THE BIOSYNTHESIS OF PORPHYRINS BY SPECIES OF ATHIORHODACEAE

A Thesis Submitted for the Degree
of Doctor of Philosphy
in the University of Oxford.

by

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DEPOSITED
THESIS
28. iii. 60

December, 1959

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ABSTRACT

PART ONE : INTRODUCTION

Chapter 1. PORPHYRINS AND RELATED COMPOUNDS

The rapid advance in knowledge of porphyrin metabolism in recent years had led to the recognition of intermediates in their biosynthesis which are not themselves porphyrins but which nevertheless contain the pyrrole structure. This chapter is therefore concerned with those naturally occurring substances with one or more pyrrole groups in their molecules, which are connected with the present investigation. Some important formulae are shown in fig. 1.

A. General.

(i) The tetrapyrrole structure.

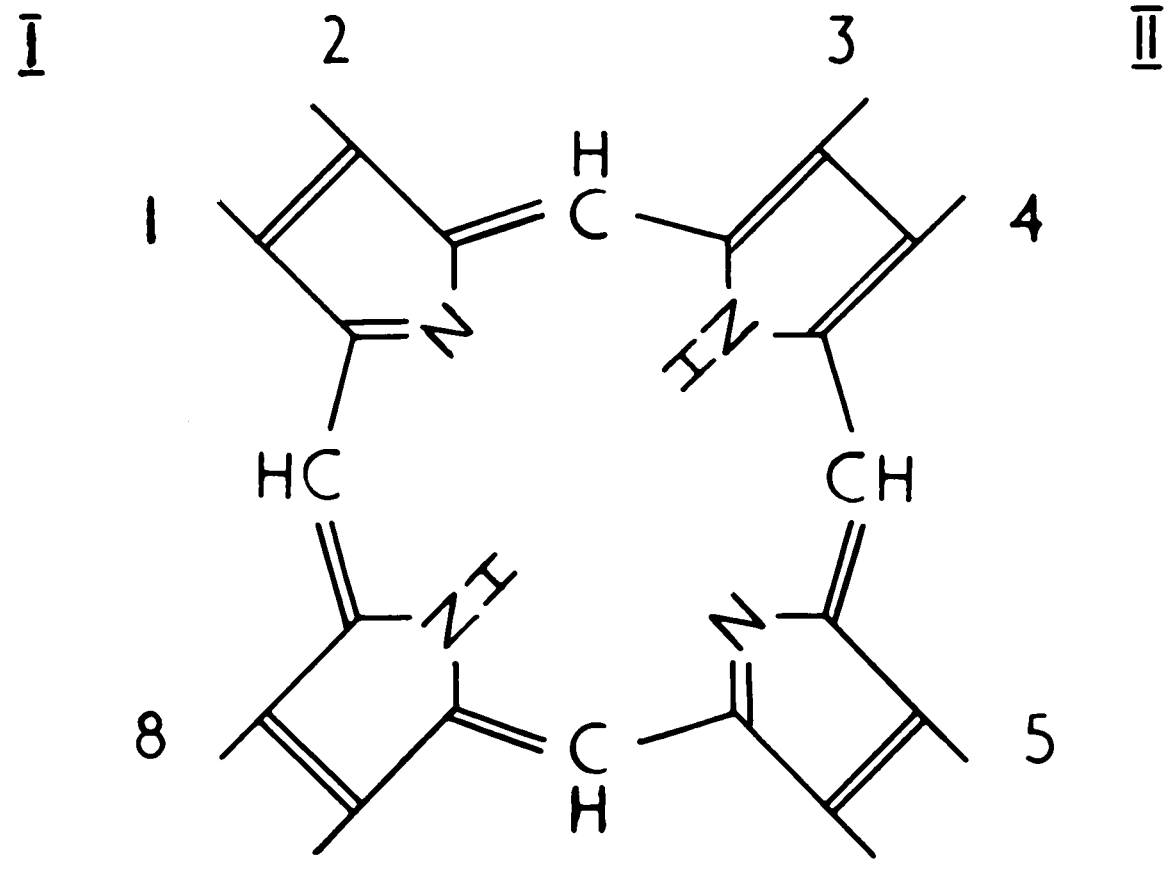
Porphyrins may be defined as cyclic tetrapyrryl methenes, being theoretically derived from the parent, unsubstituted compound called porphin. All of the known, naturally occurring porphyrins are substituted in all of the pyrrole β-positions (see fig. 1); some are also substituted at the methene bridges between the pyrrole groups. The structure is remarkable for its stability; indeed when first suggested by Küster in 1914, it was rejected as implausible. This stability is conferred by the circuit of alternating double and single bonds which constitute the whole molecule, excluding fully saturated side-chains (see Lemberg & Legge, 1949). Another way of looking at the structure is to suppose that each of the atoms taking part contribute a π-electron to a resonating electron circuit.

FIGURE 1 THE STRUCTURE OF PORPHYRINS

Key to side-chains	1	2	3	4	5	6	7	8
Uroporphyrin I	Ac	Pr	Ac	Pr	Ac	Pr	Ac	Pr
Coproporphyrin I	Me	Pr	Me	Pr	Me	Pr	Me	Pr
Uroporphyrin III	Ac	Pr	Ac	Pr	Ac	Pr	Pr	Ac
Coproporphyrin III	Me	Pr	Me	Pr	Me	Pr	Pr	Me
Protoporphyrin IX	Me	V	Me	V	Me	Pr	Pr	Me

Ac: - CH_2COOH Me: - CH_3

Pr: - CH_2CH_2COOH V: - CH : CH_2



This situation is of fundamental importance in the biosynthesis of porphyrins, since the possibility of spontaneous, non-enzymic reactions in the later stages of the process has to be considered. Indeed, porphobilinogen (PBG) gives rise to uropopphyrins during non-enzymic incubation, and even δ-aminolaevulic acid (ALA) can be condensed non-enzymically to porphyrins, though in both cases small yields are usually obtained (Cookson & Rimington, 1954; Scott, 1956) (Formulae in fig. 2)

(ii) Occurrence and function

Porphyrins or their complexes with iron have been found in all animals, plants and aerobic microbes so far examined. However, some anaerobic bacteria have been reported as sompletely lacking in cytochromes, haem and catalase activity (see Stephenson 1949). The cobalt cyclic tetrapyrrole complex, vitamin B_{12}, is probably very widely distributed amongst animals and microbes, though it has not been reported in plants apart from algae.

As regards the function of porphyrins, a quotation from Granick & Gilder (1947) is relevant: "It is only when porphyrins form metal co-ordination compounds that they appear to develop properties of great physiological significance." Such metal complexes are the iron porphyrins, the magnesium phorbins (chlorophylls) and a cobalt cyclic tetrapyrrole (vitamin B_{12}). Important pyrrole compounds which are not porphyrins are the bile pigments and other excretory products, and the phycobilins which form part of the photosynthetic pigment system of the algae Rhodophyceae and Myxophyceae. All of these compounds (except excretory products) are probably intimately concerned in oxidation-reduction processes in living organisms.

B. Free Porphyrins.

(i) Side-chain configurations.

The arrangement of the sidechains of porphyrins is indicated by a numerical system invented by Fischer (see Fischer & Orth, 1937). For example, in protoporphyrin there are four methyl, two vinyl and two propionic acid side chains (fig. 1). The number of possible isomers, due to different arrangements of side chains (assuming a planar molecule), is fifteen; Fischer gave each of these a Roman numeral. The naturally occurring isomer is protoporphyrin IX. Similarly, these are four possible isomers of coproporphyrin and four of uroporphyrin; the I and III isomers are found in nature. However, uroporphyrin I and coproporphyrin I are now thought to be side products of the synthetic pathway leading towards metalloporphyrins, whereas the corresponding III isomers are probably very closely related to the intermediates (see Chap. 2) Therefore it is noteworthy that protoporphyrin IX has a similar configuration to that of uropopphyrin III and coproporphyrin III (see Fig. 1; note the arrangement of the methyl side chains), and moreover a similar, though rather more complex configuration is found in haem compounds, cytochromes, chlorophylls, vitamin B_{12}, and all the known naturally occurring open-chain tetrapyrroles. This suggests a common plan of synthesis of all these compounds, and all of the rapidly accumulating evidence on the subject supports this idea.

(ii) Uroporphyrin, coproporphyrin and protoporphyrin.

These three porphyrins are often found in relatively large amounts in nature and are generally predominant when mixtures of porphyrins are formed. There is a number of reviews of the many observations of their occurrence: (Fischer & Orth, 1937; Dobriner & Rhoads, 1940; Granick & Gilder 1947; Watson & Larson, 1947; Lemberg & Legge, 1949; Rimington, 1954, 1957; Vanotti, 1954; Granick, 1954; Weatherall, 1954; Falk, 1954).

