglobin had already been recognized. Haems are utilized for the release of energy for the maintenance of the living cell, from the organic compounds built up by photosynthesis.

The two processes may be summarized by the equation



$$O_2 + 4H^+ + 4e^- \underset{\text{Mg-porphyrin}}{\overset{\text{Fe-porphyrin}}{\rightleftharpoons}} 2H_2O + \text{energy}$$

Cells contain other haem enzymes such as catalase and peroxidase.

Porphyrin pigments are found almost universally in nature. In this review discussion will be confined to the porphyrins occurring in microorganisms.

Derivatives of porphyrin origin have been found in fossilized excrements of crocodiles, in petroleum, oil shales, earth waxes, asphalts and coal and also from silurian deposits more than 30 million years old (Lemberg and Legge 1949, Hodgson and Peake 1961.). These findings would indicate that porphyrins originated quite early in the earth's history, and attempts have been made to show evolutionary trends by their absence or presence in primitive organisms (Lemberg and Legge 1949).

The cells of most aerobic organisms have a considerable potential for the synthesis of the porphyrin nucleus. In only a few cases must the porphyrin be added in the food as a vitamin. Very little is known of the catabolic changes of the porphyrins, other than that of haemoglobin.

STRUCTURE OF THE PORPHYRINS

Confusion in nomenclature has arisen because of the number of independent workers who have studied the porphyrins, and also because of the varied sources of material. For instance, protoporphyrin has been assigned no fewer than six names. The system of nomenclature followed in this thesis is that used by Lemberg and Legge.

The basic unit of the porphyrin structure is porphin.

Four pyrrole rings (Fig. 1) are joined by four single unsaturated carbon atoms (methene) in the form of a closed planar ring system (Fig. 2). This is a highly resonating

Fig. 1. PYRROLE RING

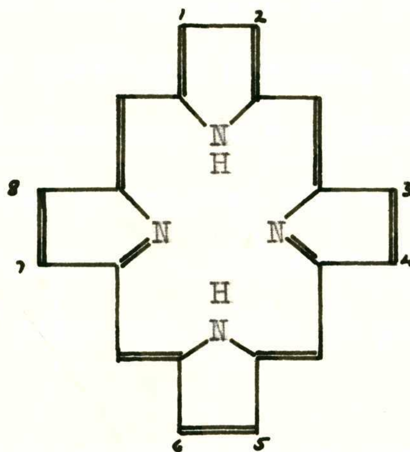


Fig. 2. PORPHIN

structure and therefore extremely stable and strongly coloured. Most of our knowledge of the structure of porphin and the porphyrins can be attributed to the work of Hans Fischer.

The various porphyrins, e.g. proto-, copro- and uroporphyrin, differ from porphin and from each other in the replacement of the H atom at the β positions on the pyrrole rings (marked 1 to 8 on Fig. 2) by different side chains. The substituents of some naturally occurring porphyrins are given

Table 1. PORPHYRIN SIDE CHAINS

Coproporphyrin	4 Methyl		4 Propionic ac.
Deuteroporphyrin	4 Methyl	2 Hydrogen	2 Propionic ac.
Protoporphyrin	4 Methyl	2 Vinyl	2 Propionic ac.
Uroporphyrin			{ 4 Propionic ac. 4 Acetic ac.

in Table 1.

A further difference within each type of porphyrin is the possible order of placement of the side chains around the porphin ring. If two types of substituents are present then four isomers are possible; if three are present, then there are fifteen possible isomers. Fortunately only a fraction of the possible number have been found in nature, the most common being isomers III and IX. In coproporphyrin III there are methyls at positions 1,3,5 and 8 and propionic acids at positions 2,4,6, and 7.

Chlorophylls are distinguished mainly by the presence of a fifth ring and by the esterification of an acid side chain with a phytol group (Figs. 3 and 4). The basic ring structure is more saturated than porphin.

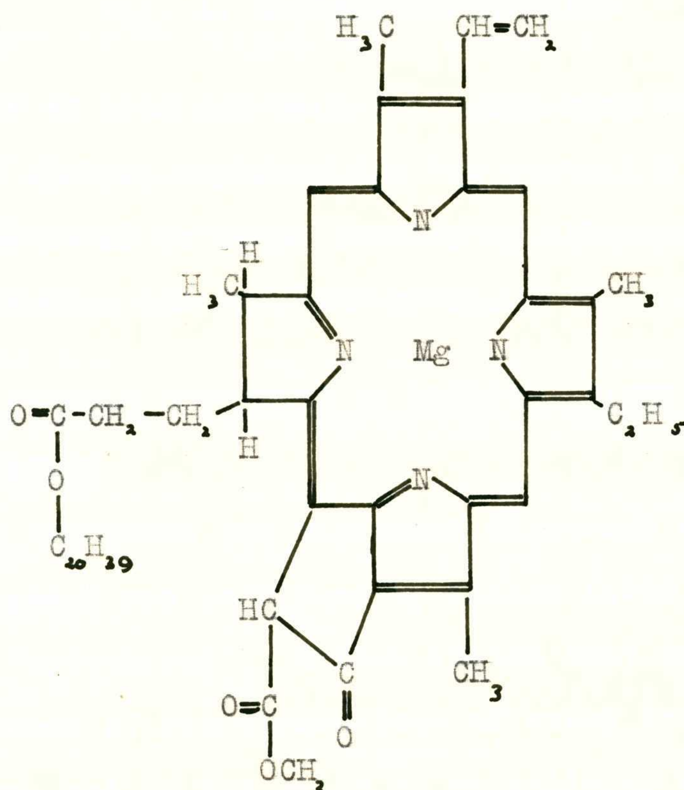


Fig. 3. CHLOROPHYLL

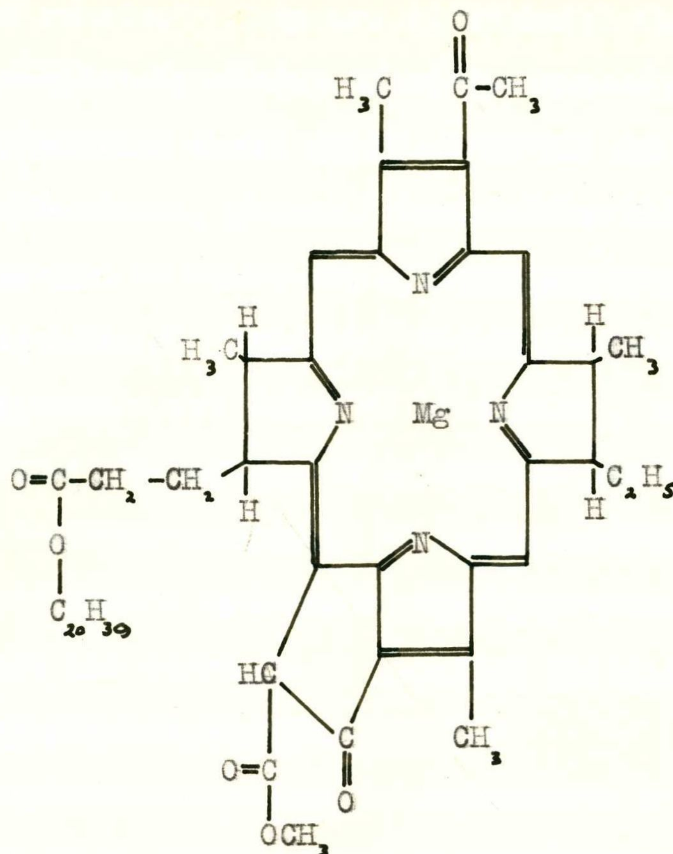


Fig. 4. BACTERIOCHLOROPHYLL

Insertion of iron (in haems) or magnesium (in chlorophyll) into the ring system, replaces the two central H atoms of Fig. 2 but the metal is bound equally by all four N atoms by ionic bonds (Fruton and Simmonds 1958).

PYRROLE COMPOUNDS IN MICROORGANISMS

i CYTOCHROMES

McMunn's important but neglected discovery of a reversibly oxidizable pigment was confirmed by Keilin (1933) in 1925, and named by him "cytochrome". Keilin stated that cytochromes exist "in tissues of representatives of every order of the animal kingdom, and in the cells of unicellular organisms such

as bacteria and yeasts." They are distributed "in aerobic cells and are completely absent in strict anaerobes."

A study by Lucille Smith (1954 a) of the difference spectra of some bacteria, provided information which she has included in a review of bacterial cytochromes found by a number of workers (Smith 1954 b). The cytochrome systems of yeast and heart muscle are found to be similar, corresponding to cytochromes $a+a_3$, b and c. The bacteria can be arranged into a number of groups according to their cytochrome content.

a) Some bacteria have a cytochrome spectrum very similar to that of yeast and heart, e.g. Bacillus subtilis, Sarcina lutea and some mycobacteria.

b) In others, cytochrome a may be replaced by a_1 or a_2 or by a mixture of a_1 and a_2. Escherichia coli has both whereas Acetobacter pasteurianum has only a_1.

c) In yet another group, both b and c are replaced by b_1, e.g. E. coli and Proteus vulgaris.

d) There are no cytochromes in a number of Streptococci and Pneumococci nor in many of the obligate anaerobes.

Contrary to the belief of Keilin (1933), haem compounds and cytochromes are found in some obligate anaerobes. A cytochrome has been found in species of Chlorobium by Kamen and Vernon (1954) and Gibson (1961), and in Desulphovibrio desulfuricans by Postgate (1954).

ii OTHER HAEM COMPOUNDS

Microorganisms contain the haemoproteins catalase and peroxidase. Formic dehydrogenase of E. coli has been shown

to be complexed with cytochrome b_1 (Wrigley and Linnane 1961). Another haem protein associated with a microorganism, is the haemoglobin produced as a result of symbiosis of Rhizobium with a legume (Virtanen 1945, Keilin and Wang 1945). Some support for the view that nitratase of Haemophilus influenzae may be a haem enzyme, is presented by Smith et al (1953).

iii CHLOROPHYLL

The principal chlorophyll-containing members of the microorganisms are the algae, and the purple, green and brown photosynthetic bacteria of the order Rhodobacteriales (Thimann 1955).

iv FREE PORPHYRINS IN MICROORGANISMS

Free porphyrins (containing no metals and not joined to protein) are found to occur naturally in many yeasts, protozoa and bacteria. The reports following indicate that, in the porphyrin mixture present in most, coproporphyrin predominates. Coproporphyrin is excreted by Corynebacterium diphtheriae, the amount depending on the iron content of the medium (Rawlinson and Hale 1949), and it has been isolated from an anaerobic culture medium of Bacillus cereus (Schaeffer 1952). Coproporphyrin has been found in yeast (Lemberg and Legge 1949) and in Mycobacterium tuberculosis avium (Patterson 1960). It is excreted by Candida guilliermondii (Shawlovskii and Bogatchuk 1961), by Propionibacterium shermanii (Pronyakova 1960) and by Tetrahymena vorax (Lascelles 1957).

By irradiating Chlorella, Granick (1945, 1948) and

Bogorad and Granick (1953 a) obtained mutants which synthesise porphyrins with 2 to 8 carboxyl groups.

A relationship between the free porphyrin and other porphyrin components of the cell, is often indicated when growth conditions are varied. An excess of iron in the culture medium of C. diphtheriae decreases porphyrin and toxin excretion and increases cellular haem(Pappenheimer 1947 a,b). Iron deficiency increases porphyrin production from δ-aminolaevulinic acid and decreases bacteriochlorophyll content in Rhodopseudomonas sphaeroides (Lascelles 1956). Schaeffer (1952) demonstrated the relationship between porphyrin excretion and cytochrome content of B. cereus. A control mechanism by cobalt and iron on porphyrin and vitamin B_{12} synthesis, was shown in Propionibacterium by Pronyakova (1960). Stich and Eisgruber (1951) studied a similar relationship in yeast. In M. tuberculosis avium the amounts of catalase and porphyrin vary inversely (Patterson 1960).

v TRIPYRROLES

So far, the only tripyrrole found in nature is the pigment prodigiosin of Serratia marcescens, and related pigments from the order Actinomycetales. Dietzel (1949) extracted from Actinomyces, a red pigment which resembled prodigiosin in absorption spectrum, melting point and composition. From a species of Streptomyces, Narni and Nicolaus (1959) isolated a red pigment which they showed to differ from prodigiosin only in the length of one of the side chains attached to the β position of a pyrrole ring. An orange-red pigment, whose spectrum

corresponded with that of prodigiosin and which could be fractionated chromatographically in the manner used by Williams et al (1956) for prodigiosin, was extracted from another strain of <u>Streptomyces</u> by Perry (1961).

The structure and biosynthesis of prodigiosin will be dealt with in Section 3.

SECTION 2. PORPHYRIN BIOSYNTHESIS

Studies of the mechanism of synthesis of the porphyrins have occupied workers for many years. Reviews of the advances in understanding have appeared at intervals, the more recent ones coming from Shemin et al (1955a), Rimington (1957) and Margoliash (1961). Investigations have shown that glycine condenses with an asymmetric four carbon compound to give, after decarboxylation, an amino-keto-monocarboxylic acid. Two molecules of this acid, δ-aminolaevulinic acid, condense to a pyrrole ring, porphobilinogen. In a way as yet undetermined, four PBG molecules are converted first to uroporphyrinogen, then by way of coproporphyrinogen to protoporphyrin. The porphyrinogens are tetrapyrroles in which the carbon bridge is saturated. The corresponding porphyrins are derived from this main synthetic route by oxidation.

PRECURSORS.

That glycine is involved in porphyrin synthesis in humans and rats was demonstrated by Shemin and Rittenberg (1945, 1946 a, 1946 b). Although the four N atoms and eight of the C atoms of the porphyrin arise from glycine, the carboxyl group of glycine is not utilized (Radin et al 1950 b, Grinstein et al 1948). It has been demonstrated that a succinyl intermediate arising from the citric acid cycle is the source of the remaining twenty-six C atoms of protoporphyrin (Shemin and Wittenberg 1951, Shemin and Kumin 1952, Wriston et al 1955). The use of isotopic tracers also showed that acetate (Radin et al 1950 a, Shemin and Wittenberg 1951) but not pyruvate (Granick 1958) is a precursor.

Shemin et al (1955 b) visualized the condensation of glycine and "active" succinate as an aspect of the metabolism of glycine, and formulated the Succinate-Glycine Cycle Fig. 5.

The isolation of ALA and carbon dioxide from an incubation mixture of glycine and succinate with chicken erythrocytes (Laver et al 1958, Gibson et al 1958), established the condensation product as ALA. The other intermediate in this scheme, α-amino-β-ketoadipic acid, cannot be isolated since it is spontaneously decarboxylated, but its diethyl ester has been shown (Shemin et al 1955 b) to be converted to PBG. Kikuchi et al (1958) found that the "active" succinate is probably succinyl CoA.

The suggested mechanism for the condensation is the formation of a Schiff's base between glycine and PALPO

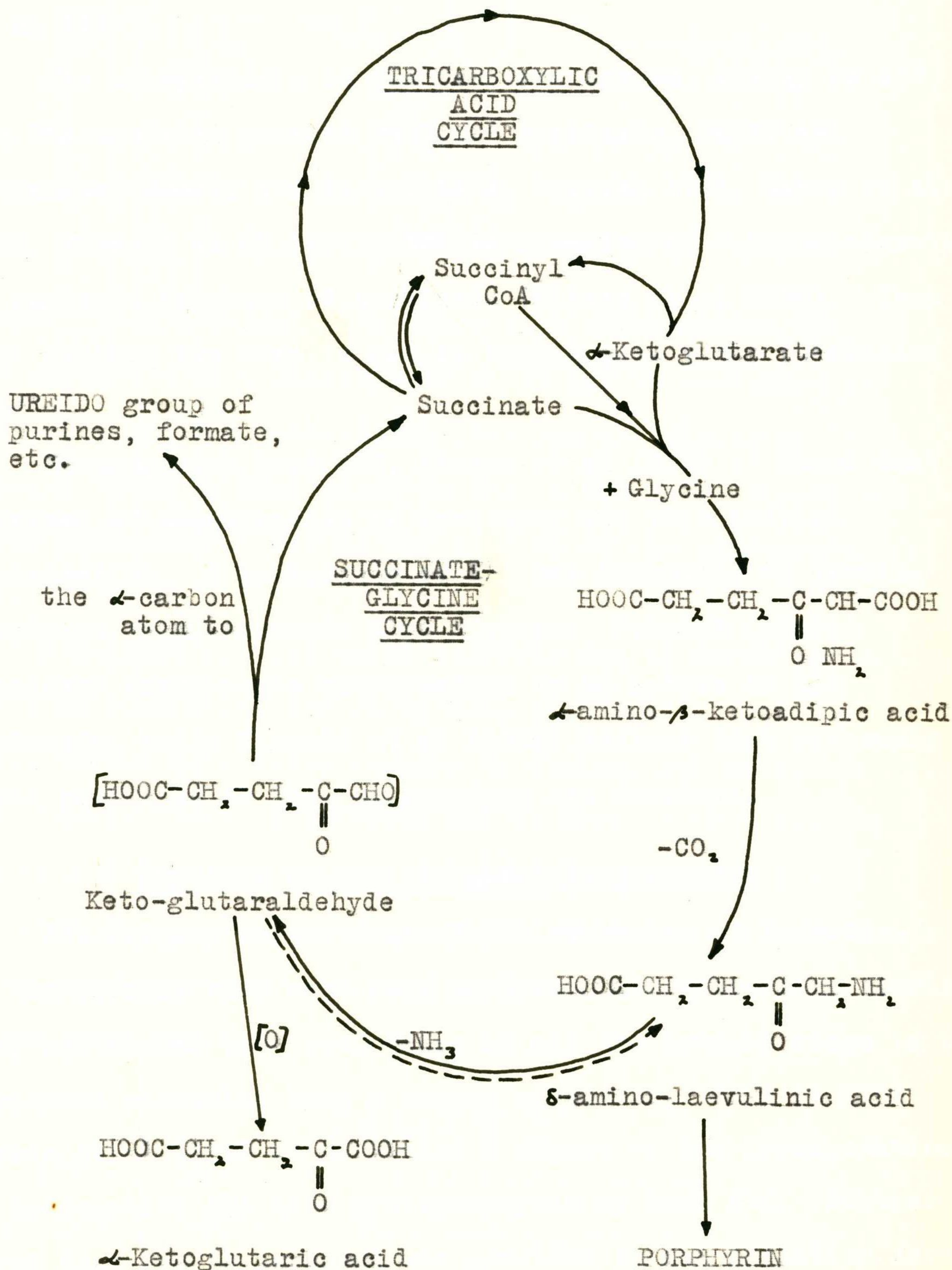


Fig. 5. THE SUCCINATE-GLYCINE CYCLE

(Gibson et al 1958). This would then condense with succinyl CoA to give α-amino-β-ketoadipic acid. A kinetic analysis using purified enzymes has supported the hypothesis (Kikuchi et al 1959).

The biosynthesis of ALA requires oxygen, energy as ATP, the TCA cycle to provide "active" succinate, PALPO and glutamine (Dresel and Falk 1956 a, Granick 1958, Laver et al 1958, Kikuchi et al 1959). DPN is required if α-ketoglutarate is employed in place of succinate (Gibson et al 1958). Iron will inhibit the step to ALA in _Tetrahymena vorax_ (Lascelles 1957).

Working with duck RBC, Shemin et al (1955 b) found that a degree of organization is necessary for this initial condensation. A homogenate of RBC was incapable of forming porphyrin from succinate plus glycine. Granick (1958) considers erythrocyte mitochondria to be active in ALA synthesis, while Kikuchi et al (1958) believe that extremely small particles in _Rps. sphaeroides_ are involved.

THE ROLE OF ALA IN PORPHYRIN SYNTHESIS

Preliminary suggestions of the mechanism of formation of the pyrrole ring were made by Shemin and Wittenberg (1951). They postulated that both sides of all the pyrrole rings in the porphyrin molecules have a common precursor. Then in 1955 Shemin et al (1955 b) published a report on the incorporation of radioactive ALA into protoporphyrin by duck RBC. Further proof that ALA is involved was provided by its isolation from an incubation mixture with chicken erythrocytes (Laver et al

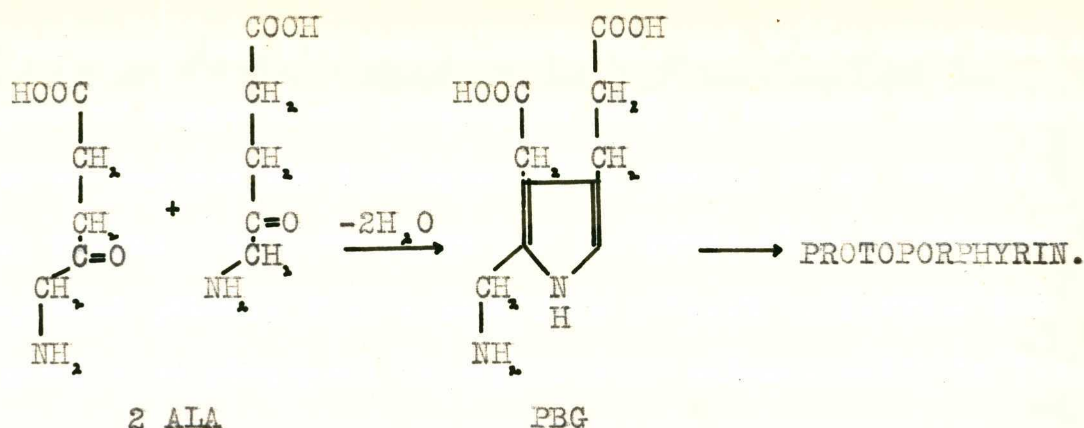


Fig. 6. CONDENSATION OF ALA

1958). Shemin suggested a Knorr type condensation (Fig. 6). The structure of a monopyrrole which can be converted to uroporphyrin, was established (Cookson and Rimington 1953, 1954, Kennard 1953) as that given in Fig. 6 for PBG. Dresel and Falk (1953) showed that PBG is the product of the condensation of ALA.

A soluble extract which converts ALA to COPRO'gen was isolated from erythrocytes by Granick (1958). This was fractionated into three enzymes by Granick and Mauzerall (1958). The ALA-ase fraction converts ALA to PBG; it is a sulphhydryl enzyme and does not require oxygen. Gibson et al (1955) isolated from a wide distribution of tissues, a single enzyme which catalyses both condensations necessary for the formation of PBG from ALA.

THE ROLE OF PBG

Although the four rings of a porphyrin may contain different substituents, all four have a common precursor, (Shemin and Wittenberg 1951). The actual mechanism for the condensation

to porphyrin has not been elucidated, but there have been a number of suggestions (Rimington 1952 a, Bogorad and Granick 1953 b, Cookson and Rimington 1954, Shemin et al 1955 b). All such suggestions must allow for the production of isomers. One suggested pathway from monopyrrole through tripyrrole and dipyrrole to porphyrin is represented schematically in Fig. 7.

In actual fact the product of PBG condensation is a porphyrinogen; this process has been found not to require oxygen (Falk et al 1953, Granick 1958, Granick and Mauzerall 1958, Bogorad 1958 a, 1958 b, Lockwood and Benson 1960).

Some relevant enzymes have been isolated. Granick and Mauzerall (1958) isolated from rabbit RBC haemolysates PBG-ase which deaminates and condenses PBG to URO'gen III. PBG-deaminase from spinach leaf (Bogorad 1958 a) produces URO'gen I and III and ammonia. It is inhibited by formaldehyde and -SH poisons and unlike PBG-ase, is thought to be localized on cell particles. Bogorad (1958 b) isolated another enzyme, URO'gen isomerase, from wheat germ. In combination with PBG-deaminase this enzyme produces URO'gen III only, from PBG.

PORPHYRINOGENS AND PORPHYRINS AS INTERMEDIATES

Early workers thought that uro- and coproporphyrins were on the direct path to protoporphyrin (Salomon et al 1952, Falk et al 1953, Bogorad and Granick 1953 b). More recent work has shown that this is not so (Shemin et al 1955 b, Dresel and Falk 1956 b, Mauzerall and Granick 1958, Bogorad 1958 c). However Bogorad and Granick (1953 a) and Granick et al (1953)

have claimed isolation of a hydrated iron protoporphyrin IX and other unidentified porphyrins, which they consider to be precursors of haem and chlorophyll.

Another early suggestion from Shemin and Wittenberg (1951) was that decarboxylation to produce the less carboxylated porphyrins occurred at the monopyrrole stage. Shemin et al (1955 b) then suggested an intermediate tetrapyrrole in which at least some of the bridge atoms are methylene, and that it is in these intermediates that decarboxylations occur. These intermediates would correspond to the porphyrinogens.

Conversion of URO'gen to COPRO'gen or the porphyrin has been demonstrated in rabbit reticulocytes by Granick and Mauzerall (1958), Mauzerall and Granick (1958) and in Chlorella by Bogorad (1958 c), and in some cases the enzymatically active fraction has been isolated. The enzymes converting ALA to COPRO'gen are soluble but the step from COPRO'gen to protoporphyrin requires a particulate enzyme (Granick 1958).

Steps in the synthesis between ALA and COPRO'gen do not require oxygen (Bogorad 1958 c, Granick 1958) but the step from COPRO'gen to protoporphyrin is oxygen dependent although cytochromes and ATP do not take part (Granick 1958). Autocatalytic conversion of URO'gen and COPRO'gen to the corresponding porphyrin takes place in the presence of oxygen. An intermediate which absorbs at 500 mμ can be detected. It is thought to be a cyclized tetrapyrrole at an oxidation level between URO'gen and uroporphyrin (Bogorad 1958 a, Mauzerall and Granick 1958). This compound is not involved in the

enzymatic decarboxylation.

ISOMER FORMATION

Attempts to explain the occurrence of isomers have been made by a number of workers (Rimington 1952 a, Bogorad and Granick 1953 b, Cookson and Rimington 1954, Shemin et al 1955 b). Discussion of these hypotheses, together with evidence which discredits them, is given by Margoliash (1961). One of the suggestions is represented by Fig. 7. Condensation of two molecules of compound A would produce isomer I, while one mole

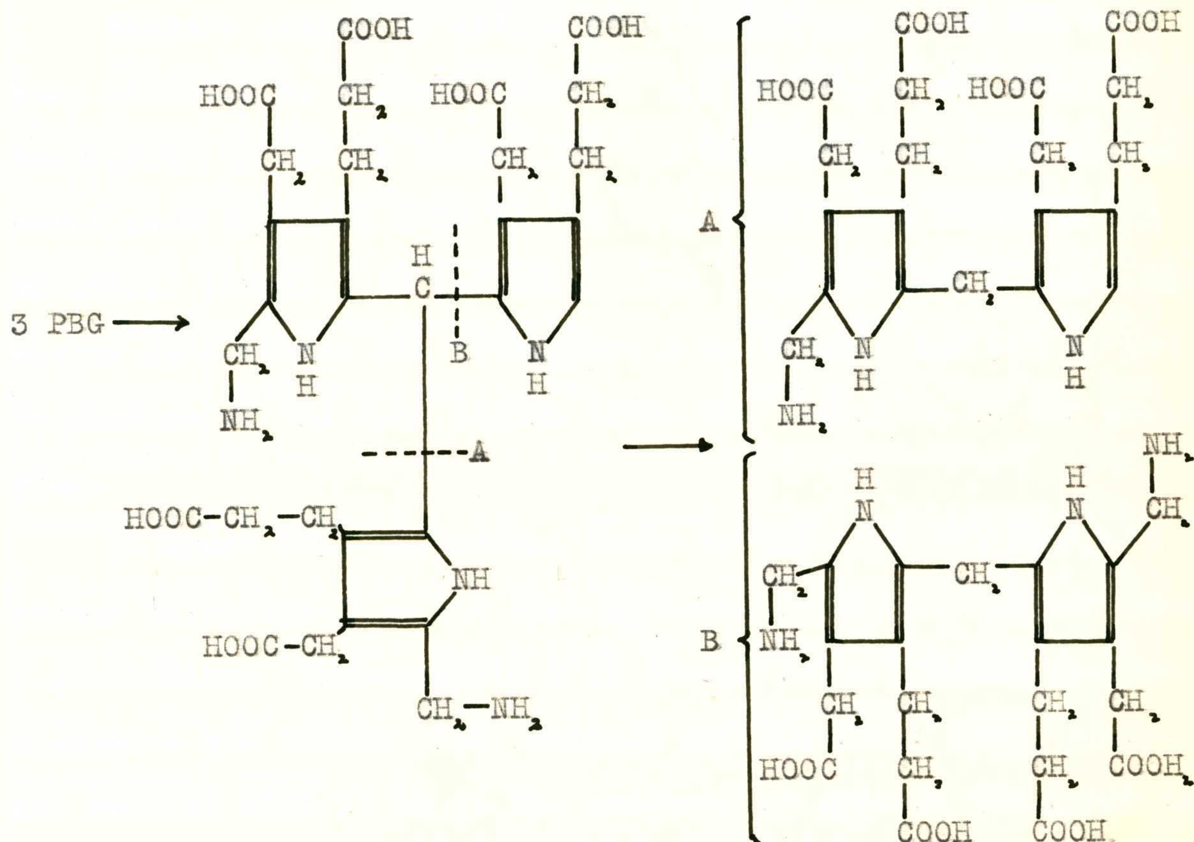


Fig. 7. A MECHANISM OF PORPHYRIN FORMATION FROM A MONOPYRROLE.

of B would give isomers III, when condensed with a mole of A.

In the formation of isomers III, if the formulation of Fig. 7 were correct, then a one C compound would have to be lost. This could well be formaldehyde (Shemin 1955). Shemin et al (1955) demonstrated the enzymatic formation of formaldehyde during conversion of PBG to porphyrins. However Bogorad and Marks (1960) and Lockwood and Benson (1960) claim that the incorporation or the production of formaldehyde is non-enzymatic. Moreover, Bogorad (1958 a) found inhibition of PBG-deaminase by formaldehyde. These findings tend to disprove the hypothesis of Fig. 7.

Enzymes acting on PBG to produce URO'gen I and III have been isolated by Bogorad (1958 a) and Mauzerall and Granick (1958). Conversion of PBG to isomer III has been achieved with enzymes from wheat germ (Bogorad 1958 b) and from rabbit RBC (Granick and Mauzerall (1958). It has been shown (Bogorad 1958 b) that the enzyme acts before the tetrapyrrole is formed, and not by inter-conversion of the side chains after the formation of the porphin ring.

It seems that the normal route of the reaction leads to isomer III production but that abnormal conditions favour isomer I production. Uroporphyrin I is formed after heating enzymes (Lockwood and Benson 1960, Granick and Mauzerall 1958, Bogorad and Granick 1953 b) or in the presence of cyanide (Lockwood and Benson 1960).

INTRODUCTION OF THE METAL

Granick et al (1953) isolat ed haematoporphyrin IX from a mutant of <u>Chlorella</u>. They claim that this iron-containing

compound is a precursor of protoporphyrin in the biosynthetic pathway to haem and chlorophyll. The metal must be incorporated during or after oxidation to the porphyrin since the porphyrinogens cannot bind metals (Mauzerall and Granick 1958). More recent studies of the problem have been made by Nishida and Labbe (1959), Minakami et al (1959), Lockwood and Goldberg (1961) and Lowe and Phillips (1961). The enzyme concerned with the incorporation of iron is located in the mitochondrial fraction; the process is favoured by anaerobic conditions.

CHLOROPHYLL BIOSYNTHESIS

Della Rosa et al (1953) showed that glycine and acetate are precursors of chlorophyll in *Chlorella vulgaris*. Granick claims that the paths of porphyrin and chlorophyll biosynthesis are identical as far as protoporphyrin. He isolated metal-free and Mg complexes of protoporphyrin, vinyl pheophytin a_5 (Granick 1945, 1948) and haematoporphyrin IX (Granick and Bogorad 1953), and suggested that they are intermediates in chlorophyll synthesis. Mauzerall and Granick (1958) observed in an incubation mixture, a substance which absorbs at 635mμ. They suggested that if this by-product of the photooxidation of porphyrinogen were a chlorin rather than an oxyporphyrin, it might represent a step in chlorophyll synthesis.

PORPHYRIN SYNTHESIS IN MICROORGANISMS

Probably the pathway of porphyrin synthesis is similar in the microorganisms to that in plants and animals. Glycine-^{15}N is incorporated into haems of *C. diptheriae* (Hale et al 1950) and

chlorophyll of Chlorella (Della Rosa et al 1953). ALA is converted to porphyrins by Rps. sphaeroides (Lascelles 1956, Kikuchi et al 1958) and PBG to porphyrins of Chlorella (Bogorad and Granick 1953 b). Other aspects of porphyrin metabolism in microorganisms are presented by Lascelles (1957), Kikuchi et al (1959), Pronyakova (1960), Maitra and Roy (1960) and Shawlovskii and Bogatchuk (1961).

SECTION 3. SERRATIA MARCESCENS

Because of its striking colour, the genus Serratia was one of the first species of bacteria observed by man. The red pigment has been the object of most studies on the organism, investigations made of growth requirements and metabolism being largely directed to effects on pigment production.

CLASSIFICATION

Since its discovery by Bizio in 1823, Serratia has been called by a number of different names (Breed and Breed 1924). The classification of S. marcescens now accepted in the Bergey manual (Breed et al 1957) follows:

Order: Eubacteriales

Family: Enterobacteriaceae

Tribe: Serratiae

Genus: Serratia

Type species: Serratia marcescens Bizio.

The *Serratia* are small rods, varying in length from 1 to 3μ and breadth 0.5 to 0.7μ. They are described as cocco-bacilli but their size is very variable. They are gram negative aerobes with peritrichate or polar flagella (Wilson and Miles 1947). Colony types have been described by Reed (1937). They occur naturally in water and air and are saprophytic on decaying plant or animal material. There are five species : *S. marcescens*, *S. indica*, *S. plymouthica*, *S. kilensis* and *S. piscatorium*.

GROWTH AND METABOLISM

Nutritional and physical requirements for growth and pigmentation are reviewed by Bishop (1959). The organism is aerobic and requires as carbon source a compound with at least three carbon atoms. A concentration of iron of 0.1mg/ml for pigmentation and 0.03mg/ml for growth is necessary (Waring and Werkman 1942 b). Ammonia is a satisfactory nitrogen source (Bunting 1940). Pigmentation is maximal in a slightly acid medium (Dewey and Poe 1943). The organism grows over a wide temperature range and pigments up to 34°C. There is generally no pigmentation at 37°C in *S. marcescens* but a mutant strain which pigments quite strongly at 37°C has been isolated (Bishop 1959).

Metabolic studies till 1959 have been reviewed by Gould (1959). These studies are few and unrelated since most interest has centred on the pigment. Evidence for a TCA cycle in *S. marcescens* has been presented by Green and Williams (1959). This fact is of interest in this work since porphyrin synthesis

requires an active citric acid cycle (Shemin and Wittenberg 1951).

PYRROLIC COMPOUNDS

Serratia has been demonstrated to contain cytochromes a$_1$, b and c (Fujita and Kodama 1934). The relationship between catalase, a haem containing enzyme, and prodigiosin was studied by Crichton and Lazarus (1948). The pigment prodigiosin has been shown to be tripyrrole (Wrede and Rothaas 1933). Santer and Vogel (1956) isolated a colourless precursor of prodigiosin, containing at least one pyrrole ring, from a white strain of S. marcescens.

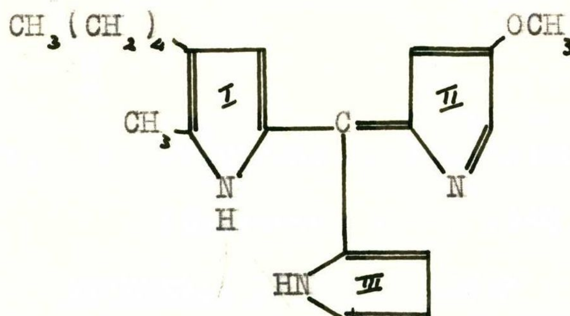
COLOUR VARIABILITY

Strains of S. marcescens differ greatly in colour and in the frequency with which they yield colour variants. Unstable colour variants of white, through pink to deep red have been isolated from single colonies (Bunting 1942, 1946, Daddi 1932, Reed 1937). More variants appear when cultures age. When cultures of S. marcescens were allowed to age in a synthetic broth medium at 30°C, the populations were observed to change colour in a reproducible manner(Bunting and Ingraham 1942, Bunting 1946). Studies on the effect of chemical and physical agents on colour stability were made by Bunting (1940, 1950), Bunting et al (1949, 1951), Dewey and Poe (1943) and Kharasch et al (1936).

STRUCTURE OF PRODIGIOSIN

Wrede and Hettche (1929) established the empirical

formula of prodigiosin as $C_{20}H_{25}N_3O$. Wrede and Rothaas (1933 a, 1933 b) suggested a tripyrrylmethene structure where ring III is attached to the carbon between rings I and II. They gave the structure as (Wrede and Rothaas 1934)---



Spectral studies of prodigiosin by Hubbard and Rimington (1950) confirmed this structure. However prodigiosin has since been shown to contain a number of fractions (Weiss 1949, Williams et al 1956).

In more recent years evidence has been presented that the three pyrrole rings of prodigiosin are joined linearly. From degradation and chromatographical studies Narni and Nicolaus (1959) proposed alternate structures I and II Fig. 9.

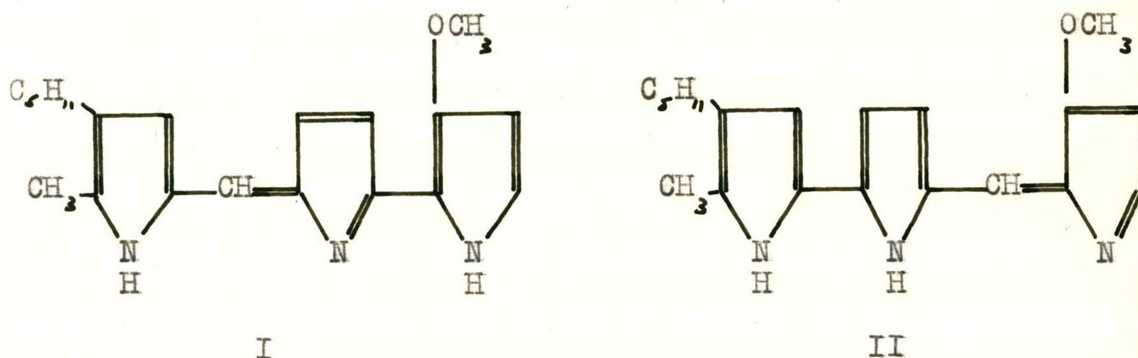


Fig. 9. SUGGESTED STRUCTURES FOR PRODIGIOSIN.
(Narni and Nicolaus 1959).

Synthetic studies (Wasserman et al 1960 a) and spectral and nuclear magnetic resonance studies (Wasserman et al 1960 b)

lead to the now accepted formula Fig. 10.---

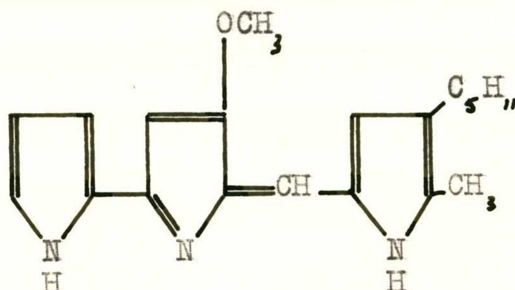


Fig. 10. STRUCTURE OF PRODIGIOSIN.

(Wasserman et al 1960 a, b).

SYNTHESIS OF PRODIGIOSIN

Hubbard and Rimington (1950) proposed that the biosynthesis of prodigiosin may be related to the mechanism of porphyrin formation. They found that both C atoms of acetate and the α-C and the N of glycine were incorporated into prodigiosin. Crichton and Lazarus (1948) postulated the origin of prodigiosin as

tripyrrylmethanes ⟶ prodigiosin

Substrate ⟶ dipyrrylmethenes ⟶ porphin ⟶ catalase.