Much of the work on free porphyrins has been in connection with their excretion by human patients suffering from any one of the three forms of porphyria, or from various anaemic conditions or lead poisoning; a recent reveiw is by Rimington (1957). Many normal plant and animal tissues contain small amounts of uroporphyrins, coproporphyrins and protoporphyrin (Vanotti, 1954).

Similarly, many microorganisms produce uroporphyrins and coproporphyrins (Vanotti, 1954; Lascelles, 1956a). Coproporphyrin I is formed normally in Saccharomyces anamensis and other yeasts (Fischer & Fink, 1925), though coproporphyrin III predominates in brewers' yeast when the supply of carbohydrates is restricted (Kench & Wilkinson, 1945). A small proportion of uroporphyrin, probably type I, accompanies the coproporphyrin III formed in cultures of Corynebacterium diphtheriae. (Gray & Holt, 1948). The major component of the mixture of porphyrins formed by suspensions of Rhodopseudomonas spheroides, incubated in the light, was coproporphyrin III; however, here again a small proportion (3 - 5%) of the total was uroporphyrin, though in this case a mixture of types I and III was produced (Lascelles, 1956a). By contrast, uroporphyrin III accounted for 50 - 60% of all the porphyrin formed in suspensions of Tetrahymena vorax (Lascelles, 1957).

Protoporphyrin has been observed in microorganisms less often than coproporphyrin. Low concentrations are found in yeast (Vanotti, 1954), and larger amounts in cultures of Tetrahymena gelei (Rudzinska & Granick, 1953), in suspensions of Rps. spheroides and T.vorax (Lascelles, 1956a, 1957), and in cultures of a mutant strain of Chlorella unable to synthesize chlorophyll (Granick, 1948a).

(iii) Other free porphyrins.

The development of paper chromatographic techniques has accelerated the discovery of porphyrins other than uropopphyrin, coproporphyrin and protoporphyrin (see Rimington, 1954, 1957:

Weatherall, 1954). Porphyrins with 1 to 8 carboxyl groups have been detected in normal animals and cases of porphyria, and in cultures of corynebacteria. The various porphyrins produced by a Chlorella mutant are discussed in chapter 2. Suspensions of Rps. spheroides formed traces of tri- and hexa-carboxylic porphyrins (Lascelles, 1956a).

In general, free porphyrins are often formed copiously in living organisms and when so produced, appear to have no important physiological function; very often they are excreted. They probably represent intermediates or stabilised derivatives of intermediates in the biosynthesis of metallo-porphyrins, produced by metabolic disturbances which may be innate or imposed by experimental conditions.

C. Porphyrin Precursors

The term "porphyrin precursor" in this thesis is used in the same sense as in the "Porphyrin Biosynthesis and Metabolism" (1955) by various authors, that is to say a compound spontaneously giving rise to porphyrin on incubation in air. Until recently, most of the observations on such precursors had used human urine as the source.

(i) Porphobilinogen (PBG)

This is the only precursor whose structure is known for certain (fig. 2). It was first detected by Sachs (1931) using acute porphyria urine, and was found to react with Ehrlich's reagent (p-dimethylaminobenzaldehyde in acid solution). It was further characterised by Waldenström (1934), Vahlquist (1939) and Waldenström & Vahlquist (1939), who called it porphobilinogen becuase it gave rise to a non-porphyrin pigment, porphobilin, on incubating such urines. Porphyrins are also formed, usually in small yield, under these conditions. (Waldenström & Vahlquist, 1939; Prunty, 1945; Gibson & Harrison, 1950; Brockman & Gray, 1953; Lockwood, 1953; Cookson & Rimington, 1954). The isomers formed are uroporphyrin I and

uroporphyrin III, in different proportions according to the pH and temperature at which the urine or PBG solution has been stored. Uroporphyrins II and IV may also be formed. (Cookson & Rimington, 1954; MacDonald & Michl, 1955).

The properties of the yellow pigment, porphobilin were investigated by Brockman & Gray (1953). On the basis of its absorption spectrum and other properties it was suggested to be dipyrryl methene or an open-chain tetrapyrrole containing a dipyrryl methene group. Lemberg & Legge (1949) state that it is probably a urobilin b, that is a urobilin (see Discussion) with the acetic and propionic acid side chains of uroporphyrin.

(ii) Other uroporphyrin precursors.

Another uroporphyrin precursor has been distinguished from PBG by its solubility in organic solvents and failure to react with Ehrlich's reagent. Moreover, it forms no porphobilin on incubation in air (Herbert, 1952; Hawkinson & Watson, 1952; Watson, 1954). It was said to be somewhat reminiscent of uroporphyrinogen I (see below), but there was "reason to believe they may not be indentical", (Watson, 1954). The uroporphyrin precursor was compared with a similar coproporphyrin precursor by Watson et al. (1951), (see below).

A similar compound may be intermediate between PBG and uroporphyrin in the chemical transformation. Evidence was obtained for a slow formation of such a uroporphyrin precursor when urine containing PBG was incubated anaerobically in the dark; there was a rapid appearance of porphyrin when air was admitted. Porphobilin was probably formed directly from PBG by a side reaction in the presence of air (Lockwood, 1953). Similar results were reported by Cookson & Rimington, 1954.

(iii) Coproporphyrin precursors.

Probably the first indication of a porphyrin precursor was by Saillet (1896) who described a pigment in normal urines which he called "urospectrin", and which was probably coproporphyrin.

A light sensitive chromogen of urospectrin was present, with absorption bands at 580 and 543 mμ in acidified, aqueous suspension. Fischer mentioned in Fischer & Orth (1937, p. 472) that the coproporphyrin isolated from the faeces of a case of congenital porphyria was present as a porphyrinogen. Other early, brief reports of the detection of porphyrin precursors are reviewed by Watson et at (1951).

Absorption bands have been associated with coproporphyrin precursors by other workers. Gray (1951) and Raine, quoted by Falk (1954), have found bands at 575 and 540 mμ in an ether extract of urine; coproporphyrin appeared on adding strong HCl. These properties were said to be suggestive of a metal complex. A compound with bands at 576 and 544 mμ was reported by De Langen (1948), but in this case by incubating coproporphyrin with a liver extract. In a recent footnote, Schwartz & Ikeda (1955) state that the coproporphyrin precursor in urine appears to be coloured.

Other reports do not mention an absorption spectrum. Other properties observed include: lack of fluorescence; extraction from ether by 1% HCl; partial extraction from ether or ethyl acetate by 1.5 N - HCl; greater stability in ether than in ethyl acetate; conversion to porphyrins accelerated by ultraviolet light, quinhydrone, iodine, acid or alkali; and a variable yield of porphyrin according to the conditions, indicating that there were side reactions; (Raine. 1947; Eriksen, 1951 A, b, 1952); Weatherall, 1952; Watson et al, 1951). Coloured, non-fluorescent zones on chromatograms were interpreted as intermediate, partially dehydrogenated compounds. Separate precursors for coproporphyrins I and III were probably present in urines. A comparison was made with coproporphyrinogen I prepared by reducing coproporphyrin I with sodium amalgam; iodine oxidised this compound back to the porphyrin but the percentage recovery was less than when the precursor was similarly oxidised. Finally, the ether-soluble uroporphyrin

precursor mentioned above was found to have similar properties, though it was more rapidly converted to porphyrin than was the coproporphyrin precursor (Watson et al, 1951).

Thus the evidence for the nature of the ether-soluble porphyrin precursors in urine is inconclusive. They bear some resemblance to the porphyrinogens of Fischer, though at least one difference has been reported.

D. Porphyrinogens

This term was coined by Fischer to denote the colourless compounds which he obtained by vigourous reduction of porphyrins. Six hydrogen atoms are taken up by each molecule of porphyrin, and the molecular weight of the product suggests that four pyrrole groups are still present (Fischer & Zerweck, 1924). Hence the porphyrinogens are fully reduced tetrapyrryl methanes; the question of whether their structure is still cyclic seems to be undecided. They revert to porphyrins on standing in air, so behaving as "porphyrin precursors", in the sense defined above. Mention of their properties is made in the Discussion, in connection with the properties of the coproporphyrinogen produced by Rhodopseudomonas capsulatus in the present study.

E. Open-chain pyrrole compounds

This class of pyrrole derivatives, the most important naturally occurring members of which are the animal bile pigments and the phycobilins of algae, is reviewed by Lemberg & Legge (1949) and by Gray (1953). They are of interest in the present study becuase of the oxidation-reduction series which they present, in analogy with a similar series probably occurring amongst porphyrinogens (see Discussion).

F. Metalloporphyrins.

(i) Iron porphyrins.