However Marks and Bogorad (1960) found that ALA was not incorporated, so that the three pyrrole rings of prodigiosin must be derived from a precursor other than PBG.

Santer and Vogel (1956) isolated a $C_{10}H_{10}O_2N_2$ pyrrolic precursor of prodigiosin from a white mutant of S. marcescens (9-3-3). Wasserman et al suggest that this precursor has the structure of compound I of Fig. 11. They found (Wasserman et al 1960 a) that prodigiosin is formed by acid catalysed condensation of methylamylpyrrole (II) with the aldehydic precursor (I). They formulated the structure of prodigiosin

as III Fig. 11. Exposure of strain 9-3-3 to methylamylpyrrole vapours caused formation of prodigiosin within minutes. They suggest that prodigiosin synthesis *in vivo* occurs through a coupling stage similar to that represented in Fig. 11.

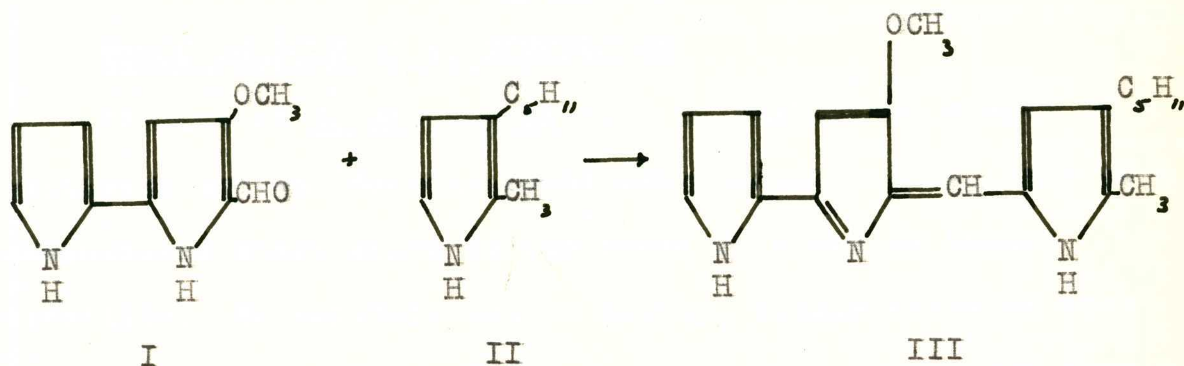


Fig. 11. SYNTHESIS OF PRODIGIOSIN.

(Wasserman et al 1960 a).

CHAPTER 2. EXPERIMENTAL AND DISCUSSION OF METHODS

ORGANISMS

STOCK CULTURE OF S. MARCESCENS

Strains of *S. marcescens* were maintained on slopes of a synthetic medium (Bunting 1940) and stored at 0°C. They were subcultured every eight to ten weeks in order to ensure viability. To maintain colour purity, single colonies were selected at intervals. Culture flasks were inoculated from slope cultures grown for 16-24 hours. Four strains of *S. marcescens* were employed, designated in this laboratory 211 (a white mutant isolated from a red strain), 200 (white), 251 (yellow) and 330 (red).

OTHER BACTERIA

Cultures of *Pseudomonas fluorescens* KB1, *Aerobacter aerogenes* and *Escherichia coli* were available in this laboratory. Cultures of *Corynebacterium diphtheriae* and *Bacillus subtilis* were supplied by Dr. Y. T. Tchan of the Department of Microbiology.

CULTURE METHODS

Growth temperature was 30°C unless otherwise stated.

MEDIA

Aglycerol-citrate-salts medium (Bunting 1940) was used routinely for the growth of *S. marcescens*. Buffered at pH 6.8 it has been shown to give best pigmentation of coloured strains. Glycerol is more stable than glucose in the presence of large

concentrations of phosphate, ammonia is a satisfactory N source, citrate keeps inorganic salts in solution and stimulates growth, and Mg^{++} and Fe^{+++} are essential for growth of the organism (Waring and Werkman 1942 b). Solid medium contained 1.5% agar.

C. diphtheriae and B. subtilis are more exacting in growth requirements. They were cultured in a cooked meat medium.

"F" medium (Adams 1950) supplemented with 0.5% peptone, and Norris medium (Norris and Jensen 1957) modified by the addition of $CaCl_2$ (0.5mM) and ammonia (2.0ml of 0.88S.G. per litre) were used to check some nutritional requirements for porphyrin synthesis.

For special tests used in the characterization of S. marcescens, peptone water and glucose phosphate medium were made according to Mackie and McCartney (1953) and semisolid medium according to Edwards and Ewing (1955). The liquid media were dispensed in $6 \times \frac{5}{8}$" test tubes.

For large scale culture, 70ml of the medium were dispensed in 250ml Ehrlenmeyer flasks which were incubated on a rotary shaker to ensure maximum aeration. For preliminary experiments, tests on "other bacteria" and tests for requirements for porphyrin synthesis, still cultures containing 10ml of medium in 30 or 50ml conical flasks or 15-20ml in 100ml conical flasks gave adequate aeration.

STERILIZATION

All flasks and tubes were autoclaved for ten minutes at 10lb pressure. Substrates added after autoclaving (e.g. ALA is decomposed by heat) were first passed through a small sterile Seitz filter fitted with a number 9 filter pad.

REMOVAL OF IRON

The method of Waring and Werkman (1942 a) was followed for freeing glassware and the medium of iron. Three extractions with 8-hydroxy-quinoline were usually sufficient to remove all traces of iron.

TYPING OF SERRATIA MARCESCENS

The metabolic tests used in the characterization of *S. marcescens* were suggested by Edwards and Ewing (1955) and Davis et al (1957), namely i gas from sugars, ii fermentation of glucose, lactose, sucrose, maltose, mannitol and rhamnose, iii indole production, iv methyl red reaction, v motility on semisolid medium and vi Voges-Proskauer test for acetoin. Gas from sugars was indicated by a Durham tube and fermentation by acid production. Observation and tests were carried out on the second and sixth days after inoculation.

ASSAYS

Optical densities were measured on a Zeiss Spectrophotometer PMQ11.

PORPHYRIN

The porphyrin solution was suitably diluted in 0.5N HCl so that the maximum optical density (OD) was less than 1.0. The OD was measured at the Soret maximum and at 430 and 380 mμ and the correction for impurities was made according to Rimington and Sveinsson (1950). Data for k and the extinction coefficients for the various porphyrins are given by Rimington (1960). The optical density at the Soret peak for the pure porphyrin is

given by:

$$ODmax. = \frac{2.Dmax. - (D430 + D380)}{k}$$

Porphyrins in the culture medium were measured after centrifuging off the cells. Cellular porphyrins were estimated after extraction and purification (see below). In each case the crude mixture of porphyrins was estimated as uroporphyrin since electrophoresis and chromatography have shown this to be the predominant porphyrin (80% of the total).

The correction formula is said to be valid if the impurities have a straight line absorption over this range 430 to 380mμ. A check was made on the medium in which S. marcescens was grown in the absence of ALA. The absorption line of the medium in 0.5N HCl satisfied the condition.

δ-AMINOLAEVULINIC ACID

The procedure according to Elliot (1960) was adopted. The assay solution follows Beer's law between 0.05 and 0.8 μmoles of ALA. The ALA concentration is proportional to the OD of its orange complex with picric acid, measured at 495mμ. Standards were prepared from a fresh dry sample of ALA.HCl (Sigma) stored at -20°C. For assay of ALA in the culture medium, an optically clear sample was obtained by centrifuging off the cells at 12,000g for 10 minutes.

PORPHOBILINOGEN

Cookson and Rimington (1954) gave a new interpretation of the data of a method first published by Vahlquist (1939). Further modifications were suggested by Gibson et al (1955).

The PBG reacts with p-dimethylaminobenzaldehyde in 5N HCl to give a pink complex absorbing at 552 and 530mμ. When assaying in the presence of porphyrin it was necessary to set up a blank cell containing the solution for assay and 5N HCl, since the acidified porphyrin as well as the PBG complex, absorbs in this range. The PBG was calculated as the monohydrate using the given extinction coefficient.

This "Ehrlich" reaction can be used also for qualitative detection of pyrrole rings provided an α-C (nearest the N atom) is free. Porphyrins therefore will not interfere until they are broken down by fusion with concentrated alkali.

IRON

The method followed was that of Gibson(1961). After oxidation of organic matter with H_2O_2 the iron is reduced by glucose and forms a pink compound with αα'dipyridyl. The extinction at 520mμ was compared with standards of ferrous ammonium sulphate. The tubes were freed from iron before the assay by soaking overnight in dilute acetic acid (approx. 3%).

PURIFICATION OF THE PORPHYRINS

Areview of this subject is given by Rimington (1957).

The porphyrins are soluble in the culture medium and are retained to a small extent (1-2%) in the cells. The cells were centrifuged off at 10,000g for 15 minutes; the medium was acidified slightly (pH 4) so that iron was not complexed into the porphyrin, and evaporated to dryness under vacuum at 40°C. The porphyrins were separated from the solid residue by esterification, and extraction of the ester into chloroform.

ESTERIFICATION AND PURIFICATION OF THE ESTERS

Methods are given by Kennedy (1956), Vannotti (1954), Lemberg and Legge (1949). The most convenient was a modification of the methods of With (1958) and Laverack (1960).

The dry solid was suspended in H_2SO_4 -methanol (5:95 v/v) overnight at room temperature. After dilution with three volumes of water, followed by adjustment to pH 3 to 5 with acetic acid/sodium hydroxide, the esterwas transferred into chloroform by several extractions. A brown porphyrin-containing solid at the interface was collected by centrifugation, dissolved in glacial acetic acid and extracted into chloroform as above. The combined chloroform extracts were washed with water at pH 4 or above, and evaporated under vacuum to a small volume. The ester was extracted into constant boiling HCl from a mixture of chloroform/ether (1:6 approx.). A number of HCl extractions were necessary to remove the ester completely. The esters were saponified to the free porphyrins by standing in the acid at room temperature for 24 hours.

For separation of the various porphyrins the acid solution was evaporated to dryness under vacuum, and a solution in dilute ammonia was subjected to electrophoresis.

Where necessary, the esters of the separate porphyrins were crystallized. The water-washed chloroform solution was dried over calcium chloride and evaporated as above. Crystallization was achieved from a minimum volume of hot dry chloroform by the addition of absolute methanol (With 1958). The ester was washed with absolute methanol.

All solvents were purified.

Assay of the medium when harvested, and of the free porphyrins after esterification showed recoveries on four occasions of 91%, 86%, 100%, and 82%. Thus it seems that something better than 80% recovery can be expected with this method. With (1958) claims only 10% recovery of porphyrin from urine using his method.

OTHER ISOLATIONS

Porphyrins adsorb onto talc or kieselguhr (With 1958) and onto calcium phosphate (Sveinsson et al 1949). They are precipitated by 12% barium chloride (With 1949). Uroporphyrin is extracted into cyclohexanone at pH 1.5 (Dresel et al 1956, Kennedy 1956). These methods, as well as adsorption onto magnesium trisilicate at pH 2, were found to be less convenient and less efficient than direct esterification of the evaporated solution.

EXTRACTION FROM THE CELLS

The crude porphyrins were extracted by the method of Rawlinson and Hale (1949). The acid acetone extract was evaporated to dryness. The porphyrins were esterified as already described.

ELECTROPHORESIS

Separation of porphyrins by electrophoresis has been achieved (Heikel 1955, With 1956, Rimington 1957).

Two types of apparatus were used in the present work: the Spinco Model R and the Spinco Model CP continuous flow. The solvent for both methods was a solution of sodium carbonate

(0.04M, pH 11) containing versene (10^{-4}M) to chelate free metal ions. The porphyrins were applied in 2N ammonia.

a) Spinco Model R. Whatman 3MM paper in strips or sheets was used. A current of 30ma was applied for approximately two hours and the bands were detected under UV light.

Five fractions moved towards the positive electrode. The fast moving red (1) was not separated from the following brown (2). A pale pink band (3) ran uncontaminated. Behind this was another pink band (4), mingled at its slower edge with a green strip (5) which reached back to the origin. Distances moved relative to the leading component were of the order of (1) 1.0, (2) 0.9, (3) 0.5, (4) 0.3 and (5) 0.1.

Buffers of phosphate 0.05M pH 6.0 and pH 7.0, and acetate 0.1M pH 5.1 and pH 3.6 gave poorer separation of the bands.

b) Spinco Model CP. The curtains and wicks were those recommended by Spinco. A suitable solvent flow rate was achieved with a setting of 6.7 on the reservoir overflow. A current of 100ma was applied, giving a potential difference of 340 volts. The porphyrin solution (0.6 µmoles/ml) was applied with a feed rate setting of 3. Unfortunately the fractions 3 to 5 of the small apparatus were not obtained; further trials would be necessary. However this method was useful in separating fractions 1 and 2 in quantity.

CHROMATOGRAPHY

A review of methods is given by Falk (1961).

PAPER

a) Preliminary circular chromatograms. The chromatograms

were run on 13" squares of Whatman 3MM paper according to the method of Rappoport et al (1955). Separation into six fractions (only two of which fluoresced red) was achieved using as solvent, acetone containing 0.0005% HCl.

b) Chromatography with lutidine. A modification of the

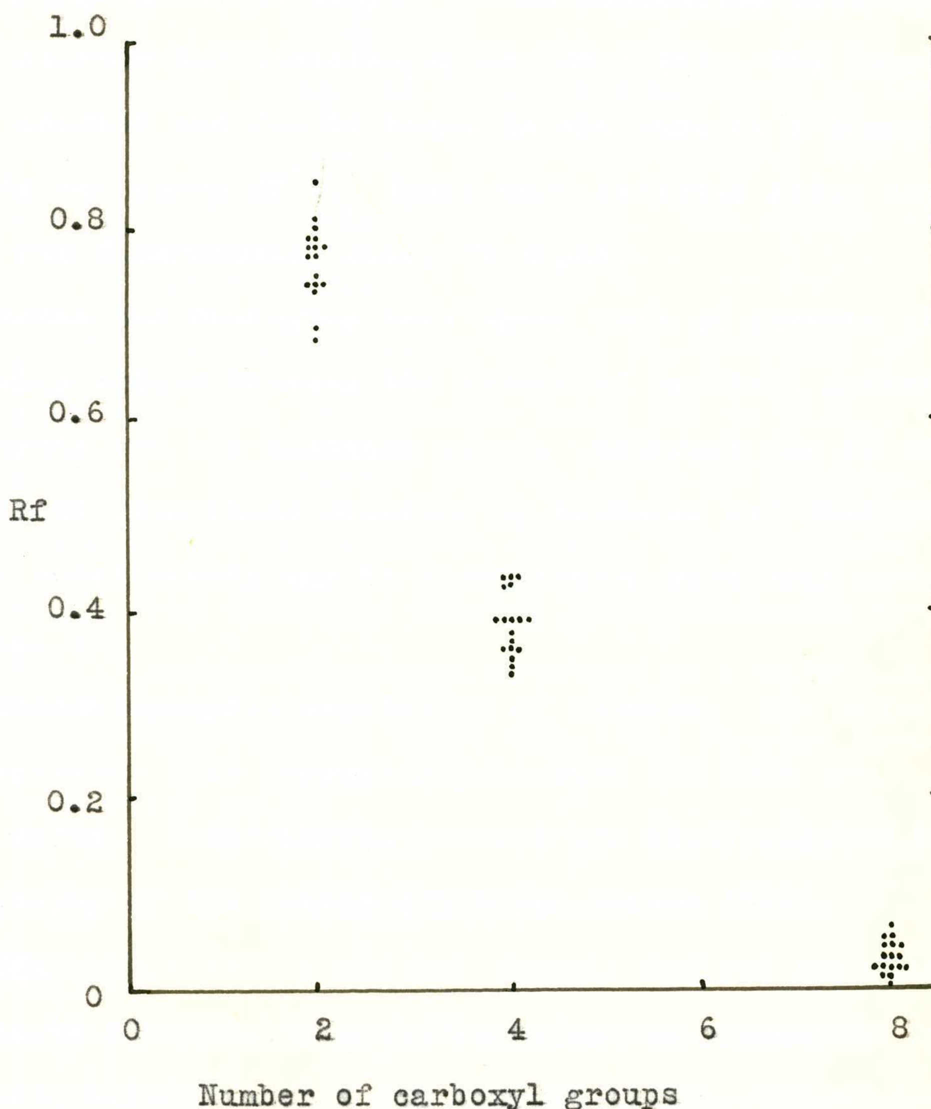


Fig. 1. Relationship between the number of carboxyl groups and the Rf value. Proto-, copro- and uroporphyrin standards separated on a chromatogram by 2,6 lutidine/water (35 : 27).

methods of Nicholas and Rimington (1951 a), Kennedy (1953) and Eriksen (1953) was used. Samples of free porphyrins in 2N ammonia were applied to Whatman No. 1 paper. After drying, the papers were suspended in the tank to equilibrate for one hour in an atmosphere of the solvent. No ammonia was used. The chromatogram was developed by the descending technique, using as solvent 2,6 lutidine/water (35 : 27). The development was carried out for 16 hours in the dark at a temperature preferably not above 22°C. Spots were detected after drying, by their red fluorescence under UV light.

Nicholas and Rimington have shown that an inverse linear relationship exists between the number of carboxyl groups of the porphyrin and the distance moved. Although the Rf values are different from those reported by Nicholas and Rimington, a similar relationship can be demonstrated with this method above (Fig. 1).

c) Chromatographic separation of isomers. The above method separates free coproporphyrin Iand III into two distinct spots. Uroporphyrin isomers are distinguished by chromatography of the esters (Falk and Benson 1953).

COLUMN

Attempts at separating the fractions on magnesium trisilicate, talc (Nicholas and Comfort 1949), alumina and powdered cellulose were unsuccessful. The bands did not separate evenly, nor were they sharply defined.

Uroporphyrin obtained from electrophoresis is contaminated by the brown fraction (2). Purification of the

uroporphyrin in its ester form could be achieved when it was passed through a column of grade III MgO (Nicholas 1951). The brown substance was firmly adsorbed at the top of the column; the pure uroporphyrin was then eluted with chloroform/ methanol (100 : 0.5 v/v).

SPECTRA

Spectra between 650mμ and 370mμ were measured in the Cary Recording Spectrophometer, Model 14. Aqueous solutions were in acid (1N HCl) or alkali (1N NaOH). The esters were dissolved in dry chloroform.

The porphyrins are characterized by the presence of a very strongly absorbing band in the range 400 to 410mμ. The optical density of this "Soret" peak is 10 to 15 times as great as peaks in the range 450 to 650mμ. The solutions being examined were therefore diluted for measurement between 430 and 370mμ. Infra-red spectra were run in a Perkin-Elmer Recording Spectrophotometer, Model 221. The samples were prepared in a Nujol mull.

MELTING POINTS

The porphyrin esters are not clearly defined crystals, but rather an amorphous mass. Uroporphyrin esters tend to blacken before they melt. The melting point was taken as the appearance of clear molten edges around the mass. Measurements were made on the Kofler Bloch apparatus and are uncorrected.

DECARBOXYLATION

Uroporphyrins in the free form were decarboxylated by heating in 1% HCl v/v in evacuated, sealed tubes, at approx. 180° C for 2 hours. The solutions were evaporated, and the resulting coproporphyrin was extracted into ether from an aqueous solution at pH 4. Small amounts of constant boiling HCl extracted the coproporphyrin from the ether. This solution was evaporated for chromatography.

CHEMICALS

ALA was obtained from Sigma chemicals. Samples of uroporphyrin III octamethyl ester (human origin) and uroporphyrin I octamethyl ester (animal origin) were obtained from Light and Co. Samples of uroporphyrin, coproporphyrin and coproporphyrin III were kindly donated by W.H.Lockwood of the Institute of Medical Research, Royal North Shore Hospital. All other chemicals were AR. Solvents were purified.

CHAPTER 3 RESULTS AND DISCUSSION

SECTION 1. PIGMENT PRODUCTION

CHARACTERIZATION OF S. MARCESCENS

COLONY APPEARANCE

There were 68 strains of S. marcescens available for study. The majority produced colonies which conformed to the following description ---

Size	1-2mm	Surface	smooth
Shape	circular; smooth edge	Moisture	glistening
Elevation	convex	Colour	variable

These colonies corresponded to the "smooth" type, one of four S. marcescens colony types described by Reed (1937). About 10% had drier, "rough" colonies while 5% were quite wet in appearance.

METABOLIC TESTS

In addition to the metabolic tests noted in the methods section, the growth rate, pigmentation at 30° and 37° C and stability of colour were observed for each of the 68 strains. A summary of results is presented in Table 1. The percentage of the strains giving a positive result (column 2)is compared with the findings from 50 strains of Davis et al (1957) (column 3), from which they gave a definition of the Serratia group (column 4). The results from the four strains used in later work are shown in columns 5 to 8.

The test for indole was unsatisfactory since the

Table 1. METABOLIC TESTS ON SERRATIA

TEST	RESULT %	DAVIS %	DEFINITION	211	200	251	330
Growth rate	96			+	+	+	+
Stability of colour	72			±	±	+	+
Pigment 30°C	60	32				±	±
37°C	2	32					
Gas from sugars	71	64	slight	+	-	+	+
Glucose	100	100	+	+	+	+	+
Lactose	41	58	- (or wk)	-	-	+	-
Sucrose	90	100	+	+	+	+	+
Maltose	96	100	+	+	+	+	+
Mannitol	93	100	+	+	+	+	+
Rhamnose	7	0	-	-	-	-	-
Motility	100	100	+	+	+	+	+
Indole	0	0	-	-	-	-	-
M.R.	0	0	-	-	-	-	-
V.P.	78	100	+	-	+	+	+

rosindole reagent released the pigment from red strains to give a false positive. However, all strains appeared to be negative after 24 hours' growth.

The most striking variations from the results of Davis et al were the smaller number of acetoin producers (V.P. test) amongst our cultures and the fact that the strains of Davis et al pigmented at 37°C. They also reported very small volumes of gas produced from glucose, whilst Breed et al (1957) maintained that no gas is produced. In many cases, our strains produced large volumes of gas.

The slow growth of some strains coincided with little or no fermentation of the sugars tested. Almost all strains metabolized glucose and mannitol, but when carbon atom number six was reduced (rhamnose) the fermentation was considerably restricted. When the cultures were grown on disaccharides, it was found that the link in sucrose (1-2α) and maltose (1-4α) was broken by almost all strains, whereas lactose (1-4β) could be used by only half the strains.

Included in the <u>Serratia</u> group by the definition of Davis et al (1957) then, are the strains 200, 211, 251 and 330. Results differ only in the fact that 211 is V.P. negative and that the coloured strains do not pigment at 37°C (in agreement with most other workers).

PIGMENT INDUCTION BY CHEMICALS

The mutant white <u>S. marcescens</u> 211 was grown in the presence of some pyrrolic compounds and of some known precursors of pyrrole rings, in an attempt to bypass the blocked step on the biosynthetic path to prodigiosin. The results are shown in Table 2.

None of the chemicals used could induce the cells to produce prodigiosin in visible quantity. Marks and Bogorad (1960) demonstrated the incorporation of both glycine-2-^{14}C and L-proline-^{14}C into prodigiosin. They found that ALA-^{14}C was not a precursor and concluded that the three pyrrole rings of prodigiosin must be derived from a precursor other than PBG. Strain 211, even when supplied with a concentration of glycine, proline and ALA far in excess of that required for

maximum pigmentation of red strains, did not produce prodigiosin. The blocked step is therefore later in the biosynthetic pathway than the incorporation of these acids.

Wasserman et al (1960 a) passed vapours of methyl amyl pyrrole over a strain of <u>S. marcescens</u> which was a susceptible member of a Rizki pair (Santer and Vogel 1956, Rizki 1954). Prodigiosin was formed. The pyrroles used above, although bearing reactive side chains, proved ineffective with strain 211, which also is a member of a Rizki pair.

<u>Table 2. CHEMICAL PRECURSOR OF PIGMENT</u>

SUBSTRATE	GROWTH TIME (Days)	CONC^{N} (M) IN MEDIUM	PIGMENT*
Control (no substrate)	6	-	-
2-carboxypyrrole	"	1×10^{-3}	-
DL-proline	"	1×10^{-3}	-
Acetyl DL-proline	"	1×10^{-3}	-
L-arginine pyrrolidone carboxylate	"	1×10^{-3}	-
Oxamic acid	"	1×10^{-3}	-
Serine	"	1×10^{-3}	-
Glycine	5	1×10^{-3}	-
Succinate	"	1×10^{-3}	-
Succinate plus glycine	"	each 10^{-3}	-
ALA	5	1×10^{-3}	+ (brown)
Control (not inoculated)	"	1×10^{-3}	-

* A negative result indicates that no pigment was formed.

Shemin and Rittenberg (1946 a) quote a suggestion of E.Aberhalden and of G.Lusk that proline and pyrrolidone carboxylic acid may be protoporphyrin precursors. The cultures grown in the presence of proline and L-arginine pyrrolidone carboxylate showed no visible evidence of porphyrin production with strain 211. However, another porphyrin precursor, ALA, was converted to a red-brown pigment.

The pigment formed in the presence ofALA was soluble in the medium and present also in the cells. It could not be washed from the cells with water, ethanol nor acetone. The strain had not been caused to mutate since the brown cells were unable to continue producing the pigment in fresh medium in the absence of the ALA. The pigment production required the mediation of the bacterium; an incubated sterile control containing the culture medium and ALA failed to show any colour change.

PRELIMINARY IDENTIFICATION OF THE PIGMENT

SPECTRA

After removal of the cells by centrifugation, the spectrum of the culture medium was examined. The neutral, acidic and alkaline spectra are reproduced in Fig. 1. The spectra, particularly the very strong absorption in the range 410 to 400mμ, are typical of porphyrins. The peaks contained in the acid and alkaline spectra are compared with spectra for copro- and uroporphyrins (Table 3). Absorption at 480mμ is not found in any porphyrin spectra. The peak positions of the acid spectrum suggest coproporphyrin but it must be

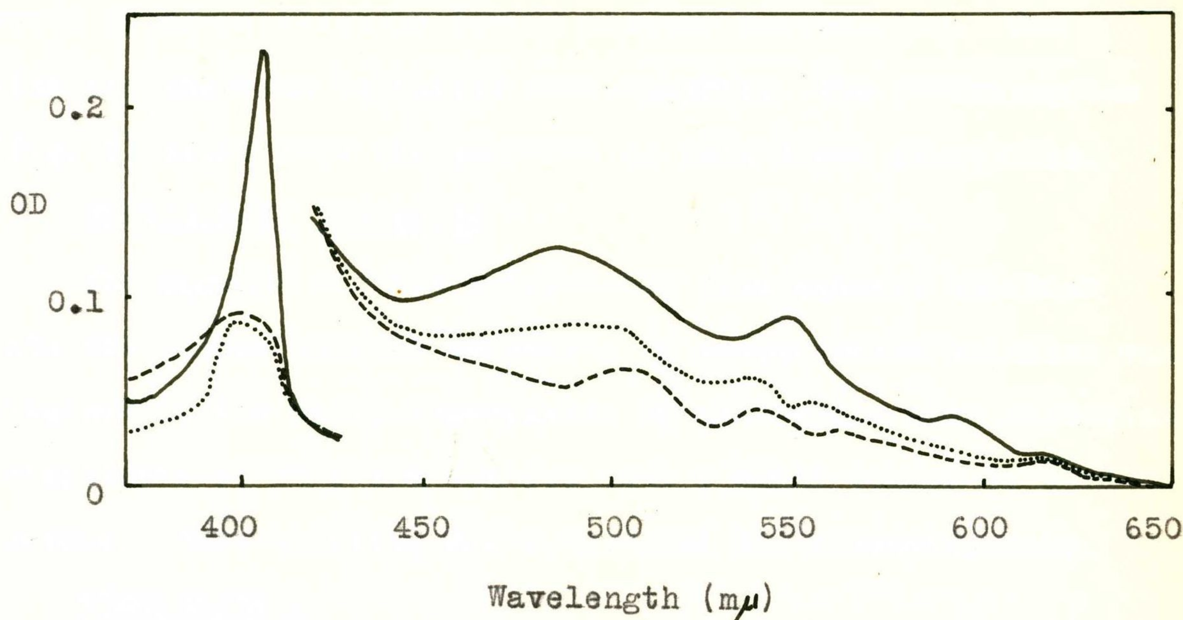


Fig. 1. Spectrum of the medium after growth of S. marcescens in the presence of ALA. Acidic ———, alkaline -----, neutral········.

Table 3. PEAKS IN VISIBLE SPECTRA

PIGMENT FROM SERRATIA		COPRO.	URO.	COPRO.	URO.
ACID†	ALKALI†	ACID*		ALKALI**	
591mμ	612mμ	591mμ	597mμ	617mμ	612mμ
550	560	548	553.5	568.5	560.5
480	539			538.5	539
	502			503.9	503.7
405	398	401 (0.1N HCl)	406 (2N HCl)	?	?

* From Lemberg and Legge (1949). Spectra in 25% HCl.

** From Fischer (Vannotti 1954). Spectra in 0.1N KOH.

Concentrated acid moves peaks slightly to longer wavelengths.

† Spectra in 1N HCl or 1N NaOH.

noted that the latter are taken in 25% HCl. Peaks in 1N HCl would be moved slightly toward shorter wavelengths. The alkaline spectrum indicates uroporphyrin. The chromatograms (Fig. 2) below show the presence of both these porphyrins.

PRELIMINARY ISOLATION

The pigment was not extractable from aqueous solution into the common organic solvents. A concentrated solution was obtained in the early experiments by adsorption onto magnesium trisilicate at pH 2 and elution into a small volume of acid acetone. This solution was evaporated for chromatography.

FLUORESCENCE

The concentrated pigment was fractionated on a circular chromatogram. Six bands, two of which fluoresced bright red under UV light, were apparent. This fluorescence, together with the appearance of the spectra, suggests the presence of porphyrin compounds, although Vannotti (1954) notes that non porphyrins such as derivatives of bilirubin and certain alkaloids have a similar fluorescence. A solution of the pigment in water fluoresced a bright yellow-red. When acidified, the fluorescence became more intense and blue-red. In alkali the fluorescence was a weaker orange-red. This variation is also typical of porphyrins (Vannotti 1954). Accordingly, the pigment was chromatographed in lutidine as suggested for porphyrins.

CHROMATOGRAPHY IN LUTIDINE

Fig. 2 shows the type of separation when the total pigment was chromatographed. The appearance was the same

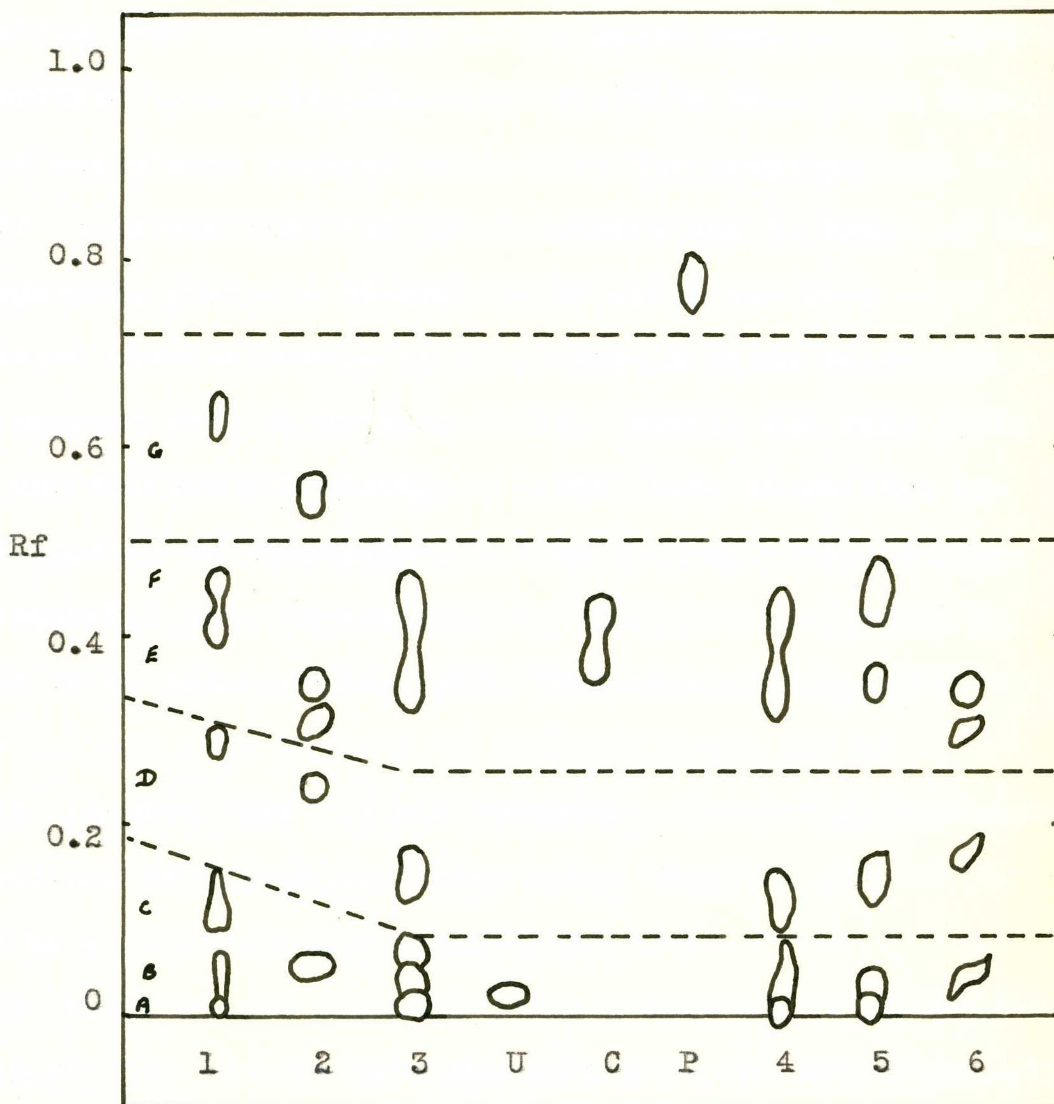


Fig. 2. Chromatographic separation of six samples of the whole pigment. Solvent 2,6lutidine/water (35 : 27), run 16 hours at 15-20°C. U,C and P : uro-, copro- and protoporphyrin standards.

when the pigment was purified subsequently by esterification.

There was a maximum of seven spots, two of which appeared only in the more concentrated applications.

a) The slowest moving spot (A) corresponds to uroporphyrin and in each chromatogram this spot predominated, indicating that uroporphyrin is the major component. This was verified by electrophoresis. For this reason the total pigment was always assayed using data for uroporphyrin.

b) A second spot (B) moved immediately in front of the uroporphyrin, giving a "double spot" which has been considered characteristic of uroporphyrin alone (Nicholas and Rimington 1951 b, Eriksen 1953). Dresel and Falk (1956 c, d) and Canivet and Rimington (1953) have shown that the second spot is an octacarboxylic non uroporphyrin. Another octacarboxylic porphyrin, which runs behind uroporphyrin is claimed to be octa-aceticporphin (Nicholas and Rimington 1951 b).

c) Another spot (C) was observed only when concentrated applications of the pigment were made. Judging from the Rf, it may be a heptacarboxylic porphyrin.

d) There always appeared next, a spot (D) below copro-porphyrin. Its various Rf's indicate a 5-, 6- or 7-carboxylic porphyrin. Porphyrins of this type have been found by Dresel and Falk in the incubation mixture for the synthesis of haem from ALA or PBG. (Dresel and Falk 1956 d).

e) The next spot (E and F) is a mixture of two copro-porphyrin isomers, possibly I and III. Sometimes the two spots were separate.

f) A porphyrin (G) appeared, again in the more concentrated applications, between the copro- and protoporphyrin

markers, indicating a tricarboxylic porphyrin. A similar trace was observed by Dresel and Falk (1956 d).

The Rf values from lutidine chromatography are not constant but appear to increase with an increase in the amount of porphyrin applied, e.g. (1) and (2). A balance must be found so that there is sufficient of the minor components to separate from adjacent spots, and yet excess of the major components must be avoided so that streaking does not occur. These facts must be kept in mind when interpreting a chromatogram. Nicholas and Rimington (1951 c) claimed an inverse linear relationship between the number of carboxyl groups and the Rf. The present method shows a similar relationship (see page 35). However, it has been found (Falk 1954) that the four isomers of coproporphyrin can be separated by lutidine into three spots. This, together with the double octacarboxylic spot renders more difficult the exact identification of porphyrins on these chromatograms. In addition, uroporphyrin isomers do not separate in lutidine, so that factors other than carboxyl groups and isomeric configuration determine separation.

<u>S. marcescens</u>, grown in the presence of ALA, produces a mixture of porphyrins.

In the acid spectrum there is a very broad peak with a maximum at 480-490mμ. Mauzerall and Granick (1958) observed at 500mμ absorption by a slightly oxidized porphyrinogen. They stated further, that the corresponding non cyclic compounds absorb intensely at 460-490mμ. The peak in the acid spectrum

may be by such a compound, provided the conditions of aeration would not have caused its complete oxidation.

Marks and Bogorad (1960) grew *Serratia* in the presence of ALA without observing porphyrin production. Their culture was on a solid medium of a casein hydrolysate, so that any porphyrin formed would have diffused into the medium and been disguised by the colour of the hydrolysate.

Strain 211 forms predominantly uroporphyrin. During most syntheses of porphyrins, coproporphyrin is noted as the major component. For example there is the natural excretion by *Candida* yeast (Shawlovskii and Bogatchuk 1961), and by *B. cereus* (Schaeffer 1952) and synthesis in the presence of ALA by *Rps. sphaeroides* (Lascelles 1956).

CONDITIONS FOR PIGMENT PRODUCTION

To obtain maximum production of the porphyrins, investigations were made on the effects of temperature, aeration and substrate concentration on the resulting porphyrin concentration, and the time taken to reach this maximum was determined.

TEMPERATURE

Duplicate flasks were set up with and without ALA (1×10^{-3}M) and incubated with intermittent shaking at 30° and 37° C. Aliquots of the medium were assayed at intervals. The control flasks showed no porphyrin content. The results for the flasks containing ALA are shown graphically in Fig. 3, for nine days. A further estimation on the 20th day showed a very slight decrease in the porphyrin content of the flasks

at 37°C, while that of the flasks at 30°C increased to 18.5×10^{-3} µmoles porphyrin per ml. Thus it is apparent that porphyrin synthesis is faster initially at 37°C but that the total conversion of substrate is less. The reason for this is not clear.

The temperature effect on porphyrin synthesis in S. marcescens was observed over 9 days in a growing culture, so that it cannot be compared with work reported by others.