These include iron protoporphyrin (haem), which forms

an essential part of haemoglobins, catalase, peroxidase, and cytochromes b, c and f, and the various iron porphyrins and chlorins (dihydroporphyrins) of the cytochrome a complex, in which side chains have been modified, (see Granick & Gilder, 1947; Lemberg & Legge, 1949; Hill & Hartree, 1953; Lemberg & Stewart, 1955, Postgate, 1956).

Cytochromes and a pigment resembling haemoglobin (though unable to combine reversibly with oxygen), are abundant in members of the Athiorhodaceae and Thiorhodaceae and in Chlorobium (Elsden, 1954; Vernon & Kamen, 1954; Kamen & Vernon, 1955; Kamen, 1955; Newton & Kamen, 1955; Gibson & Larsen, 1955). These compounds probably mediate in the electron transport process of bacterial photosynthesis (Kamen, 1955).

Suspensions of Rps. spheroides incubated in the light excrete free haem into the incubation medium (Lascelles, 1956,b); see Chapter 2.

(ii) Magnesium porphyrins.

Under this heading come the chlorophylls of plants and photosynthetic bacteria (strictly, magnesium phorbins), and magnesium porphyrins behaving as precursors of chorophylls a and b in Chlorella (see chapter 2 for discussion and formulae). Bacteriochlorophyll is distinguished from plant chlorophylls by its greater state of reduction, there being two extra hydrogen atoms in one of the pyrrole groups. It is found in the Thiorhodaceae and Athiorhodaceae (van Niel, 1944), but a different type of chlorophyll is present in the Chlorobacteriaceae (Larsen, 1953; Goodwin, 1955). The chemistry of plant chlorophylls and bacteriochlorophyll is reviewed by Fischer & Stern (1940); see also Willstätter & Stoll (1913)

(iii) Other metalloporphyrins.

Metals, other than magnesium and iron, complexed with

porphyrins in living organisms include copper and zinc. Large amounts of copper uroporphyrin III are found in the feathers of the Turaco bird; otherwise only small amounts of such metalloporphyrins have been detected (see Granick & Gilder, 1947; Vanotti, 1954).

From the above summary, it is evident that studies with members of the Athiorhodolaceae have contributed to knowledge about free porphyrins, iron porphyrins and chlorophylls. The present study extends this range to include porphyrin precursors and magnesium protoporphyrin.

CHAPTER 2. THE BIOSYNTHESIS OF PORPHYRINS.

A. General.

Possibly the first idea about the biosynthesis of porphyrins was that haem and chlorophyll might share a common origin, their chemical similarity having been established by Hoppe-Seyler (1880). However, no significant advance was made in understanding the synthesis of the porphyrin ring until Shemin & Rittenberg (1945) showed the specific utilisation of ^{15}N - glycine for haem formation in man. Since then progress has been rapid and many intermediates have been recognised; furthermore many of the main points have been confirmed in experiments with animals, plants and micro-organisms.

Most of the information on the early intermediates in porphyrin biosynthesis has come from work with red blood cells or haemolysates, since such material has active systems for the biosynthesis of haem, is easily available, and is of interest in the understanding of the porphyria diseases. However, the results have been corroborated in most respects when different biological material has been used. Recent reviews on porphyrin biosynthesis are by Granick (1954),, Rimington (1954, 1956, 1957), and various authors in "Porphyrin Biosynthesis and Metabolism" (1955).

In this account, emphasis will be put on observations made with member of the Athiorhodaceae, and on the role of porphyrin precursors and the formation of metallorporphyrins.

A. The Initial Substrates.

The studies of Shemin and his co-workers on isotope incorporation into haem by whole or haemolysed red cells from the duck have eventually made it possible to account for each atom of the protoporphyrin molecule as derived from a particular atom of either glycine or a derivative of α-oxoglutarate containing four carbon atoms and behaving as if unsymmetrical. Such work

is reviewed by Shemin (1955, 1956) and Granick (1954). Contributions to this problem were also made by Altman et al (1948) and Muir & Neuberger (1949, 1950).

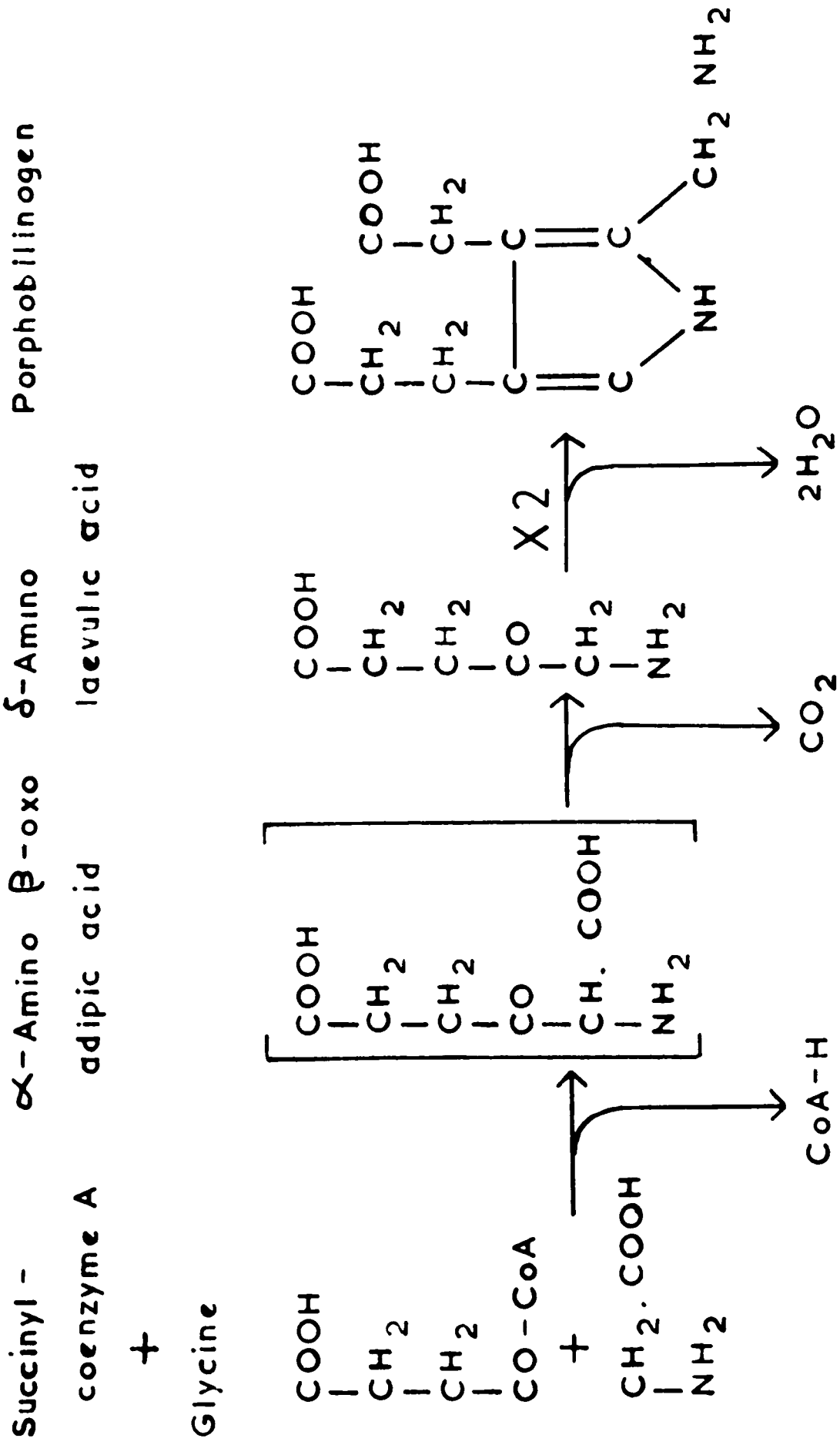
Glycine and carboxylic acid serve as substrates for porphyrin formation, also in micro-organisms. ^{15}N - Glycine is incorporated into porphyrins and haem pigments in C.diphtheriae (Hale et al. 1950). ^{14}C - Glycine and ^{14}C - acetate are incorporated into the pheophorbid skeleton of chlorophyll in growing cultures of Chlorella vulgaris. (Della Rosa, Altman & Salomon, 1953). Porphyrins are formed by suspensions of T.Vorax incubated aerobically with glycine and fumarate (Lascelles, 1957).

Suspensions of Rps. spheroides incubated in the light convert glycine and ∝-oxoglutarate to porphyrins and bacteriochlorophyll. Apart from succinate, other carboxylic and amino acids were much less active than ∝-oxoglutarate or glycine, respectively. (Lascelles, 1956a).