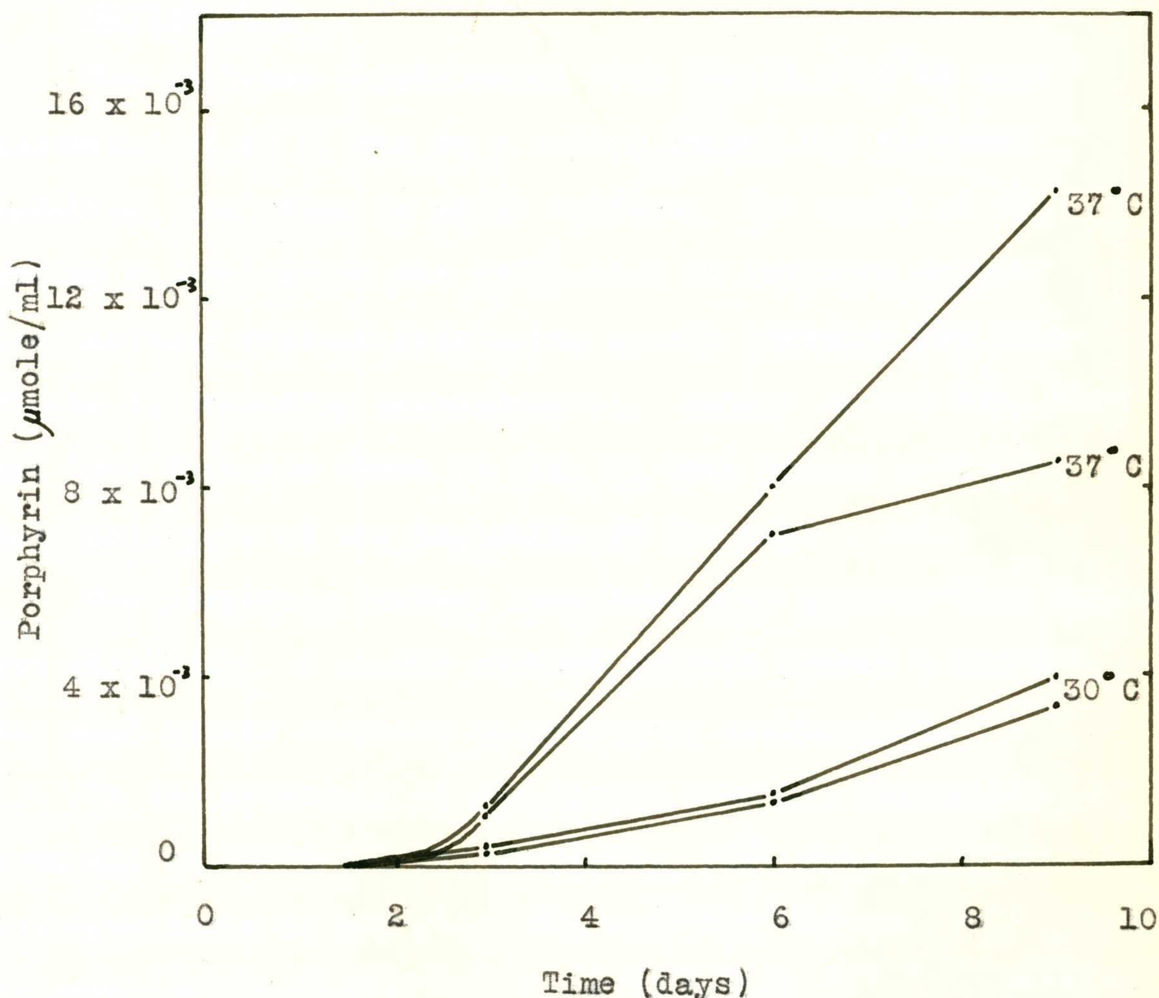


Fig. 3. Effect of growth temperature on synthesis of porphyrin from ALA (1×10^{-3}M) by S. marcescens 211. Aeration not maximal.

These reports refer usually to non-growing suspensions of bacteria or erythrocytes examined for up to 24 hours and the incubation temperatures used lie between 29° and 37°C, although in general no comment is made on the effect of varying the temperature. However Granick (1958) reports on chicken RBC incubated with glycine for 16 hours, a marked temperature effect in the range 32-38°C, corresponding to a Q_{10} of approx. 10.

OXYGENATION

Two cultures supplemented with ALA (1 x 10^{-3}M) were incubated at 30°C, one on a rotary shaker for vigorous aeration, the other in still culture. The depth of the medium in the latter was sufficient to restrict oxygen availability without decreasing growth (checked for both flasks by turbidity measurements). Assays on the 7th, 10th and 13th days are shown in Fig. 4, both having contained zero porphyrin at the beginning of the experiment.

The porphyrin content rose much more quickly when oxygen was plentiful. A poorly aerated culture showed a gradual, but much slower increase in porphyrin synthesized.

In the synthesis of porphyrins from ALA the reactions from ALA to COPRO'gen are anaerobic, that from COPRO'gen to protoporphyrin is aerobic. The uro- and coproporphyrins can be produced non-enzymatically by oxidation of the porphyrinogens. The vigorous aeration used in these experiments probably oxidized the URO'gen to uroporphyrin preferentially, making it the main component. Any COPRO'gen formed

from the URO'gen would have been oxidized to coproporphyrin. No protoporphyrin was produced. However, the same porphyrin pattern was observed on chromatography of pigment synthesized during less favourable conditions of aeration.

The still culture of Fig. 4 was aerated vigorously after the 13th day and the porphyrin content was found to increase sharply in 24 hours to equal that of the continuously aerated

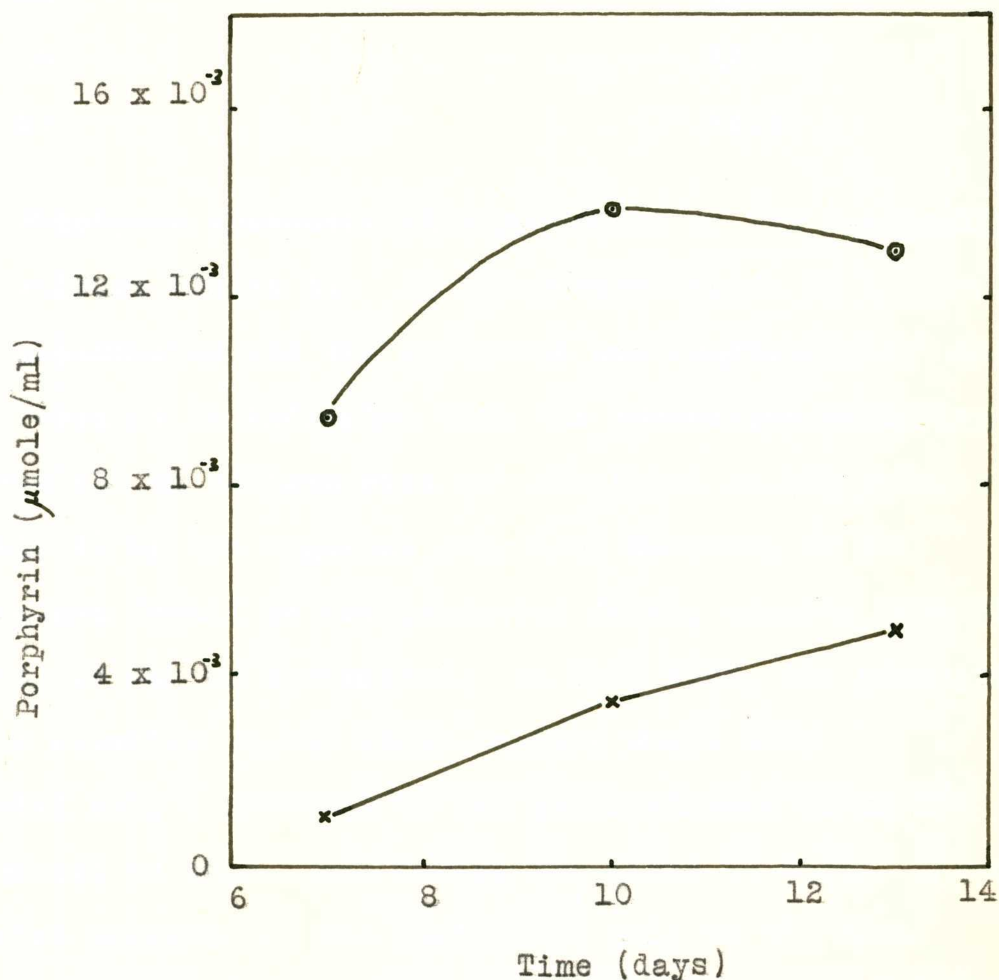


Fig. 4. Effect of aeration on synthesis of porphyrin from ALA (1 x 10^{-3}M) by S. marcescens 211.—⊙—: culture vigorously aerated;—x—: still culture. Growth temperature 30°C.

Table 4. EFFECT OF LOW OXYGEN TENSION

Porphyrin (μmoles x 10^{-3}/ml).

DAY	AERATED CULTURE (1)	STILL CULTURE (2)
0	0	0
7	7.5	1.0
10	13.9	3.6
13	12.9	5.0
14		12.6
15		17.1
20		22.2

flask. Porphyrin synthesis then proceeded as for a normally aerated culture (Table 4). If the porphyrinogen of the still culture accumulated and was converted non-enzymatically when aerated, then it is indicated that the non-oxygen requiring steps from ALA to URO'gen were not inhibited in the culture (1) (Table 4) by the vigorous aeration. However, if the porphyrinogen formed is not converted fairly quickly into the porphyrin, it may be decomposed. In this case, the porphyrin formed on aeration of the still culture would have represented only a fraction of the porphyrinogen which had been produced in that flask during the first 13 days. Since the aerated maximum (1) was equivalent only to the thus depleted maximum of the still culture (2) (12.6 x 10^{-3} μmole/ml) then some inhibition of the anaerobic conversion of ALA to porphyrinogen must have occurred in the aerated flask (1). Whichever case is true aeration causes more rapid formation of porphyrin.

SUBSTRATE CONCENTRATION

Five flasks were supplemented with ALA to 0.43, 0.71, 1.0, 1.29 and 1.57mM respectively, and incubated on the rotary shaker at 30°C. Comparison of the amounts of porphyrin present would have little meaning, hence the

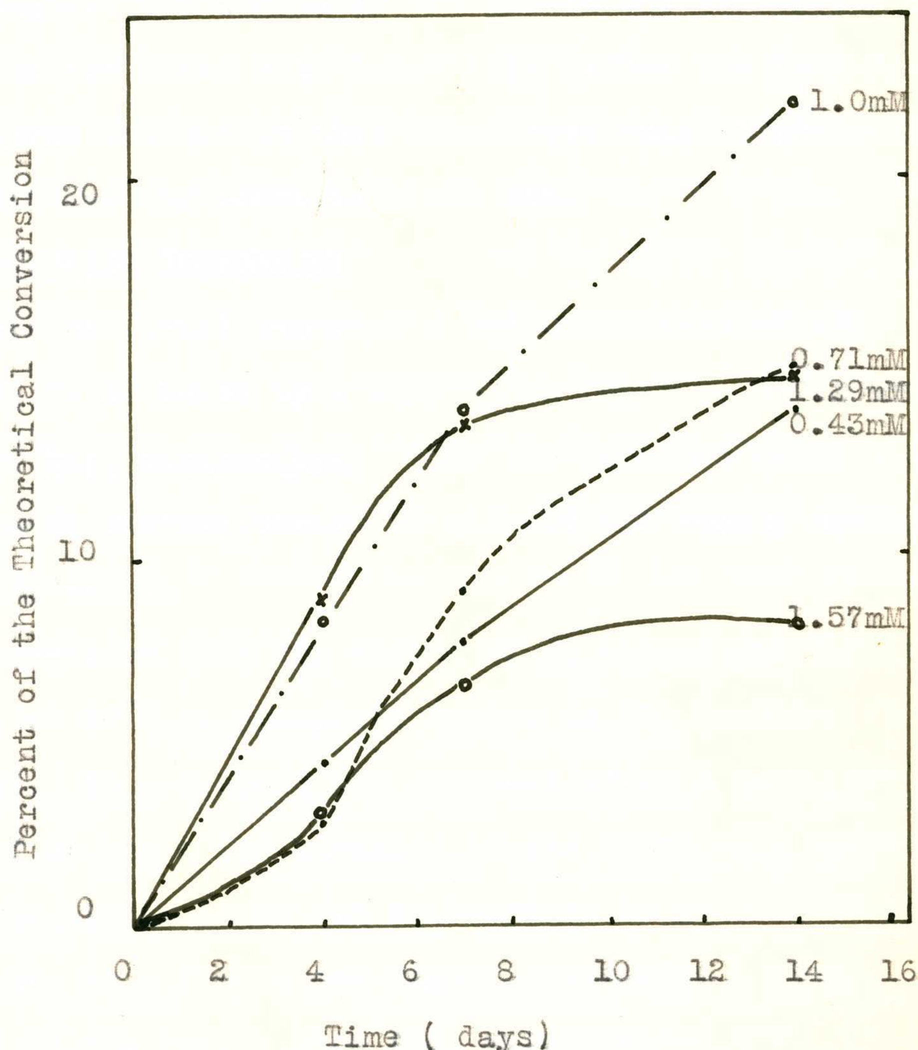


Fig. 5. Efficiency of substrate conversion. S. marcescens 211 grown with aeration at 30°C in the presence of various concentrations of ALA. 0.43mM —·— ; 0.71mM---·---; 1.0mM—·—o-; 1.29mM—x— ; 1.57mM —o—.

efficiency of the conversion was calculated. Now eight moles of ALA will condense to give one mole of porphyrin so that the theoretical yield of porphyrin can be calculated for each flask. Comparison of this figure with the porphyrin assayed, gives the efficiency as percent conversion (Fig. 5).

Throughout the course of the growth the efficiency was greatest in the flask with 1.0mM ALA. After the 14th day the porphyrin content of this flask rose only slightly till on the 54th day the conversion was 26%.

NON-GROWING SUSPENSION

Lascelles (1956) recorded a 50% conversion of ALA (2×10^{-3}M) in 24 hours by a suspension (approx. 1.0mg dry wt per ml) of Rps. sphaeroides. This was recorded under conditions which allowed photosynthesis to proceed and supply energy to the resting cells.

A suspension (2mg dry wt/ml) of S. marcescens 211 was incubated at 30°C in phosphate buffer in the presence of ALA (1×10^{-3}M). No porphyrin was apparent after 6 hours but at the end of 24 hours the contents of the flask were coloured a very pale pink. There was no increase in production when fresh cells, or a frozen and thawed suspension , were incubated in the buffer plus ALA, supplemented with glycerol or citrate as an energy source. Although no assay was performed here, it was obvious that growth of the organism would provide more efficient porphyrin synthesis.

MAXIMUM PIGMENT PRODUCTION

From the experiments on aeration and substrate

concentration, it is apparent that the time for maximum porphyrin production depends on the growth conditions. For subsequent experiments, the conditions employed were ---

ALA concentration	1×10^{-3}M
Temperature	30°C
Aeration	on rotary shaker
Time	14 days

In 14 separate preparations, the yield of crude porphyrin in the medium was of the order of 4-4.5mg per 200ml. This represents a conversion of 15-25% of the ALAin 14 days. The cells grown in the presence of ALA had an increase in porphyrin content over the controls. The porphyrin extracted and purified from the cells was equivalent to 1-2% of that found in the medium. The porphyrin composition of this extract was found by chromatography to be the same as that isolated from the medium.

PRODUCTION BY OTHER BACTERIA

Four strains of *S. marcescens* --211, 200, 251 and 330 -- and a strain each of *A. aerogenes*, *Ps. fluorescens* and *Streptomyces* were grown in small flasks of Bunting's medium. Strains of *B. subtilis* and *C. diphtheriae* and *S. marcescens* 211 as a control, were grown in cooked meat medium plus citrate. Each bacterium was grown in the presence and absence of ALA (1×10^{-3}M) for seven days, after which the medium was assayed for porphyrin. The porphyrin was then extracted by esterification in the usual way. The solutions of the free porphyrins were chromatographed in lutidine.

All cultures other than Streptomyces grew quite vigorously, so that the porphyrin content of the medium reflects the relative abilities of the various bacteria to convert ALA into free porphyrin. Since the meat does itself contain porphyrin, the medium in the absence of ALA was assayed. No porphyrin could be detected. The results of the assays are given in Table 5.

All the bacteria tested were able to convert ALA into porphyrin, and the differing efficiency was very marked in the Serratia group. In the meat medium, S. marcescens and

Table 5. PORPHYRIN SYNTHESIZED BY OTHER BACTERIA

BACTERIUM	PORPHYRIN (μmoles x 10^{-3}/ml)
Buntings medium	
S. marcescens 211	10.1
200	1.1
251	24.9
330	17.3
A.aerogenes	9.0
E. coli	1.1
Ps. fluorescens	15.8
Streptomyces	0.2
Meat medium	
S. marcescens 211	30.4
B. subtilis	29.9
C. diphtheriae	3.7

Growth at 30°C for 7 days. ALA 1×10^{-3} M.

B. subtilis produced a much greater amount of free porphyrin than C. diphtheriae. This was unexpected since C. diphtheriae is known to have an active porphyrin synthesis and excretion (Rawlinson and Hale 1949). Since natural excretion by C. diphtheriae depends on the iron content of the medium, a control mechanism may have inhibited production from ALA, or else the synthesized porphyrin may have been incorporated into cell cytochromes.

Since the porphyrin content of 10ml of medium is low, the applications to the chromatograms were necessarily small, so that after development, all porphyrins present probably did not appear. Table 6 indicates visible spots using the

Table 6. CHROMATOGRAM OF PORPHYRINS FROM OTHER BACTERIA

S. marcescens	211	A	.	.	.	E
	200	A	B	.	.	.
	251	A	B	C	D	E
	300	A	.	.	.	.
A. aerogenes		A	B	.	.	.
E. coli		A	B	.	.	.
Ps. fluorescens		A	B	C	.	.
S. marc. (meat)		A	B	.	.	.
B. subtilis		A	B	.	.	E
C. diphtheriae		A	.	.	.	.

Porphyrins extracted from media of Table 5 and chromatographed in 2,6 lutidine/water (35 : 27). Letters refer to porphyrins of Fig. 2.

letters A to F to indicate spots corresponding to those of Fig. 2.

The most notable fact is the predominance of uroporphyrin in every case. From this fact and from the spots appearing in the heavier applications, it is suggested that all the bacteria tested produce the same spectrum of porphyrins from ALA as does *S. marcescens* 211. This has been confirmed for *Ps. fluorescens*.

SECTION 2. FRACTIONATION AND IDENTIFICATION OF THE PORPHYRINS

In order to obtain sufficient porphyrin to attempt separation and accumulation of the fractions in quantity, usually six to ten flasks containing 70ml of medium were grown under conditions for maximum porphyrin production. The porphyrins were extracted as the esters and after conversion to the free porphyrins, they were evaporated to dryness. If the extract appeared greasy, a solution in 5N HCl was again shaken in several volumes of ether, and the ether was re-extracted with 5N HCl.

COLUMN CHROMATOGRAPHY

CELLULOSE

Columns of powered cellulose, 0.8 x 20cm, were packed in acetone with tamping. The free porphyrins were applied, and the column was developed with acetone. There was some movement into a wide, diffuse, pink band, with a fairly sharp brown band across its centre. However, the colours merged before they reached the bottom of the column. The addition of acid did not sharpen the bands.

MAGNESIUM OXIDE

The method of Nicholas (1951) was followed for porphyrin separation on grade III magnesium oxide. The columns were packed in chloroform, the porphyrins were applied as the esters, and the column was developed with chloroform/methanol. Separation into a number of pink and brown bands was noted

but again diffusion occurred in the lower part of the column.

ALUMINA

Columns, 0.8 x 20cm, were packed in chloroform and the porphyrins applied as the esters. Development in chloroform produced two pink fractions, and when the eluant was changed to 1% methanol in chloroform, a further pink plus yellow band was eluted. Electrophoresis and chromatography of the fractions indicated that the first two contained the less carboxylated porphyrins, and that the one eluted by chloroform/methanol contained the more highly carboxylated types. However, each fraction was found to contain a mixture of porphyrins.

Column chromatography proved to be unsatisfactory. The fractions were impure with regard to single porphyrins and the bands were ill-defined. In the case of the cellulose and MgO columns, the porphyrins were adsorbed over the whole column. Columns of alumina (McSwiney et al 1950) and calcium carbonate (Gray and Holt 1948) as well as other inert solids, have been used successfully in preparing pure porphyrins. A systematic investigation of several adsorbents has been made by Nicholas (1951). The clear separation of pure porphyrins obtained by these workers could not be duplicated in this present work.