B. The Formation of the Pyrrole Ring.

The biosynthetic pathway towards the monopyrrole porphobilinogen (PBG), which appears to operate in all systems so far examined, is shown in fig. 2.

Figure 2. THE BIOSYNTHESIS OF PORPHOBILINOGEN



(i) ∝-Amino β-oxoadipic acid.

This may be the product of condensation between glycine and succinyl coenzyme A, (see below). The free acid has not yet been isolated because of its spontaneous decarboxylation to δ-amino laevulic acid, but ethyl esters give rise to porphyrins in suspensions of Rps. spheroides, though only slightly in a cell-free extract, which actively converted δ-amino laevulic acid to porphyrins (Neuberger, Scott & Shuster, 1956)

(ii) δ-Aminolaevulic acid.

Decarboxylation of ∝-amino β-oxoadipic acid would yield δ-aminolaevulic acid (ALA), whose recognition as a precursor of haem came when it was labelled with isotopes and found to be incorporated in systems from red cells (Shemin & Russell, 1953; Neuberger & Scott, 1953).

Recently, the formation of ALA from glycine and succinyl coenzyme A has been shown in cell free extracts of Rps. spheroides (Kikuchi, Shemin & Bachmann, 1958; Gibson 1958; Kikuchi, Kumar, Palmage & Shemin, 1958; Sawyer & Smith 1958). Pyridoxal phosphate is needed for the reaction.

The final step in the biosynthesis of PGB is the condensation of two molecules of ALA to form one of PBG (see fig. 2). The reaction is catalysed by ALA - dehydrase, first prepared by Gibson, Neuberger & Scott (1955) from acetone-dried ox liver, and later found in extracts of Rps. spheroides (Neuberger, Scott & Shuster, 1956). However no formation of PBG was detected when suspensions of Rps. spheroides (whole organisms) were incubated anaerobically in the dark with ALA, but only under conditions permitting porphyrin synthesis (incubation aerobically or in the light), when free porphyrins and haem are produced, in addition to PBG. In these suspensions, no bacteriochlorophyll is formed from ALA (as it is from glycine and ∝-oxoglutarate in the light); it may be that glycine and/or ∝-oxoglutarate are required for

synthesis of the phytol residue, the phorbin methyl ester group, or a specific apoprotein of bacteriochlorophyll, as well as for the porphyrin moiety (Lascelles, 1956 a,b).

(iii) Factors affecting porphyrin formation from glycine and ALA

In general, the synthesis of porphyrins from ALA is less sensitive to experimental conditions than that from glycine. For example, suspensions of Rps. spheroides must be incubated in the light to achieve porphyrin or haem synthesis from glycine and ∝-oxoglutarate, whereas ALA can act as a substrate aerobically in the dark. Moreover, synthesis from ALA is less sensitive to inhibitors than that from glycine and ∝-oxoglutarate (Lascelles, 1956 a,b.)

The sensitivity of these reactions to the supply of B -vitamins in the organism shows a similar picture. The earlier suggestions that pyridoxin and pantothenate are implicated in porphyrin synthesis from glycine but not from ALA, in duck red cells and suspensions of T.vorax, have been borne out by the recent enzyme studies with cell-free extracts of Rps. spheroides mentioned above; (Schulman & Richert, 1955, 1956; Lascelles, 1957). The studies with T.vorax also showed a reduction in porphyrin formation from glycine if the organism was deficient in nicotinic acid, thiamin, thioctic acid or riboflavin, though in no case was the QO_2 value with glucose affected. Of the above vitamins, only riboflavin defidiency reduced porphyrin synthesis from ALA. In suspensions of Rps.spheroides, porphyrin synthesis from glycine and ∝-oxoglutarate, but not from ALA, was much reduced when the organism was deficient in thiamin, nicotinic acid or biotin, but was restored by the addition of the appropriate vitamin. (Lascelles, 1956a).

C. The Formation and Interconversion of Porphyrins.

Less is known about the reactions leading from PBG to the metalloporphyrins than about those leading to PBG. There are four main problems:

(a) The mechanism of the oxidative condensation of four monopyrroles to one molecule of a type III porphyrin.

(b) The mechanism of the modification of the side chains of PBG to those of protoporphyrin.

(c) The nature of the intermediate on the pathway to protoporphyrin; are they the porphyrins themselves or some related compounds?

(d) The insertion of the metal atom: before or after ring closure?

More evidence is now available about (b) and (c) than about (a) and (d).

(i) The conversion of PBG to porphyrins.

This conversion was first demonstrated by Falk, Dresel & Rimington (1953), using a haemolysate and supernatant fraction from chicken red cells (see also Falk, 1955; Dresel & Falk, 1956 a, b). In suspensions of Rps.spheroides incubated with ALA in the light, PBG appears in the medium and then disappears during the formation of porphyrins; however, the disappearance of PBG (but not porphyrin formation) also takes place with boiled cells. It may be that PBG does not pass into the organism; no popphyrins are formed from added PBG by Chlorella unless the cell wall has been modified by freezing and thawing (Bogorad & Granick, 1953a). Similarly, PBG is converted to porphyrin by haemolysates but not by whole red cells (Dresel, 1955).

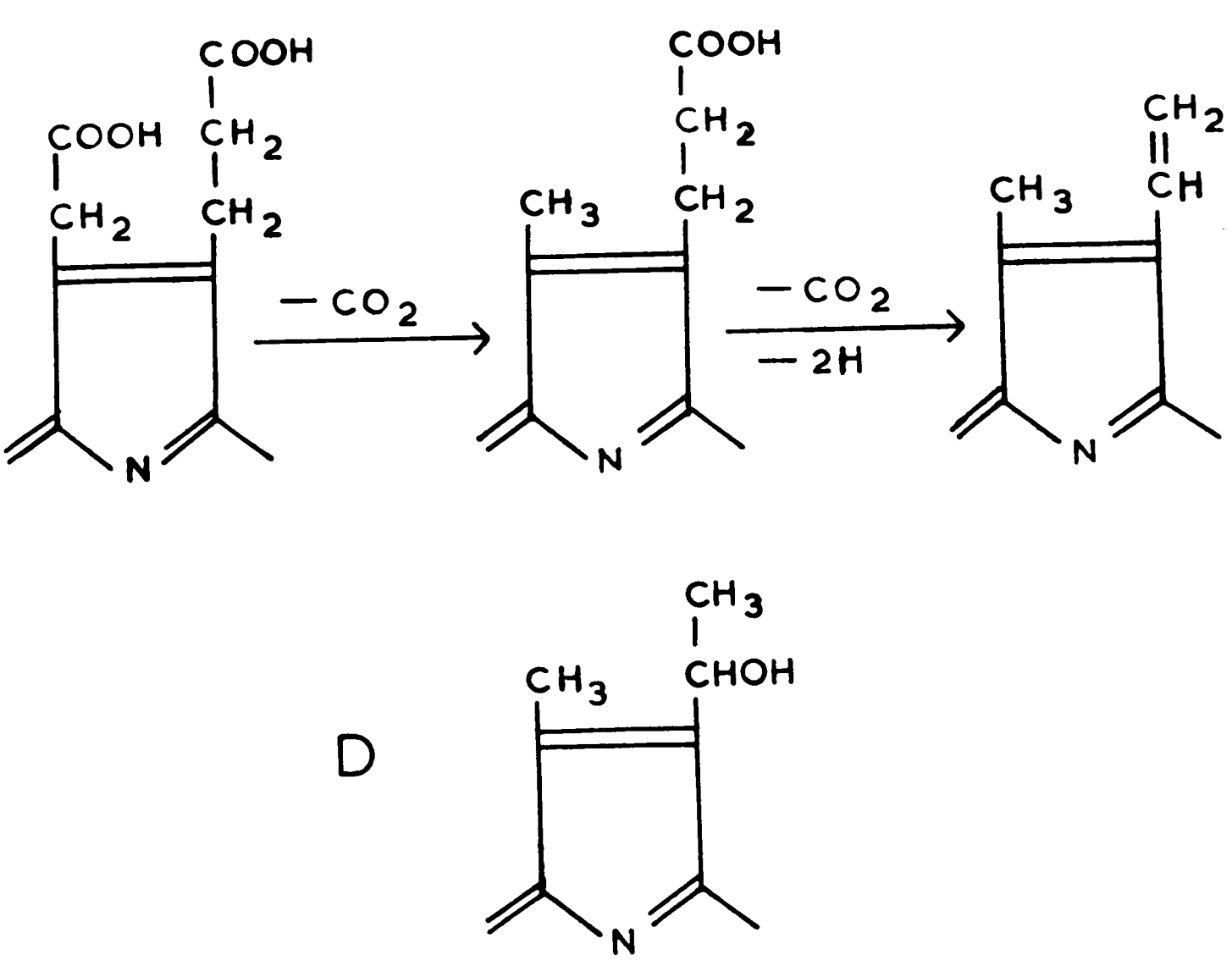
(ii) The modification of the side chains.