SOLVENT FRACTIONATION

The cells were removed from a fresh preparation and the medium was extracted to completion with ether/acetic acid (10 : 1). The ether layer was extracted, first with 0.2% HCl

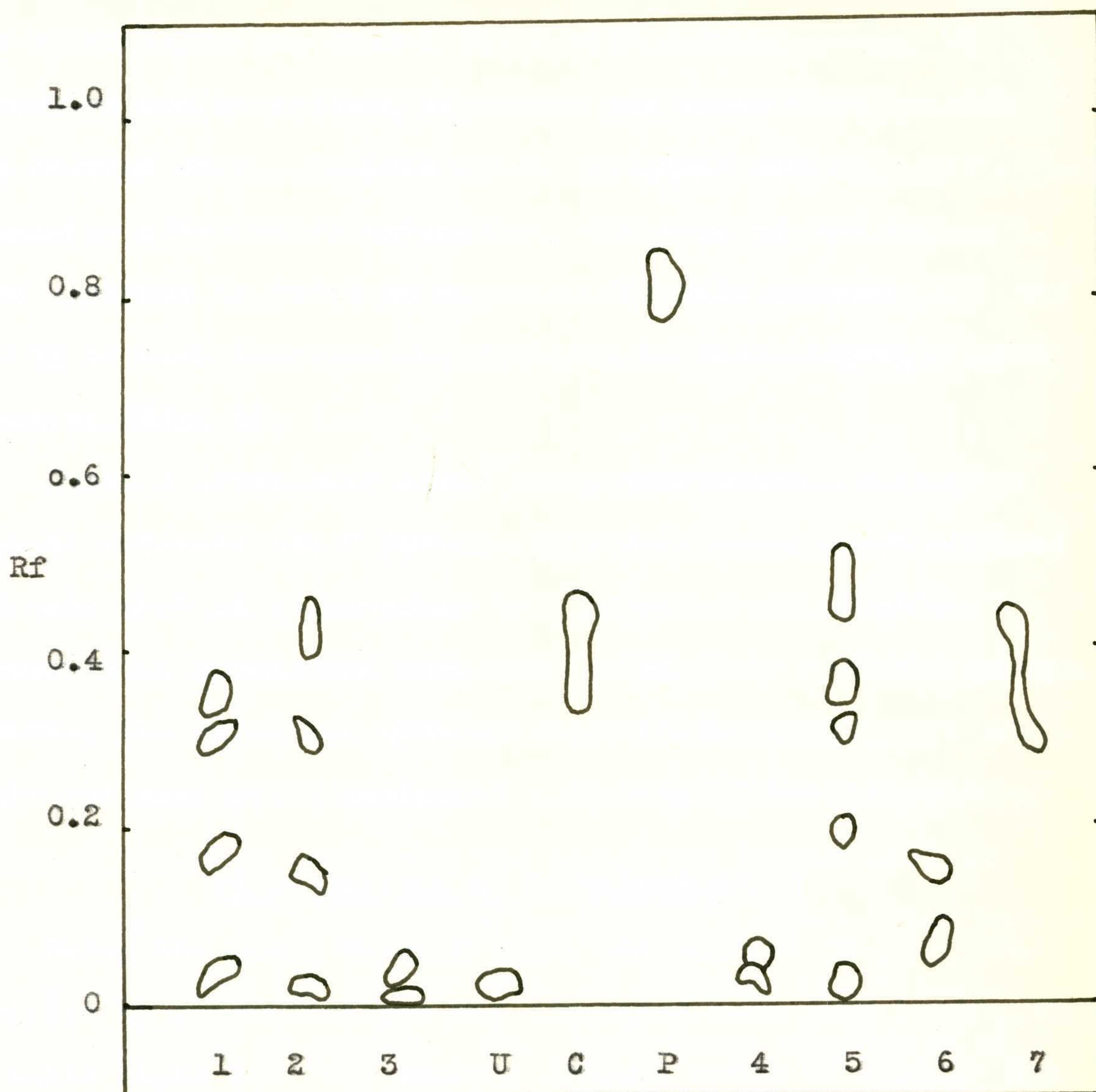


Fig. 6. Chromatographic separation of solvent fractionation of porphyrins. Porphyrins were extracted into ether (pH 4) then from ether into 0.2% and 2% HCl. Theether was also washed with sod. acetate. 1. Original medium; 2. Ether extract; 3. Remainder after ether extraction; 4. and 5. 0.2% and 2% HCl extracts respectively; 6. Sod. acetate extract; 7. Remainder in ether after sod. acetate washing; U, C and P : uro-, copro- and protoporphyrin standards.

to completion, then with 2% HCl. In another fractionation, the ether layer was washed first with a small volume of saturated sodium acetate, to remove any uroporphyrin which was carried over. Samples of the original medium, the remainder after ether extraction, and each of the extracts, were dialysed in water to decrease the salt content, and the purity of each fraction was followed by chromatography with lutidine (Fig. 6).

Spots 3 (remainder after ether extraction), 6 (sodium acetate extract) and 7 (ether after sod. acetate extraction) indicate that it is possible to fractionate the highly carboxylated porphyrins from those with fewer carboxyl groups. However, the solutions obtained were still contaminated with salts and each still contained a mixture of the porphyrins.

FRACTIONATION BY ELECTROPHORESIS

Using the small Model R cell, the mixture of porphyrins purified by esterification was separated into five bands, visible in white light but very sharply defined under UV light. The fractions could be cut out and eluted from the paper by washing in constant boiling HCl or by esterification. The Model CP was useful in preparing only fractions 1 and 2 in quantity. The porphyrins were obtained in sodium carbonate solution and were purified by esterification.

The electrophoretic method of fractionating porphyrins has several advantages over other methods tried. It is speedy and simple. The small model was particularly useful

because of the number of fractions obtained immediately. There was no loss of total porphyrin nor of any single fraction as was possible using the column or solvent fractionations; the bands were clear and there was no fluorescence in the intervening spaces. As noted in the methods section, some bands overlapped. However it was possible to cut pure fractions by sacrificing the yield slightly; no whole fraction was lost.

Initially the bands were cut to obtain total yield rather than pure fractions. Each was eluted into acid and prepared for chromatography and assay of porphyrin content. The result of chromatography is shown in Fig. 7; the letters A to G refer to porphyrins as in Fig. 2. Each time, there was only one spot from fractions 1 (A), 2 (A), and 3 (D). Fraction 4 always contained the single or double spot of coproporphyrin (E and F), the lower spot D often being absent, and occasionally the higher spot (G) between copro- and protoporphyrins appearing. When sufficient quantities of fraction 5 were obtained for chromatography, the coproporphyrin spot was always present; spots D and G were visible occasionally.

The overall picture was as expected, with the more highly carboxylated porphyrins running further on the electrophoresis strip. Fraction 3 was well separated from the other bands, so that its single porphyrin could be obtained pure. Fraction 2 contained only one spot, uroporphyrin. Since it was mixed with fraction 1, it is

likely that the brown is not due to a porphyrin. The appearance of a D spot in fraction 4 may be due to adsorption. Fraction 5 fluoresced green and not red as the porphyrins do; the porphyrins appearing on chromatography of this may have been contaminants from fraction 4.

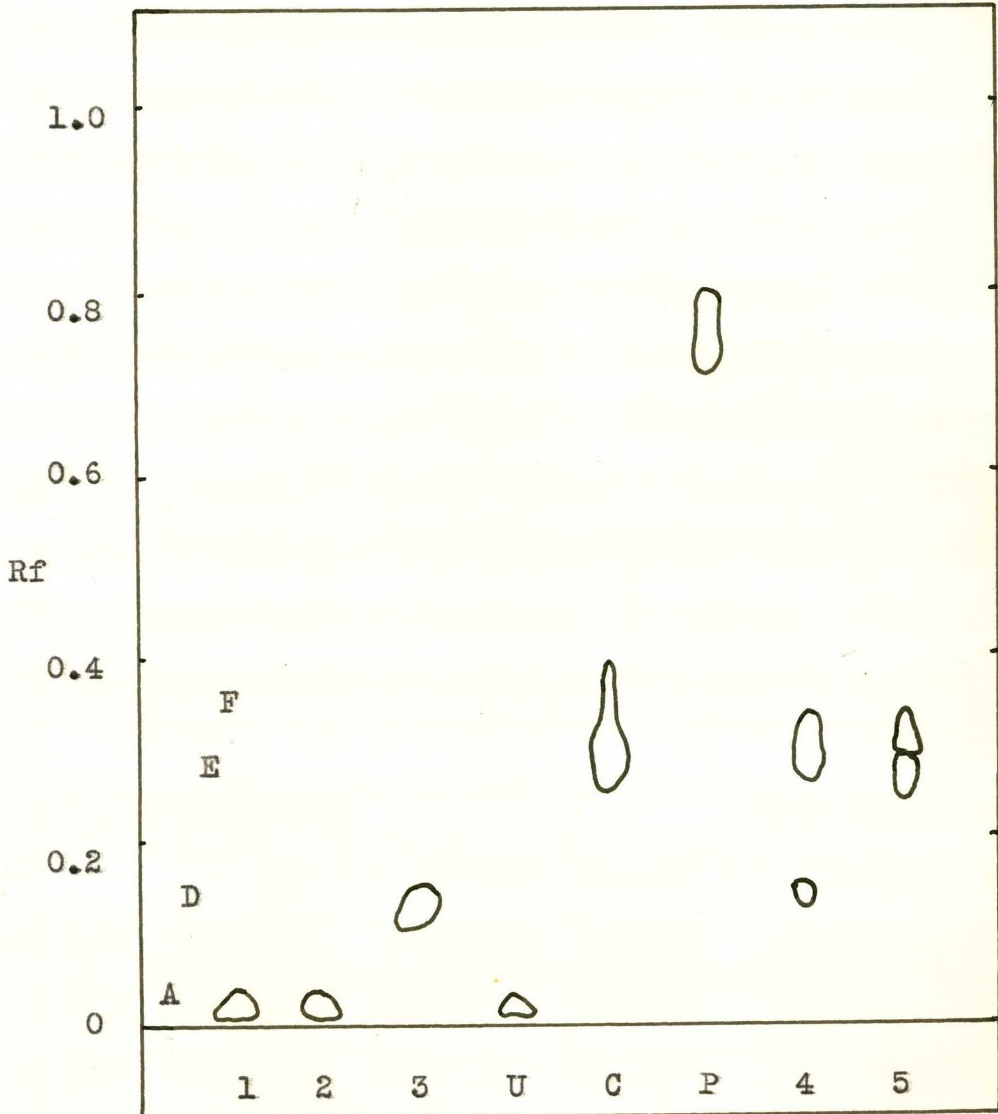


Fig. 7 Chromatogram of electrophoretic fractionation. Fractions 1 to 5. U, C, P : uro-, copro- and protoporphyrin standards.

The fractions 1 to 5 obtained above were assayed separately, the data of Rimington (1960) for uroporphyrin being applied to fractions 1 and 2, and that for coproporphyrin being applied to fractions 3, 4 and 5. The separate results from seven electrophoresis runs of five preparations were expressed as a percentage. The results varied considerably as follows --- 1. 42-80%, 2. 4-37%, 3. 1-3%, 4. 5-16% and 5. 2-7%. Bearing in mind the fact that fractions were mixed, 1 with 2 and 4 with 5, (Fig. 7), the results were regrouped and presented in Table 7. Fraction 1 + 2 is uroporphyrin, which represents 79-88% of the total porphyrin produced. The "mid" fraction represents 1-3%, while fraction 4 + 5 contains 10-18% of the total porphyrin.

The fractions were next identified more fully. They were cut so as to prevent contamination with adjacent fractions.

Table 7. ASSAY OF ELECTROPHORESIS FRACTIONS

	Porphyrin % of total.		
PREPARATION	1 + 2	3	4 + 5
a	84	3	13
a	84	3	13
b	87	1	12
b	88	2	10
c	87	2	11
d	79	3	18
e	87	2	11

Fractions eluted from paper with acid.

IDENTIFICATION OF THE PORPHYRINS

FRACTION 1.

Solutions of fraction 1 from the Model CP and Model R cells were combined, the methyl ester was prepared, washed and crystallized from chloroform/methanol. The ester was redissolved in chloroform and purified on a grade III MgO column under pressure, by the method of Nicholas (1951). It was recrystallized twice from chloroform/methanol and dried under vacuum. The ester was a dark red solid, soft and quite fibrous in texture, and not crystalline in appearance.

Remaining on the column after elution of the main component, was a narrow brown band at the top with a pink band immediately below it. The brown band was contamination from the brown fraction 2. An attempt was made to remove the pink band with more polar solvents. Only water, after extrusion and cutting of the column, was partly successful, but the amount was so small that no attempt could be made at identification. It is thought that it may correspond to porphyrin B or C of Fig. 2, or since it was slightly water soluble, that it may be incompletely esterified uroporphyrin of fraction 1.

A small amount of the ester was converted to the free porphyrin and chromatographed in lutidine in several concentrations, so that any contaminant would be revealed. Each application showed that the preparation was pure uroporphyrin. It will now be called URO F1 for convenience.

The visible absorption spectrum of the ester in pure

chloroform was examined (Fig. 8). The peak positions are compared in Table 8 with the uroporphyrin spectrum recorded by Lemberg and Legge (1949). The spectra coincide very well except for the absence of the peak at 581.5mμ. This peak appears from Fig. 8 to be incorporated into the unsymmetrical peak at 570.5mμ.

The porphyrin was shown to be uroporphyrin by electrophoresis, chromatography on MgO and in lutidine, and by its spectrum. The melting point of the ester was found and

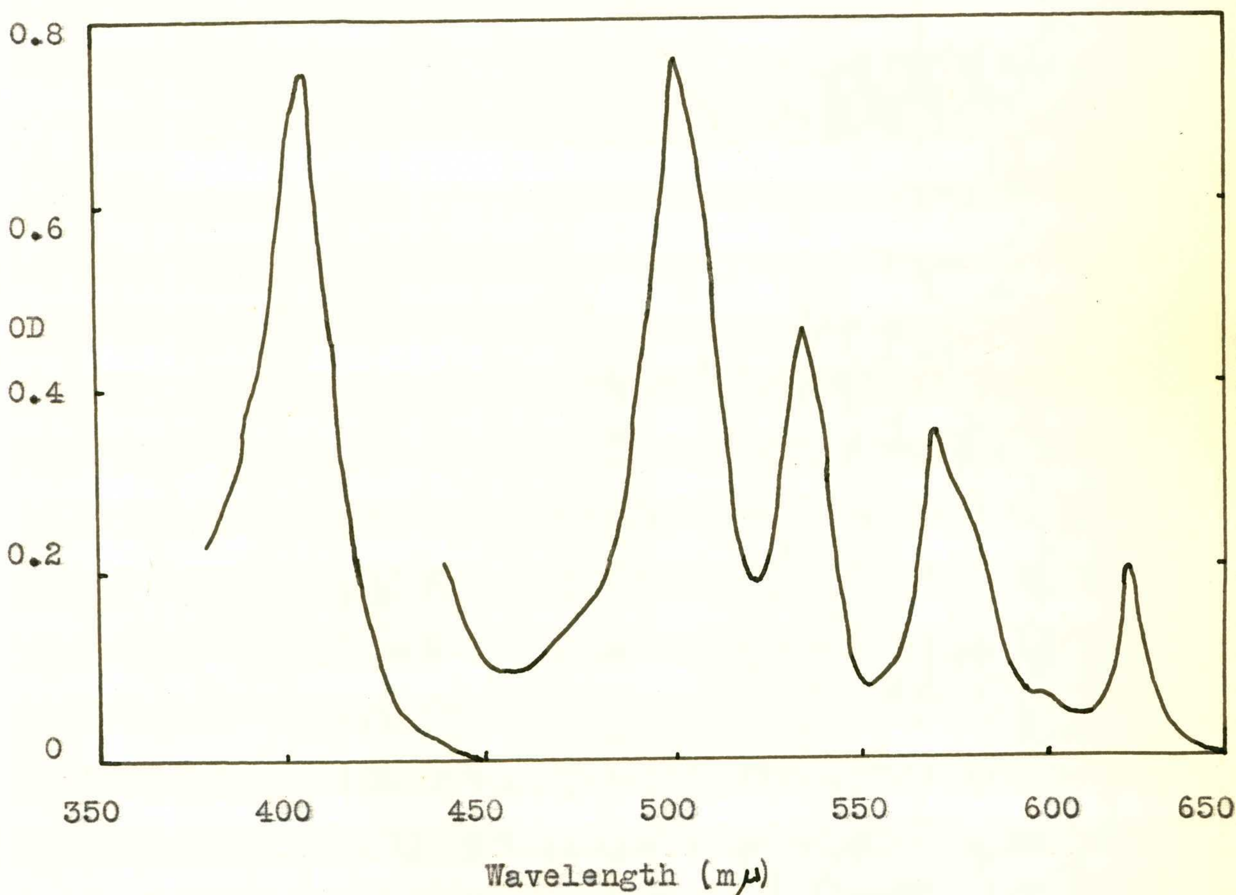


Fig. 8. Visible absorption spectrum of fraction 1 from the *electrophoretic separation of the pigment. Purified ester in* chloroform. Solution diluted 15-fold between 450 and 380mμ.

Table 8. SPECTRA OF UROPORPHYRIN ESTERS

Peak positions mμ. Solvent chloroform.

FROM LEMBERG AND LEGGE (1949).	URO F1
626	625
(599)	(597)
581.5	
570.5	570.5
536	535.5
501	501
408	409

Table 9. UROPORPHYRIN ESTER MELTING POINTS

		COMPOSITION OF EACH SAMPLE ǂ	
		URO. I (%)	URO. III (%)
UROF1	258-262°C	80	20
		15	85
URO. I*	286°C	90	10
URO. III*	256°C	80	20
		25	75
URO. I xx	292-294°C	100	
URO. III xx	264°C		100

* Experimentally determined on samples from Light and Co.

According to Dawson et al (1959).

ǂ According to Nicholas and Rimington (1953).

compared with melting points determined on samples of uroporphyrins I and III (URO. I and URO. III respectively) (Table 9). During heating the appearance of the URO F1 changed from red to black at 200-210°C, and at about 230°C the thinner patches of the mass resolved into fine, short black needles. This change to needle-like forms was observed also in the URO. I standard. These phenomena made the melting point difficult to determine. The difficulty is experienced by other workers. The melting point was taken as the temperaturewhen the edges of the mass became clear and began to spread. The URO F1 had completely melted at 280-285°C.

Neither the URO F1 nor the standard samples had the melting points of pure uroporphyrins, and yet on lutidine chromatography all three ran in a single spot. Nicholas and Rimington (1953) have determined the melting points of mi mixtures of uroporphyrins I and III in varying proportions. From the presentation of a curve of melting point against proportion of each isomer, an indication of the isomer composition of each sample may be obtained (Table 9) (two because of the shape of the curve).

Three methods were used to determine the isomer composition of the URO F1 and, since they appeared to be impure, also of the standard samples. The methods were the double solvent chromatography technique of Falk and Benson (1953), infrared spectroscopy, and partial decarboxylation of the uroporphyrin to coproporphyrin followed by lutidine

chromatography.

A tracing of a typical Falk and Benson chromatogram is given in Fig. 9. The lower spot in each case is uroporphyrin I, Rf 0.03-0.06, and the upper is uroporphyrin III, Rf 0.54-0.58, the value being calculated from the positions of the spots before and after the second (kerosene/dioxane) development. On a few chromatograms, the standard URO. I showedtraces of uroporphyrin III. The chromatograms

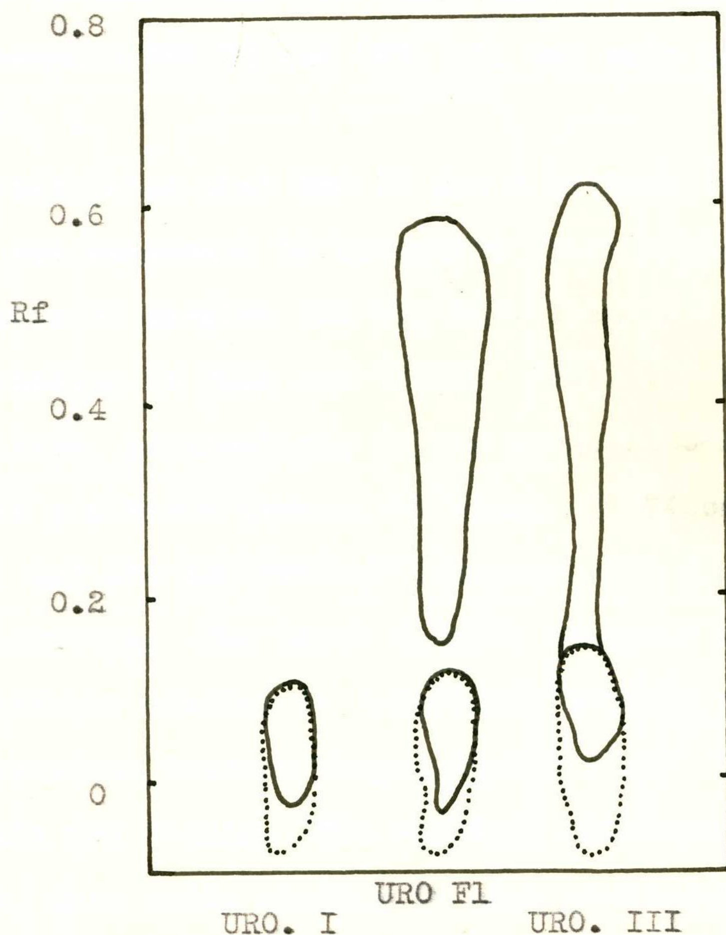


Fig. 9. Identification of uroporphyrin isomers by chromatography (Falk and Benson 1953). URO. I and URO. III : standard uroporphyrins I and III; URO F1 : uroporphyrin of fraction 1 from electrophoresis. Dotted lines show spot positions after development with the first solvent.

compared reasonably well with those of Falk and Benson, who gave Rf values for uroporphyrins I and III as 0.02 and 0.5 respectively. They claim that in mixtures of the pure isomers, a 15% admixture of either was easily distinguished. Falk and Benson (1954) refuted a claim of Watson and Berg that the method was much less sensitive. If this is so, and if the indication of the isomer composition given by melting point data was correct, then one would expect to see both isomers from samples URO F1 and URO. III but only uroporphyrin I from URO. I.

Having established that URO F1 was a mixture of isomers I and III, it was necessary to determine which predominated. Infra-red spectra of samples URO F1, URO. I and URO. III were examined. According to Falk and Willis (1951) the spectra of the isomers differ, particularly in the range 1100-1300cm^{-1}. The actual peak positions correspond except at 740cm^{-1} (uroporphyrin III) and 850 and 1260cm^{-1} (uroporphyrin I), but the heights are different. The three samples examined had spectra (Fig. 10) remarkably similar to eachother, showing none of the decided variation between uroporphyrins I and III recorded by Falk and Willis. The spectra, although not identical with, were quite similar in peak positions and relative heights to that of uroporphyrin I given by Falk and Willis. Thus all three samples URO F1, URO. I and URO. III appeared to be mainly uroporphyrin I.

Coproporphyrins I and III are separable above a 5% contamination by the technique of lutidine chromatography, the III isomer running ahead of the isomer I (Falk 1955).

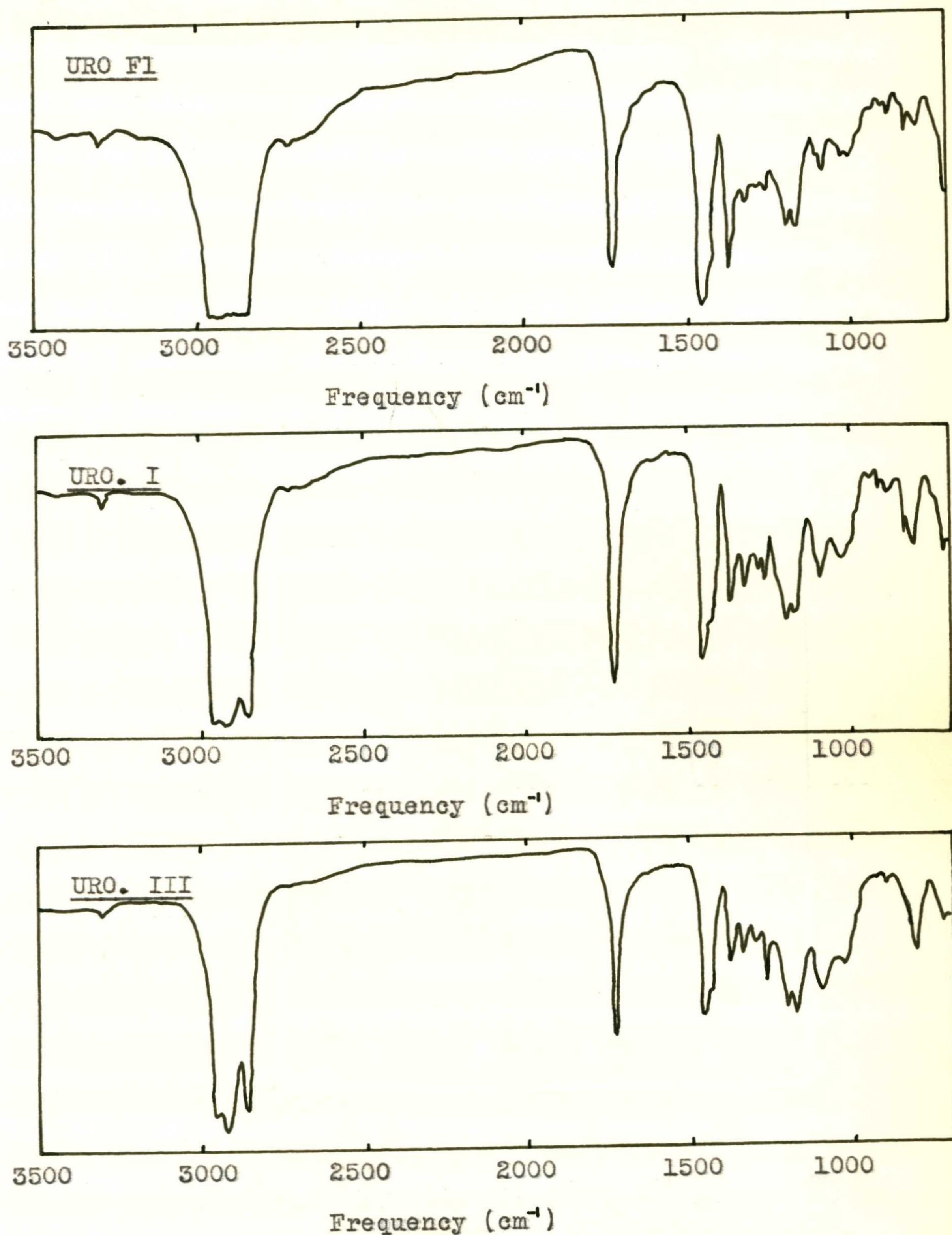


Fig. 10. Infrared spectra of esters of uroporphyrin (URO F1) from electrophoresis and "standard" uroporphyrins I and III.

The three uroporphyrin samples were decarboxylated partially to their corresponding coproporphyrin isomers. The acetic acid side chains of uroporphyrin are unstable in 1% HCl at 180°C and are decomposed to methyl groups. After heating, the samples were prepared for chromatography with lutidine (Fig. 11). The coproporphyrin III standard was apparently not pure. The URO. I sample contained isomer I only, and the URO. III and URO F1 were predominantly isomer I but showed signs of the presence of isomer III.

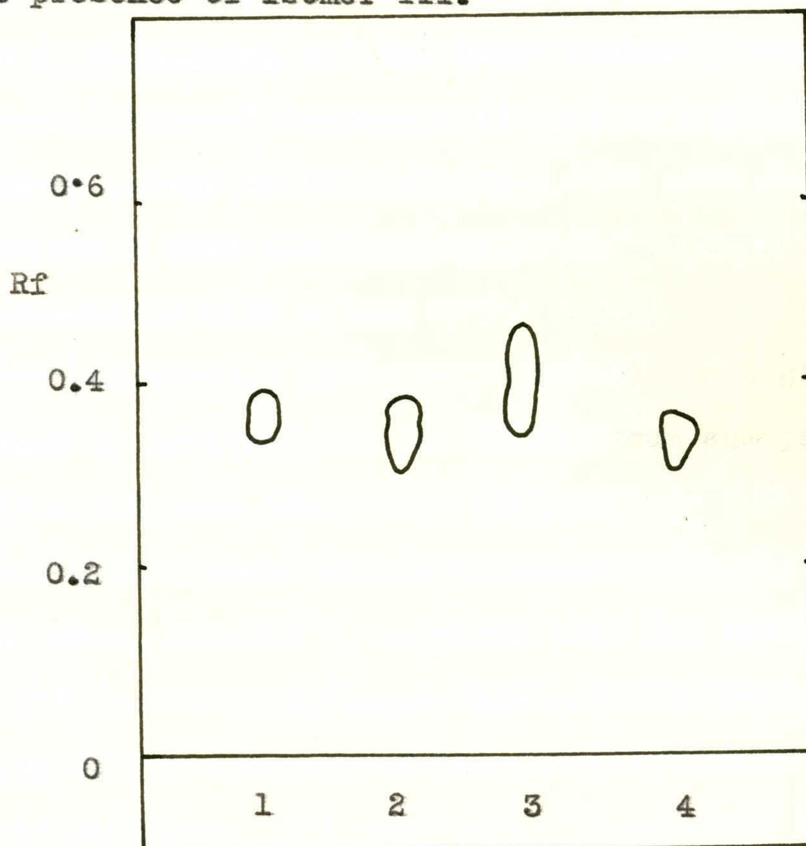


Fig. 11. Identification of uroporphyrin isomers. Samples decarboxylated to coproporphyrin; chromatogram developed with 2,6 lutidine/water (35 : 27). Samples were : 1 and 4 -- URO. I and URO. III originally, 2 -- URO F1 originally, 3 -- sample of coproporphyrin predominantly isomer III.

The excess formation of uroporphyrin I by S. marcescens 211 in the presence of ALA is interesting when considered in relation to general porphyrin synthesis. Enzymes isolated from various tissues (Bogorad 1958 a, b, Mauzerall and Granick 1958) tend to form isomer III while under abnormal conditions isomer I predominates (Bogorad and Granick 1953 b, Granick and Mauzerall 1958, Lockwood and Benson 1960). The abnormal conditions of some human porphyrias cause excretion of uroporphyrin I (Rimington 1952 b). No correlation can be made between S. marcescens and the other tissues, but there may be a "detoxification" mechanism at work to deplete the abnormally large amount of ALA present.

The fraction 1 from electrophoresis then, is uroporphyrin, possibly contaminated with a very small amount of a 7- or 8-carboxylated porphyrin. It was proved by chromatography to be a mixture of isomers, and the IR spectrum and partial decarboxylation indicate that it is predominantly uroporphyrin I with a small amount of isomer III. The melting point of the ester, 258-262°C, suggests that the ratio of isomers I : III is 4 : 1, although this ratio is possibly low, depending on the accuracy of the criterion used for the temperature of melting.

FRACTION 2

The coloured material separated on the Model CP cell was obtained in 15 adjacent fractions of the total 32; those moving more slowly to the positive electrode were yellow, or dilute solutions of the brown fraction 2. The three slowest,

when assayed, showed a complete absence of the porphyrin Soret peak.

In order to ascertain if this brown fraction was or was not porphyrin, the acid spectrum of a concentrated solution was examined. There were no absorption peaks at all. Absorption rose gradually from zero optical density at 650-600mμ and then more quickly to an O.D. of 2.0 at 350mμ. This is not a typical porphyrin spectrum.

A concentrated sample was tested for the presence of pyrrole rings. Porphyrins as such give a negative result but fusion with alkali frees the α-position on the pyrrole ring so that it reacts with Ehrlich's reagent (see page 31). The brown sample tested before and after alkaline fusion, gave negative results.

Fraction 2 contains a brown component and is contaminated with trailing uroporphyrin from fraction 1 (see Fig. 7). The absorption spectrum , porphyrin assay and test for pyrroles, proved the brown component is non porphyrin.

FRACTION 3

This fraction constitutes only 1-3% of the total porphyrin produced, so that insufficient was obtained to attempt an exact identification. Its spectrum, fluorescence and chromatographic behaviour indicate its porphyrin character.

A very small amount of its methyl ester was crystallized from chloroform/methanol, washed in methanol and its spectrum in chloroform examined. The peak positions were almost identical with those of fraction 4 below, and corresponded very closelyto values for coproporphyrin (Table 10).

Because of its chromatographic and electrophoretic mobilities, it cannot be coproporphyrin.

Chromatography of the free porphyrin in lutidine, indicates that it may contain 5, 6 or 7 carboxyl groups (Figs. 2 and 7), most probably 6 or 7. Penta- and hexa-carboxylic porphyrins have been isolated from porphyria urines by Canivet and Rimington (1953). The spectra of the esters of these porphyrins show α-bands at 622.5mμ and 623.6mμ respectively. The ester of fraction 3 had an α-band at 622.5mμ but the measurement was not accurate enough to distinguish between the two. In any case, spectral data is an inefficient means of identification when considered alone, since workers report so many different results. For instance Canivet and Rimington (1953) give a value of 621.4mμ for the α-band of copro-

Table 10. SPECTRA OF ESTERS IN CHLOROFORM

Peak positions (mμ).

COPROPORPHYRIN*	PURIFIED FRACTION 3	PURIFIED FRACTION 4
622.5	622.5	621
(596.3)	(597)	(596)
(577.5)		
568	567	568
533	532	533
499	498-502	499-502
405	402	402

* From Lemberg and Legge (1949).

porphyrin ester, Lemberg and Legge (1949) give 622.5mμ.

Fraction 3 could be identified only as a single porphyrin with 5, 6 or 7 carboxyl groups.

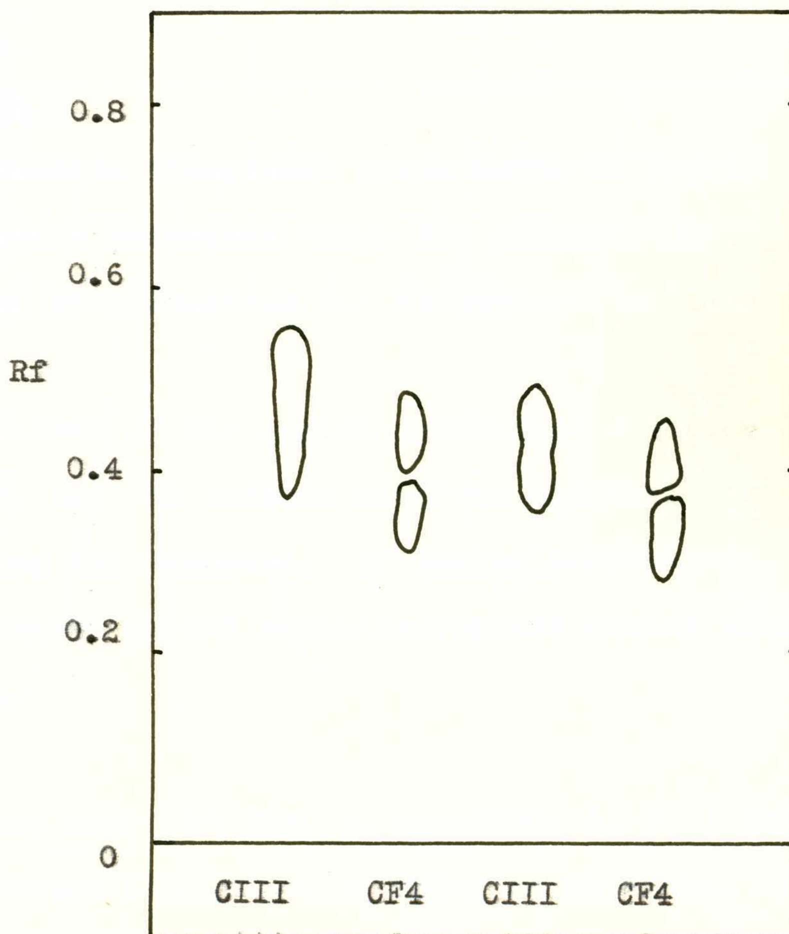
FRACTION 4

A quantity of this porphyrin was obtained from the Model R cell by cutting papers well away from the following fraction 5. It was purified by two crystallizations of the ester from chloroform/methanol, the product being dark red and non crystalline. It ran as pure free coproporphyrin on a lutidine chromatogram.

The spectrum of the ester in chloroform was of the Etio type (Granick and Gilder 1947) similar to the spectrum of uroporphyrin (Fig. 8). The peak positions was compared with those reported for coproporphyrin (Table 10). The peak recorded at 577.5mμ was incorporated into the peak at 568mμ causing its asymmetry.

Again in this fraction, sufficient material was available to attempt identification of its isomeric composition. Its ester melting point was determined using the criterion taken for uroporphyrin, i.e. the point when the edges of the mass melted. The melting point was determined as 175-179°C. Jope and O'Brien (1945) have constructed a melting point-composition curve for the esters of coproporphyrins I and III. They found that 10-15% of one isomer may remain undetected in the other if the melting point is the sole criterion; the resolidification point was thought to be the more sensitive criterion of isomer purity. A

confusing factor is the reported double melting point of coproporphyrin III; after melting partially between 130-153°C, it resolidifies and melts at 150-170°C again. The melting point of fraction 4 (one only) indicates that its composition of isomers I : III is 15 : 85. However, the ratio is most certainly lower than this; the ester was crystallized from chloroform/methanol, and since coproporphyrin I is much more insoluble in methanol than the isomer III, the relative



Fig, 12. Identification of coproporphyrin isomers by chromatography with 2,6 lutidine/water (35 : 27). CIII : standard, predominantly coproporphyrin III; CF4 : purified fraction 4 from electrophoresis.

concentration of coproporphyrin I would have been increased.

Chromatography of the free porphyrin with lutidine showed the presence of both isomers I and III (Fig. 12) and these were, surprisingly, in approximately equal concentration as judged from the size and fluorescence of the spots. Before esterification also, the concentrations appeared to be equal.

Fraction 4 then has been shown to be pure coproporphyrin. Both isomers I and III are present but isomer III predominates.

FRACTION 5

This fraction fluoresced green under UV light. It was shown to contain porphyrin (Fig. 7), possibly through contamination with fraction 4. No attempt was made to isolate it.

The spectrum of the impure esterified fraction 5 is shown in Fig. 13. No further work was directed towards characterizing the compound. It may be substance(s) formed through breakdown of the other porphyrins during their purification.

Referring again to Fig. 2, compounds A, D, E and F have been isolated and identified as fully as possible. Compound G would probably be found in fraction 5 from electrophoresis, while a preliminary finding was made that compounds B and C may be separable from fractions 1 plus 2 as esters on a magnesium oxide column. Their number of carboxyl groups

indicate that they would run behind uroporphyrin in fraction 2. It was found that compounds B, C and G were present in such small amounts that further identification was not practicable.