It must here be emphasised that, though the free porphyrins are themselves probably not the true intermediates on the pathway from PBG towards metalloporphyrins, it is very probable that

FIGURE 3.

THE FORMATION OF THE SIDE-CHAINS OF PROTOPORPHYRIN.

Key: Pyrrole groups (as in fig.1) ⟶	I	II	III	IV
Uroporphyrin	A	A	A	A
Coproporphyrin	B	B	B	B
Protoporphyrin	C	C	B	B
Monohydroxyethyl deuteroporphyrins (Granick, Bogorad & Jaffe, 1953)	C	D	B	B
	D	C	B	B
	D	D	B	B



they are very closely related to the true intermediates, especially in respect of the configuration of their side chains. Thus it is in order to refer to the "uroporphyrin configuration" and the "coproporphyrin configuration", and so on, in discussing the way in which the side chains may be modified. Skeleton formulae are given in figure 3.

Early theories supposed that protoporphyrin was the first to appear and that copro- and uro-porphyrins were produced from it by successive decarboxylations (see Lemberg & Legge, 1949). There is evidence that this may in fact occur in autolysing yeast cells and putrefying bacteria (Kench, 1954; Vanotti, 1954). However, the main body of evidence is that the uroporphyrin configuration is the first to appear (by condensation of four molecules of PBG), and successive decarboxylations lead through the coproporphyrin configuration to the protoporphyrin configuration (see fig. 3) (e. g. Neuberger, Muir & Gray, 1950; Schmid, Schwartz & Watson, 1950; Shemin & Wittenberg, 1951). The occurence of porphyrins with 7, 6, 5 and 3 carboxyl groups (see chapter 1) suggests that the six decarboxylations may proceed successively, (see fig. 4).

The last two decarboxylations, from the coproporphyrin to the protoporphyrin configurations (fig. 3) each requires the removal of two hydrogen atoms as well as one molecule of carbon dioxide. Some information about the intermediate configurations has come from Granick, Bogorad & Jaffe (1953), see also Granick (1955a), working with mutant strains of Chlorella unable to synthesise chlorophyll. Amongst the porphyrins excreted by one such mutant were protoporphyrin itself and

three monohydroxyethyl deuteroporphyrins (fig. 3). Evidence was given that these deuteroporphyrins may be formed by spontaneous hydration of the corresponding acrylic or keto acid derivatives, which may be similar to the true intermediates.

D. The Role of Porphyrin Precursors.

The terms "porphyrin precursor" and "porphyrinogen" are used here in the same sense as in chapter 1, porphyrinogens being defined as the colourless porphyrin precursors which may be obtained by vigourous reduction of porphyrins *in vitro*.

(i) Porphyrin formation.

There are a few reports of the synthesis of porphyrin precursors, rather than the porphyrins themselves, in experiments in which tissue homogenates or cell-free preparations are incubated with ALA or PBG; (precursors appearing in urine have already been mentioned in chapter 1). After incubation of homogenates or cell-free extracts of rat liver with PBG, precursors of uro-, copro- and protoporphyrin, converted to the porphyrins by iodine or irradiation with ulraviolet light, were present (Schwartz & Ikeda, 1955). A protein fraction from chicken red cells converted PBG to a colourless compound which yielded uroporphyrin III (or IV) on autoxidation; a similar precursor of coproporphyrin III (or IV) was formed with another fraction (Granick, 1955b).

However, in other systems synthesising porphyrins, no precursors could be detected. In experiments on the formation of porphyrins from ALA or PBG in haemolysed chicken red cells, no increase in porphyrin content could be found on treatment with iodine (Dresel & Falk, 1956b). Similarly, no precursors have been detected amongst the porphyrins formed when suspensions of *Rps. spheroides* are incubated anaerobically with glycine and α-oxoglutarate or with ALA (Lascelles, unpublished).

Some observations of Bogorad (1955 a,b) suggest an

explantion for these apparent discrepancies. The starting material for his enzyme fractionations was an aqueous extract of spinach leaf acetone powder. One purified fraction converted PBG in either the presence or absence of air to a colourless product, which slowly formed uroporphyrin I in air. Another fraction converted the colourless product rapidly to uroporphyrin I (via. an intermediate with a strong absorption maximum at 500 mμ). The whole extract produced uroporphyrin I from PBG, but if 0.1 M cysteine was added the porphyrin did not appear. Presumably, the whole extract and the second fraction contained a hydrogen acceptor system whose oxidation of the colourless precursor could be prevented by cysteine. Thus it may be that in those cases where porphyrin precursors appear, such a system is either absent or prevented from acting by the availability of hydrogen donors other than the precursors.

(ii) Porphyrin interconversion.

There is also conflicting evidence on the role of precursors as substrates for decarboxylation towards porphyrins with fewer carboxyl groups. Experiments suggesting that porphyrins themselves can undergo such decarboxylation are discussed first. Radioactive uroporphyrin III was incubated with a preparation of non-homogenised bone marrow cells, and protoporphyrin isolated containing 50% of the added radioactivity, (Salomon, Richmond & Altman, 1952). More detailed studies were made by Falk, Dresel & Rimington (1953) with later elaboration (Dresel, 1955; Dresel & Falk, 1956c). When a small amount of uroporphyrin III was incubated with a haemolysate of chicken red cells (but not the supernatant fraction), protoporphyrin (but no coproporphyrin) appeared in 50% yield; larger amounts of substrate gave no extra protoporphyrin and it was suggested that uroporphyrin III was utilised only slowly. However, the careful use of isotope

dilution techniques suggested that uroporphyrin III, coproporphyrin III, haematoporphyrin IX and protoporphyrin IX were probably not intermediates in the synthesis of haem from glycine, and that when dilution was observed it was due to an inhibitory effect of the added porphyrin. Finally, Eriksen (1955) observed the appearance of uro-, copro- and protoporphyrin when whole red cells were incubated with glycine under nitrogen, followed by the disappearance of uro- and coproporphyrin and a steady increase in the amount of protoporphyrin when further incubation was done aerobically. No evidence was given that the disappearance of the porphyrins was not due to their degradation. Falk (1955) and Dresel & Falk (1956 b) mention that there is some evidence for such degradation in haemolysates.

Observations of a rather difference nature are reported by Schwartz & Ikeda (1955). Radioactive uroporphyrin incubated with a rat liver homogenate and other tissues was incorporated into neither coproporphyrin nor protoporphyrin, but there was up to 15% incorporation into chromatographic fractions consisting of green and brown, fluorescing and non-fluorescing zones which were probably not degradation products of haem.

By contrast some workers report that porphyrins are not utilised by their systems. Uroporphyrin was not converted to other porphyrins by a pigeon liver acetone powder which could utilise ALA or PBG (Schulman, 1955), and similarly, chemically synthesised, highly radioactive coproporphyrin was not incorporated into haem by duck red cells. (Shemin in "Porphyrin Biosynthesis and Metabolism", 1955, p.228).

Others have found that the chemically prepared porphyrinogens are utilised more readily than porphyrins. The formation of haem (as measured by the incorporation of added radioactive iron) in a haemolysate of duck red cells was

increased by uroporphyrinogen III but not by uroporphyrins I or III or by uropopphyrinogen I. Moreover, the incorporation of glycine 2 -^{14}C into haem was reduced by uroporphyrinogen III. Coproporphyrinogen III behaved like uroporphyrinogen III, though less effectively (Nevé, Labbe & Aldrich, 1956). Similarly, an enzyme fraction from rabbit reticulocytes would decarboxylate uroporphyrinogen III to coproporphyrinogen III, but was inactive with uroporphyrin. (Mauzerall & Granick, 1958). Earlier, Granick (1955 b) had found that an enzyme fraction from chicken red cells would decarboxylate a product obtained by the action of another fraction on PBG, but again was inactive with uroporphyrin. Finally, uroporphyrinogen III is converted to coproporphyrin III by haemolysates of human red cells (Rimington & Booij, 1957).

On the other hand, it seems that the chemically prepared uroporphyrinogen is probably not as active as the biologically formed porphyrin precursor, at least in the case of Chlorella. Uroporphyrinogens I or III formed much less porphyrin than did a colourless precursor of uroporphryin I (obtained by the action of an enzyme preparation from spinach on PBG), when incubated with frozen and thawed cells of Chlorella; the porphyrins thus produced had from 3 to 8 carboxyl groups, (Bogarad, 1955 a, b).

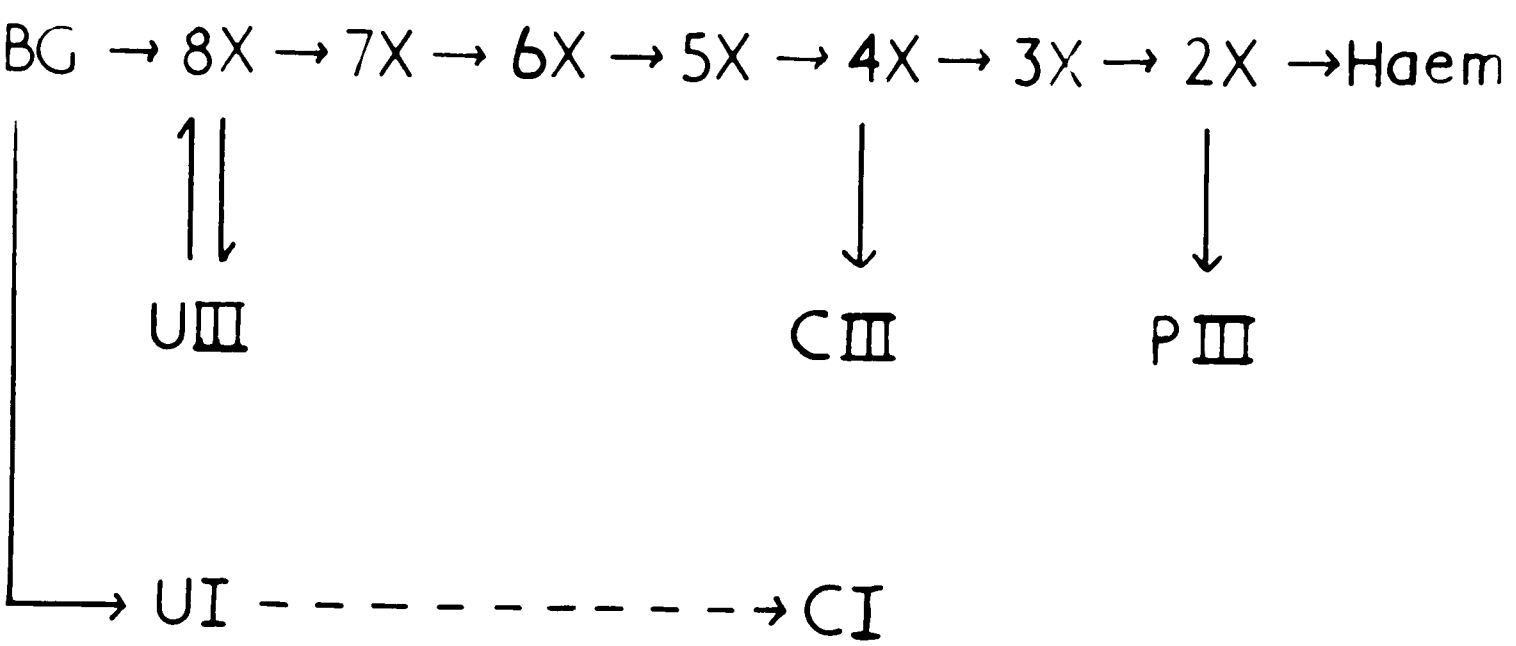
From the evidence summarised above, it seems unlikely that the porphyrins are themselves decarboxylated, though in some systems,none of which are cell free, they appear to be converted to porphyrins with fewer carboxyl groups, perhaps after preliminary reduction to the oxidation level of porphyrinogens. In the general scheme put forward by Dresel (1955), the true intermediates in the conversion of PBG to metalloporphyrins are represented as 8x, 7x, etc. according to their numbers of carboxyl groups (fig. 4).

FIGURE 4

THE RELATIONSHIP OF PBG, PORPHYRINS AND HAEM

(According to Dresel, 1955)

For explantion, see text. U = uroporphyrin,
C = coproporphyrin, P= protoporphyrin.



These intermediates are probably reduced porphyrins; the metal may be already inserted at this stage, or perhaps there is a complex with some other molecule. The situation may be analogous to that in purine biosynthesis where ribityl derivatives are probably the true intermediates (Schulman & Buchanan, 1951; Greenberg, 1951; see also Woods, 1952).

E. Studies with Enzyme Fractions.

(i) The formation of type III porphyrins

Experiments in which enzyme preparations are heated briefly at about 60° before incubation have shown that there is a heat-labile factor which directs formation of the type III isomers, that is of uroporphyrin III instead of uroporphyrin I. This has been studied with preparations from Chlorella and from red blood cells (Bogorad & Granick, 1953; Rimington & Booij, 1957; Booij & Rimington, 1957)

(ii) Stages in the conversion of PBG to coproporphyrin III

Some of the work with enzyme fractions on this problem has already been discussed in the light of the formation of porphyrin precursors rather than porphyrins themselves.

An enzyme fraction purified 45 -fold, called PBG deaminase, converted PBG to a colourless precursor of uroporphyrin I, with the release of an amount of ammonia equivalent to the PBG disappearing. The colourless product was further converted to uroporphyrin III by an enzyme fraction from wheat germ, or to porphyrins with 5 - 7 carboxyl groups by frozen and thawed cells of Chlorella (Bogorad, 1955,a,b,c).

The recent work of Granick and Mauzerall (1958) has simplified the whole situation, and as a result the reactions:-

PBG $\xrightarrow{(1)}$ Uroporphyrinogen III $\xrightarrow{(2)}$ Coproporphyrinogen III

have been well established. Two separate enzymes from animal red cells catalyse the two reactions; a third enzyme from rabbit reticulocytes catalyses reaction (2), and has no

action on ALA, PBG or uroporphyrin. However, this rabbit enzyme decarboxylates uroporphyrinogen I at half the rate with uroporphyrinogen III. It is of interest that Neuberger, Muir & Gray (1950) suggested that uroporphyrin I might be decarboxylated more slowly than uroporphyrin III (perhaps by the same enzyme), to explain the relative incidence of the isomers.

It now seems likely that the colourless precursors of porphyrins, produced and utilised by animal and plant preparations, are the same as the porphyrinogens of Fischer (see chapter 1), though the evidence was conflicting when the present study was begun (see Discussion). No study of porphyrin precursors in bacteria has been reported.

F. Some Factors Affecting Porphyrin Interconversion.

(i) Oxygen and a supply of energy.

If it is accepted that the porphyrinogens are the true intermediates on the pathway to metalloporphyrins, then none of the steps is oxidative until after coproporphyrinogen has been reached (see fig 3.) Then, however, two hydrogen atoms must be removed to form each vinyl group of protoporphyrinogen, (and eventually six more must be removed if a porphyrin or metalloporphyrin is to be produced). In accordance with this idea is the observation that the proportion of uro- and copro-porphyrins to protoporphyrin produced is increased if the incubation of animal tissues or haemolysates is done anaerobically (Falk, Dresel & Rimington, 1953; Dresel & Falk, 1956 a; Eriksen, 1955).

Oxygen may also be needed for the provision of energy for the synthetic pathway; in preparations with an intact photosynthetic system, light may take the place of oxygen. Effects of light in experiments with Rps. sph roides are related with effects of iron below.

(ii) B-vitamins.

Work with suspensions of T.vorax deficient in various B-vitamins (see section B of this chapter) has indicated that pantothenate may be required for the decarboxylation and dehydrogenation steps leading to protoporphyrin (Lascelles, 1957).

(iii) Iron.

The addition of iron salts to suspensions of T.vorax increased the proportion of protoporphyrin at the expense of the uroporphyrin in the mixture of porphyrins formed from ALA, and may thus be required for the same reactions as pantothenate, mentioned above (Lascelles, 1957). The situation with Rps. spheroides was more complex. Suspensions incubated in the light with ALA formed protoporphyrin only if iron salts and an oxidisable substrate such as fumarate were added; otherwise the major component was coproporphyrin. However, when the suspensions were incubated aerobically in the dark, the addition of iron had no effect on the types of porphyrin produced; protoporphyrin was formed by organisms which had been grown aerobically in the dark, but only coproporphyrin by light grown organisms (Lascelles, 1956 a). Hence, in conditions when photosynthesis can take place, Rps. spheroides needs a supply of iron for the steps between the coproporphyrin and protoporphyrin levels of decarboxylation.

G. The Formation of Iron Porphyrins.

(i) Relationship with free porphyrins.

There are many recorded observations of an increase in production of porphyrins when the synthesis of haem or cytochromes is depressed (for example by lack of iron, ribofavin, or vitamin B_{12}, or in mutant strains of yeast). With suspensions of Rps. spheroides incubated in the light with fumarate and ALA, formation of both haem and protoporphyrin was

increased and that of coproporphyrin reduced when iron salts were added. However, the amount of iron needed for maximum synthesis of haem was about five times greater than for protoporphyrin (Lascelles, 1956 b).