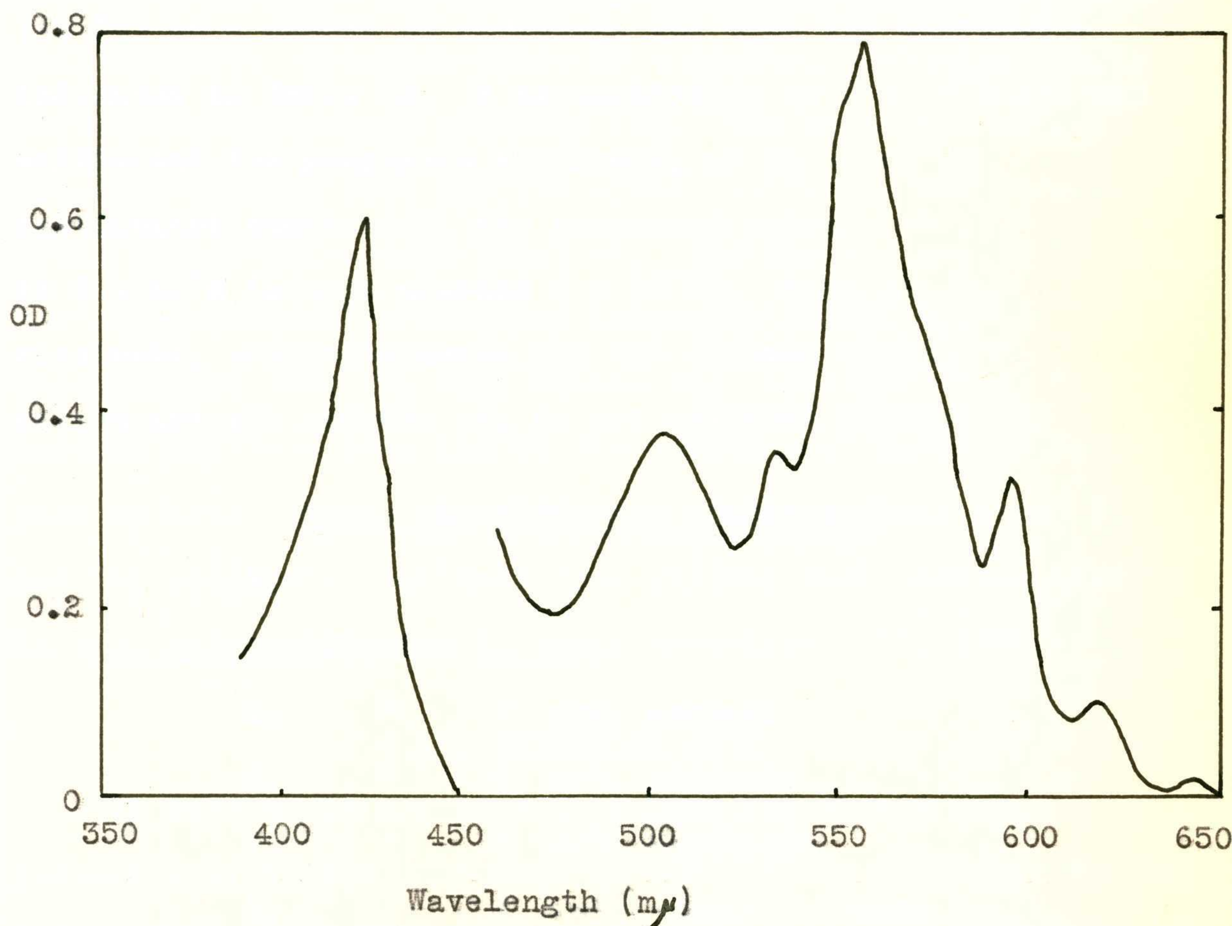


Fig. 13. Visible absorption spectrum of unpurified fraction 5 from the electrophoretic separation of the porphyrins. Ester in chloroform.

SECTION 3. FACTORS INVOLVED IN PORPHYRIN PRODUCTION

REPLACEMENT OF δ AMINOLAEVULINIC ACID

An experiment was carried out to determine if *S. marcescens* strain 211 could synthesize porphyrin from the precursors of ALA. These precursors have been shown, in animal and plant tissue and in bacteria, to be glycine and a four carbon compound derived from the TCA cycle. Flasks of Bunting's (1940) medium were supplemented with yeast extract (to 1%) as a source of cofactors and with the substrates indicated in Table 11. A concentration of 1 x 10^{-3}M ALA was sufficient for porphyrin synthesis, so to allow for inefficient conversion, the precursor substrates were added to 5 x 10^{-3}M (except alanine 3 x 10^{-3}M). Acetate, pyruvate, succinate, α-ketoglutarate and the citrate already in the medium are all members of, or are oxidized through the TCA

Table 11. ALTERNATE SUBSTRATES

1.	-		
2.	Glycine		
3.	"	+	sod. acetate
4.	"	+	sod. pyruvate
5.	"	+	sod. succinate
6.	"	+	sod. α-ketoglutarate
7.	Alanine		
8.	"	+	sod. α-ketoglutarate

cycle. Flasks were incubated with aeration at 30°C. Samples were assayed for porphyrin on the 3rd and 7th days.

No porphyrin was synthesized. It is unlikely that a permeability barrier to these molecules existed, or that acetate, pyruvate etc. could not be converted to the active four carbon unit, since S. marcescens possesses an active TCA cycle (Green and Williams 1959). It is also unlikely that the organism completely lacks the ability to condense glycine with the active unit, since it is known to synthesize the porphyrin unit for incorporation into cytochromes (Fujita and Kodama 1934). Possibly there is a control mechanism acting on the reaction which produces ALA. The synthesis of ALA requires oxygen, ATP, the TCA cycle, PALPO and DPN (Granick 1958) all of which were supplied. The enzymes may be in a very low concentration so that any porphyrin formed was incorporated into the cell substance. Alternatively, the control may have been due to the presence of some inhibitor such as iron, in the basic medium. Iron has been shown to inhibit porphyrin synthesis from glycine plus α-ketoglutarate in Rps. sphaeroides and T. vorax (Lascelles 1956, 1957).

THE EFFECT OF IRON

In several trials with S. marcescens strain 211, porphyrin synthesis increased in the absence of iron. Flasks fitted with glass caps were freed from iron, and iron-free Bunting's medium was supplemented with iron of concentrations of 0, 0.54, 1.07, 1.61, 2.68mM Fe . ALA (1×10^{-3}M) was added in duplicate to each iron concentration. All the

flasks were inoculated and incubated for 5 days at 30°C and the porphyrin content was assayed. Table 12 shows results from a typical experiment.

It is apparent that the presence of iron caused a 15-20% inhibition of porphyrin synthesis from ALA over the 5 day period. There was no increase in inhibition when the iron content was raised from 0.54mM to 2.68mM, and no relationship was found such as that observed in C. diphtheriae (Pappenheimer 1947 a, b).

Propionibacterium shermanii excretes large amounts of porphyrin and can readily convert ALA to porphyrin (giving up to a 6-fold increase) but not to vitamin B_{12}. The presence of iron decreases the amount of porphyrin produced. Pronyakova (1960) found that a very high iron concentration (3.6μg atoms/ml) completely suppressed porphyrin by this organism, and that the addition of ALA then increased vitamin B_{12} synthesis. This indicates that iron plays a regulatory

Table 12. THE EFFECT OF IRON ON PORPHYRIN SYNTHESIS

IRON CONCENTRATION (μmoles Fe/ml)	PORPHYRIN IN FLASKS (μmoles x 10^{-3}/ml)		MEAN PORPHYRIN (μmoles x 10^{-3}/ml)
0	2.13	2.18	2.15
0.54	1.59	1.86	1.73
1.07	1.74	1.88	1.81
* 1.61	1.94	1.69	1.82
2.68	1.85	1.80	1.83

Substrate ALA (1 x 10^{-3}M), 30° C, S. marcescens 211.

* Concentration in normal medium (Bunting 1940).

role. There may be a regulation by iron with S. marcescens but it is different from the above mechanism since there were no signs of complete inhibition of porphyrin synthesis with the higher iron concentrations.

Other than the paper by Pronyakova (1960), the literature shows no mention of any inhibition by iron on the conversion of ALA to COPRO'gen. Iron partially inhibits the condensation of glycine and α-ketoglutarate to ALA in Rps. sphaeroides (Lascelles 1956) but has no inhibitory effect on the conversion of ALA to porphyrins in Rps. sphaeroides or T. vorax (Lascelles 1956, 1957). In the latter cases the iron does, however, direct synthesis in favour of protoporphyrin production. This has been shown not to occur in S. marcescens since at no time was protoporphyrin detected in the growth medium or in excess in the cells.

REQUIREMENT FOR AN OXIDIZABLE SUBSTRATE

It was found that modified Norris medium, which contained glucose, potassium phosphate, magnesium sulphate, iron , sodium molybdate, calcium and ammonia, would not support the synthesis of porphyrin from ALA. Since Bunting's medium did support the synthesis, those components present only in the Bunting's medium were added separately to the modified Norris medium. These were glycerol, sodium chloride,iron , citrate. The citrate-containing flask was the only one where porphyrin was synthesized from ALA. Accordingly, S. marcescens 211 was grown in citrate-free Bunting's medium, in the presence of ALA (1×10^{-3}M) and other carboxylic acids. The flasks

were inoculated and incubated at 30°C. The porphyrin content of each flask was assayed after 9 days (Table 13). Good growth was obtained in each medium. The control flasks contained no porphyrin.

Porphyrin synthesis was considerably lower in the absence of the carboxylic acids. The presence of a compound oxidizable through the TCA cycle, increased the conversion to porphyrin 5- to 6-fold with the exception of fumarate, which caused only a 3-fold increase. The reason for the difference with fumarate is not clear.

Lascelles (1956) investigated the requirements for an oxidizable substrate for porphyrin synthesis by Rps. sphaeroides. Synthesis from glycine and α-ketoglutarate

Table 13. THE EFFECT OF OXIDIZABLE SUBSTRATES

SUBSTRATE ADDED (2×10^{-3}M)	PORPHYRIN IN FLASKS (μmoles x 10^{-3}/ml)		MEAN PORPHYRIN (μmoles x 10^{-3}/ml)
Nil	0.50	0.63	0.57
Acetate	2.84	2.93	2.89
Pyruvate	1.86	2.99	(2.43)
Citrate	3.08	2.84	2.96
α-ketoglutarate	1.54	3.45	(2.50)
Succinate	2.46	3.54	(3.00)
Fumarate	1.62	1.79	1.71
Malate (DL)	2.93	3.05	2.99

All flasks supplemented with ALA (1×10^{-3}M), inoculated with S. marcescens 211 and grown at 30°C for 9 days.

required an additional substrate; fumarate, succinate, malate, oxalacetate, lactate and pyruvate were equally effective, while acetate showed only one tenth of the activity. Synthesis from ALA required the substrate only if carried out in the dark. Rps. sphaeroides is a photosynthetic organism and apparently produces enough energy in the light for porphyrin production from ALA. Fumarate, succinate and lactate were equally efficient as oxidizable substrates for cells grown in the dark.

Serratia is not a photosynthetic organism. The results with the carboxylic acids above, indicate that an energy supply is required to convert ALA to porphyrin in S. marcescens. This fact may explain the increase in porphyrin production when the culture was vigorously aerated. Air is not directly required for the reactions between ALA and COPRO'gen in other tissues, but an increased oxygen supply to S. marcescens may increase the supply of energy from the citrate present via the TCA cycle, thereby enhancing porphyrin synthesis.

THE RELATIONSHIP BETWEEN ALA AND PORPHYRIN

It was thought necessary to demonstrate the utilization of ALA during the production of the porphyrins. Previously, it was shown merely that porphyrin was synthesized in the presence of ALA. Three flasks containing Bunting's medium were set up. Two were supplemented with ALA (1×10^{-3}M), one as the "experimental" flask, one as the "ALA control" flask. The third served as a "blank". The "experimental" and the

"blank" were inoculated with an even suspension of strain 211 and all three were incubated at 30°C with vigorous aeration. Samples (approx. 3ml) were taken at zero time and at known time intervals, and after checking turbidity at 600mμ for dry weight determination, the cells were removed and samples were assayed for ALA, porphyrin and PBG.

Growth of the organism was identical in both the "experimental" and the "blank" cultures, showing a typical growth curve after a slight lag in the first three hours. No free porphyrin was formed in the absence of ALA. There was no PBG or porphyrin formed in the "control" flask, i.e. in the absence of the bacterium. The changes during incubation are represented by Figs. 14 and 15.

The ALA was found in the "control" flask to be quite stable for about 50 hours under the conditions of incubation, then decomposed very slowly. This means that the low (15-25%) yield recorded after 14 days in the usual productions was not due to spontaneous decomposition of ALA.

In the "experimental" flask more than 75% of the ALA had been used up in 40 hours. Now it may be significant that the

Figs. 14 and 15. Growth of <u>S. marcescens</u> 211 at 30°C in the presence of ALA in aerated culture. Utilization of ALA —·—; appearance of PBG --¦-- and of porphyrin —▫—; Dry weight of <u>S. marcescens</u>--×--. The stability of ALA —o— was checked in the absence of the bacterium.

ALA (μmoles/ml)

0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0

Dry Weight Cells (mgm/ml)

5.0 4.0 3.0 2.0 1.0 0

0 20 40 60 80 100

Time (hours)

Fig. 14.

Porphyrin (μmoles x 10^{-3}/ml)

6.0 5.0 4.0 3.0 2.0 1.0 0

PBG (μmoles/ml)

0.4 0.3 0.2 0.1 0

0 20 40 60 80 100

Time (hours)

Fig. 15.

order of events in this flask was 1. rise in cell numbers and drop in ALA concentration, 2. rise in PBG concentration, 3. rise in porphyrin concentration. Such a sequence tends to suggest that the ALA was converted first rapidly to PBG, then that the PBG was incorporated into porphyrin. This hypothesis is supported by the fact that in the first 40 hours 0.375μmoles PBG/ml, which is equivalent to 0.75μmoles ALA/ml, appeared in the medium, and 0.7μmoles ALA/ml was lost. However, the loss of PBG (0.325μmoles/ml, equivalent to 0.08μmoles porphyrin/ml) over the next 60 hours was much greater than the porphyrin formed (0.0055μmoles/ml). Then too, the porphyrin continued to rise in concentration up to 10-14 days incubation, when the PBG certainly was exhausted.

An alternate suggestion which may be given, is that the PBG excreted into the medium represents a loss of ALA to porphyrin synthesis. Then the 75% of the ALA converted to free PBG leaves 25% for porphyrin synthesis. This agrees with the usual 15-25% total yield of porphyrin. In support of this hypothesis is the finding that <u>Chlorella</u> cells are not permeable to PBG (Bogorad and Granick 1953 b). Also it was found that <u>Rps. sphaeroides</u> incubated with ALA, excreted PBG into the medium during the first few hours (Lascelles 1956). The subsequent drop in PBG concentration was not due to enzyme action, and the PBG was not incorporated into free porphyrins. During the incubation with <u>S. marcescens</u>, the 75% of the ALA was converted to PBG quickly and excreted into the medium; this PBG then may have been unable to

penetrate into the cells again to the enzyme sites, but have been broken down spontaneously. The porphyrin formed would have been the product from ALA and PBG retained by the cell.

It can be concluded then, that porphyrin is formed from ALA during incubation with S. marcescens. PBG is probably an intermediate in the synthesis of porphyrin from ALA since it was excreted into the medium during the first few hours of incubation. The fact that PBG was excreted in such quantity suggests that, in S. marcescens, there is a rate limiting step on the pathway of porphyrin synthesis, whereby PBG is converted only slowly to the next intermediate.

SEQUENCE OF APPEARANCE OF THE INDIVIDUAL PORPHYRINS

Experiments were carried out to determine which, if any, of the porphyrins was synthesized first by S. marcescens under the usual conditions. Very little porphyrin was formed during the first day, so flasks of medium were harvested on the 2nd, 4th and 7th days, purified, and the free porphyrins separated by electrophoresis. Samples of the medium before extraction of the porphyrins, were concentrated and chromatographed in lutidine.

The chromatograms were poor due to the interference by inorganic salts, but even the 2nd day preparation showed the uroporphyrin and two coproporphyrin spots. The "mid" spot D of Fig. 2, was not visible due possibly to too small an application. This porphyrin was shown to be present by electrophoresis. The electrophoresis strips contained all

three of the usual porphyrin-containing bands. The bands were eluted by esterification of the porphyrins directly on the papers. The porphyrins were freed and assayed, and each was expressed as a percentage of the total (Table 14).

The proportions of the fractions may be compared relative to eachother in this set of assays, but not with the earlier results, which were obtained by elution with HCl.

It is apparent that all the major components isolated after the usual 14 day growth period, appeared simultaneously in the medium, and were present throughout the incubation.

Table 14. PORPHYRINS APPEARING PER DAY

	*FRACTIONS FROM ELECTROPHORESIS		
DAY	F 1 + 2	F 3	F 4 + 5
2	64	1	35
2	66	1	33
2	68	1	31
Mean 2	66	1	33
4	72	2	26
4	66	2	32
Mean 4	69	2	29
7	74	5	21
7	80	3	17
Mean 7	77	4	19

* Results from assays expressed as a percentage of the total porphyrin.

The simultaneous appearance of the porphyrins agrees with the idea that uro- and coproporphyrins occur as by-products from the direct pathway to protoporphyrin and haem, i.e. the porphyrins are not themselves intermediates.

The trend in the results indicates that the proportion of coproporphyrin may have been higher in the earlier period of synthesis. Further experiments would be required for proof.

SUMMARY

A number of metabolic tests were carried out on 68 strains of *Serratia*. The *Serratia* were found to conform to the definition of *Serratia marcescens* given by Davis et al (1957).

An attempt to induce prodigiosin synthesis by growing a white mutant, *S. marcescens* 211, in the presence of some pyrrole compounds was unsuccessful. However, this strain synthesized from δ-aminolaevulinic acid (ALA), a pyrrole precursor, a red-brown water soluble pigment, which was found to contain a mixture of porphyrins.

The conditions for maximum conversion of the ALA to porphyrin were determined for a 14 day growth period in Bunting's medium as ---

ALA concentration	1×10^{-3}M
Temperature	30°C
Vigorous aeration.	

A thick suspension of non-growing cells produced very little or no porphyrin from ALA in 24 hours.

Under the above conditions, yields of 4-4.5mg porphyrin per 200 ml, corresponding to 15-25% conversion of ALA, were recorded. A small amount (1-2% of that soluble in the medium) was found in the cells.

Four strains of *S. marcescens* and cultures of *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Escherichia coli*, *Bacillus subtilis* and *Corynebacterium diphtheriae* were found to synthesize similar mixtures of porphyrins from ALA. The efficiency of the conversion differed even within the four

strains of S. marcescens.

The pigment was separated into five fractions by electrophoresis. Three of the fractions showed the red fluorescence of porphyrins. These porphyrins were isolated pure and identified separately.

The major component was uroporphyrin, representing 79-88% of the total porphyrin produced. It was shown to be predominantly uroporphyrin I, containing 20% or less of the isomer III.

The next abundant component, 10-18% of the total porphyrin, was coproporphyrin., In this case isomer III predominated, with isomer I representing less than 15%.

A third "mid" porphyrin, 1-3% of the total, was identified only as having 5, 6, or 7 carboxyl groups. There were also traces of three porphyrins containing 8, 7 and 3 carboxyl groups respectively.

A green-fluorescing fraction revealed on electrophoresis was thought to be breakdown products of the above porphyrins.

A brown non-pyrrolic pigment and a compound absorbing at 480-490mμ were also synthesized concurrently with the porphyrins. It is suggested that the latter was a linear tetrapyrrole.

S. marcescens 211 was unable to synthesize water soluble porphyrins from the precursors of ALA.

The presence of iron in the growth medium caused a 15-20% inhibition of porphyrin synthesis from ALA. There was no further inhibition as the Fe concentration was increased

from 0.54 to 2.68mM. Synthesis was not directed towards protoporphyrin as in *Rhodopseudomonas* and *Tetrahymena vorax*.

Carboxylic acids oxidized through the TCA cycle, enhanced porphyrin synthesis. Energy is therefore required for ALA conversion to porphyrins. This may account for the greater synthesis in aerated cultures. Acetate, pyruvate, citrate, α-ketoglutarate, succinate and malate accomplished a 5 to 6-fold increase over the control, fumarate only a 3-fold increase.

During incubation of *S. marcescens* 211 with ALA, a drop in ALA and a rise in porphobilinogen (PBG) and porphyrin concentrations in the medium was demonstrated. The PBG concentration after 40 hours was equivalent to 75% of the added ALA, then it decreased. The porphyrin concentration in 14 days was equivalent to 15-20% of the added ALA. It is suggested that the released PBG was not converted to porphyrin but accounts for the ALA lost to porphyrin synthesis.

PBG is probably an intermediate in the synthesis since it was produced within the first few hours of incubation. The release of large quantities indicates that the conversion of PBG to the next intermediate is a rate limiting step on the biosynthetic pathway.

Uro-, copro- and the "mid" porphyrins appeared simultaneously in the medium, supporting the belief that the porphyrins are not themselves intermediates on the pathway to protoporphyrin. Coproporphyrin appeared to represent a larger proportion of the total porphyrins in the early growth stages.

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