(ii) The insertion of iron

Evidence that protoporphyrin can be utilised for the synthesis of haem comes from studies with organisms requiring haem for growth. Protoporphyrin would replace haem for the growth of Haemophilus influenzae (Granick & Gilder, 1946; Gilder & Granick, 1947), and certain trypanosomes (Lwoff, 1951) Magnesium protoporphyrin will also replace haem for H.influenzae, at only one-fifth of the concentration of haem or protoporphyrin necessary. Magnesium protoporphyrin was assumed to be more permeable to the organism. (Granick, 1948 b). Failure to penetrate the organisms could account for the inability of protoporphyrin to replace haem for the growth of a strain of Staphylococcus aureus (Jensen & Thofern, 1953), and to produce haem when incubated with suspensions of Rps. spheroides (Lascelles, 1956 b).

Recent work with radioactive iron has given further details, but it is not yet known at what stage the iron is inserted: whether into protoporphyrin, protoporphyrinogen, or even earlier in the biosynthetic pathway.

H. The Formation of Chlorophylls.

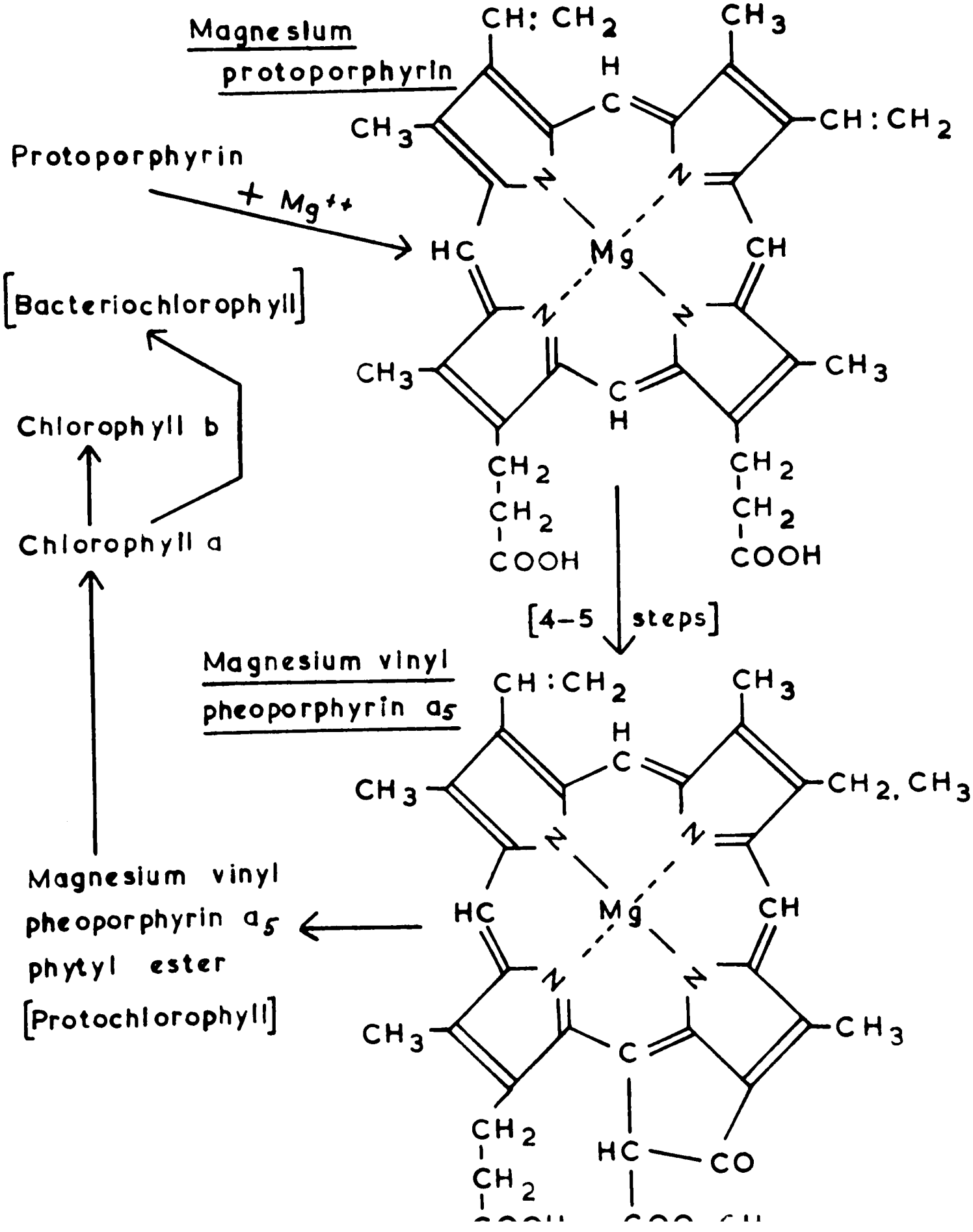
(i) The biosynthesis of chlorophyll in Chlorella.

A pathway of chlorophyll biosynthesis was suggested by Granick (see Granick, 1951), on the basis of magnesium porphyrins produced in cultures of mutant strains of Chlorella unable to form chlorophyll; no evidence was given that these compounds were not produced by side reactions. The mutants producing protoporphyrin and other porphyrins have already been described. Two yellow mutants synthesised magnesium

FIGURE 5

THE BIOSYNTHESIS OF CHLOROPHYLL BY A MUTANT STRAIN OF *Chlorella*

(According to Granick, 1948 b, 1950)



protoporphyrin (not previously observed in nature) (Granick, 1948 b), and magnesium vinyl pheoporphyrin a_5 (Granick, 1950), respectively (fig. 5). Addition of two hydrogen atoms to pyrrole ring IV of magnesium vinyl pheoporphyrin a_5, together with the phytol group, would yield chlorophyll a. The addition of the phytol group alone would yield protochlorophyll, found in higher plants grown in the dark, in which its hydrogenation is a photochemical process. However, in some plants, including algae and conifer seedlings, chlorophyll formation can proceed in the dark (see Granick, 1951).

No evidence is available on the formation of magnesium protoporphyrin, or whether bacteriochlorophyll synthesis in bacteria is similar to chlorophyll synthesis in Chlorella(see discussion (ii) The effects of iron.

The condition of iron chlorosis in plants, in which a deficiency of chlorophyll arises when a supply of iron is not available, is well known; the situation is complicated by factors in the soil which affect the availability of the iron (Hill & Hartree, 1953; Brown, 1956). The concentration of "chlorobium chlorophyll" in green sulphur bacteria is much increased by adding iron salts to the growth medium (Larsen, 1952, 1953). Bacteriochlorophyll production is also increased by iron salts in light-grown cultures of Rps. spheroides; in the absence of added iron, porphyrins are excreted in the later stages of growth when bacteriochlorophyll synthesis has almost ceased (Lascelles, 1955). In suspensions of this organism incubated in the light with glycine and α-oxoglutarate, the synthesis of bacteriochlorophyll is much reduced in the absence of added iron salts and large amounts of porphyrin are formed. However, there is no simple relationship; the production of porphyrin is much greater than the amount of bacteriochlorophyll which fails to appear. Hence the effects of iron are not fully accounted for by its stimulation of protoporphyrin formation (Lascelles, 1956 a)

(iii) The effects of light and oxygen.

As mentioned above, some plants form chlorophyll in the dark and all plants reach the protochlorophyll stage. However, no free porphyrins or bacteriochlorophyll are formed in the dark by cultures of Rps. spheroides (Lascelles, 1956a), nor is there any appreciable pigmentation due to carotenoids; however, cytochromes are formed in cells grown aerobically in the dark. Recently, suspensions of this organism have been shown to form bacteriochlorophyll in the dark, but less actively than in the light. (Lascelles, 1959)

If air is allowed access to a culture of Rps. spheroides growing anaerobically in the light, synthesis of both carotenoids and bacteriochlorophyll is inhibited, but growth continues. A blue-green mutant was obtained, lacking carotenoids, which was killed by this combined action of light and oxygen; it was suggested that carotenoids in the parent strain protect cell constituents from photosensitised oxidation (Griffiths, Sistrom, Cohen-Bazire & Stanier, 1955; Sistrom, Griffiths & Stanier, 1956). A mutant of Chlorella, lacking carotenoids, would only grow in the dark (Claes, 1954); presumably, the oxygen necessarily produced by the plant type of photosynthesis of this organism causes a similar photosensitised oxidation.

The blue-green mutant of Rps. spheroides produced less bacteriochlorophyll and grew more slowly than the wild-type, especially at low light intensities. It formed green, water-soluble pigments, one of which was bacteriopheophorbid and the others similar to pheophorbid a (a derivative of chlorophyll a, which is found only in plants). These pigments were either intermediates in the synthesis of bacteriochlorophyll or its degradation products; there was no evidence one way or the other (Griffiths et al., 1955; Sistrom et al., 1956).

These effects of light and oxygen on Rps. spheroides suggest that bacteriochlorophyll and carotenoids are affected in parallel by the fundamental changes in general metabolism induced

by light and oxygen in the Athiorhodaceae (see next chapter)

CHAPTER 3 THE ATHIORHODACEAE

A. General Metabolism

The first detailed study of the purple, non-sulphur bacteria (Athiorhodaceae) was made by van Niel (1944), who simplified their classification into two genera and six species. He also defined their differences from the purple, sulphur bacteria (Thiorhodaceae) which primarily live by photosynthetic fixation of carbon dioxide, with reduced sulphur compounds as hydrogen donors; (plants do not require such reduced compounds since they give off oxygen during photosynthesis).

On the other hand, the Athiorhodaceae primarily oxidise organic compounds during photosynthesis and at the same time assimilate the organic compounds as carbon source for growth; carbon dioxide is normally taken up only when a highly reduced substrate, such as butyrate, is being used. Some members can use reduced sulphur compounds or hydrogen instead of organic compounds, but all of these require B-vitamins in small amounts (van Niel, 1944). Thus the Athiorhodaceae are perhaps best described as photosynthetic heterotrophs.

The organic compound used as hydrogen donor and growth substrate may be any of a wide variety of fatty acids, alcohols, sugars and amino acids (Gaffron, 1933, 1935; van Niel, 1944). The work on the metabolism of such compounds in Athiorhodaceae, especially in relation to carbon dioxide fixation, has been reviewed by Elsden (1954); see also Stoppani, Fuller & Calvin (1955); Elsden & Ormerod (1956); Ormerod (1956); Coleman (1956).

B. Growth in the Dark.

Many organisms which are primarily autotrophs can also live heterotrophically. The Athiorhodaceae, in an analogous fashion, are primarily photosynthetic, but can also live by oxidation in the dark of those organic compounds which they are able to assimilate

photosynthetically (see van Niel, 1944; Woods & Lascelles, 1954)

Though van Niel (1944, 1949) observed similarities in the uptake of acetate by Rhodospirillum rubrum in the light and in the dark, it has recently been shown that metabolism in the light may differ from that in the dark. The evidence suggests that the tricarboxylic acid cycle operates in Rsp. rubrum (Elsden, 1954); indeed all the necessary enzymes are present (Eisenberg, 1953, 1955, 1957). In the dark, this cycle was probably essential in Rsp.rubrum for the oxidation of all substrates tested: in the light, some substrates (succinate, fumarate, malate and propionate)could apparently be metabolised by some other means since their photometabolism was not inhibited by fluoroacetate (Elsden & Ormerod, 1956). It has been suggested that the increased reducing power generated in the light may be responsible for such differences (Gest, 1951; van Niel, 1954); an increased supply of reducing power for processes such as the synthesis of bacteriochlorophyll may also be available in the light.

The relative contributions of these two types of metabolism when both light and oxygen are present shows variation between species. In ~~the~~ light of five times the saturating intensity, no oxygen was taken up by Rsp. rubrum, though the uptake by Rps. spheroides and Rps. capsulatus was merely reduced to 70% of that in the dark (Clayton, 1955). According to van Niel (1944), many strains of Athiorhodaceae are strictly anaerobic when first isolated; most of these (though not all) will tolerate air after repeated subculture in the laboratory. The adaptation to light in Rps. spheroides which has been grown in the dark coincides with increased powers of bacteriochlorophyll synthesis; bacteriochlorophyll formation is inhibited by oxygen. (Cohen-Bazire et al. 1957; Lascelles, 1959).

Since the Athiorhodaceae can rapidly produce bacteriochlorophyll, they must have active enzyme systems for synthesising the porphyrin nucleus. Water-soluble, red pigment was observed by van Niel (1944) in the culture fluids of many strains of Athiorhodaceae; this was probably porphyrin. Porphyrin synthesis was studied with Rps. spheroides by Lascelles (1955, 1956 a,b), thereby providing a basis for the many recent investigations which have used this organism as a tool for research on the biosynthesis of porphyrins.

CHAPTER 4. THE PRESENT INVESTIGATION

It is evident from the survey given in the previous chapters that more is known about the early stages in the biosynthesis of porphyrins than about the steps from PBG to metalloporphyrins. After the present study was begun, more evidence about these intermediate and later steps was published (see chapter 2), but the work was with enzyme systems from Chlorella or red blood cells; the detailed reactions from PBG onwards in bacteria have not yet been shown to be identical with those in animals and plants. The present study has been mainly concerned with some of the probable intermediates in these reactions as they occur in a locally-isolated member of the Athiorhodaceae.

In recent years, micro-organisms have been found increasingly useful in the investigation of biosynthetic processes, mainly because of their powerful and varied synthetic ability and their common metabolic lesions which may often easily be induced by the investigator, and by their ability to survive in a more varied environment than can animal cells, (see Woods, 1953)

In the field of porphyrin biosynthesis, micro-organisms have in the past been used relatively infrequently, despite the many observations of the appearance of porphyrin in growing cultures. Recently, this state of affairs has begun to change, and microbes have b en used increasingly.

The initial aim of the present study was to exploit the variability of micro-organisms to uncover some aspect of porphyrin synthesis in the Athiorhodaceae different from those studied by Dr. Lascelles, in this laboratory, using suspensions of Rps. spheroides (see the previous chapters).

The variability of micro-organisms may be used in two ways: the variation may be induced by imposing mutations on a selected organism (see Woods, 1953; Mitchell, 1953; Adelberg, 1953), or different natural strains or species may be selected for study. The latter method often gives less control over the situation, but has

occasionally yielded fundamental knowledge, for example on the mechanism of photosynthesis (van Niel, 1949).

A search for such different strains or species may be made amongst organisms whose known properties seem promising from the point of view of the metabolic processes under study. The early experiments in the present work were with the laboratory strain of Rhodopseudomonas palustris, which was thought to require pantothenate for growth, with the aim of throwing light on the participation of coenzyme A in porphyrin synthesis.

Alternatively, different species may be isolated from a suitable natural environment, an approach which has most often been used in studies of catabolic processes, where a strain with the appropriate nutritional needs or capabilities is isolated by the classical enrichment techniques. In studies of biosynthetic reactions, this method usually means a fairly indiscriminate selection of organisms, hoping that a useful strain may emerge. During the present study, such a search was made, and restricted to photosynthetic bacteria which must presumably be active in porphyrin synthesis for the production of chlorophyll. A member of the Athiorhodaceae was isolated and found to form much porphyrin in suspensions, under conditions similar to those used by Dr. Lascelles for Rps. spheroides. However, under anaerobic conditions little porphyrin appeared, but a colourless compound was formed which yielded porphyrin on oxidation in air. In view of the importance which was attached to such porphyrin precursors, (an opinion confirmed by subsequent reports and by the present study), and since another important compound, magnesium protoporphyrin, was excreted by the organisms the work with Rps. palustris was discontinued in favour of the locally isolated Rhodopseudomonad.

Studies at the different levels of investigation available in microbiology, as defined by Stephenson (quoted by Woods, 1953), are important to obtain a balanced picture of the life of an organism. Thus it is necessary to work with harvested suspensions, and later cell-free systems and purified enzymes for detailed appraisal of a reaction sequence, and with growing cultures (perhaps eventually in

their natural environment), for finding the importance of these reactions in the growth of the organism. Hence, though most of the present work was done with suspensions, growing cultures have also been used; this was especially important with the locally isolated organism, since initially nothing was known of its nutrition or classification.

PART TWO : EXPERIMENTAL

Chapter 1. THE SPECIES OF ATHIORHODACEAE USED.

Three members of the Athiorhodaceae have been used during the work to be described; most attention has been devoted to a locally isolated strain of Rhodopseudomonas capsulatus, which is described first.

A. Rhodopseudomonas capsulatus, strain Oxford.

The identification of the genus and species of this organism is described in section A (iii) of this chapter; it will subsequently be referred to as Rps. capsulatus, unless it must be distinguished from other strains of the species, when the description "Oxford strain" will be used.

(i) The source

The choice of a situation expected to provide a good source of photosynthetic bacteria was founded upon the report that Athiorhodaceae are most abundant in muds in which much microbial decomposition of organic matter is going on, or in the overlaying water, especially in oily situations (van Niel, 1944). A convenient pond near Oxford had precisely these properties, since into it was drained a constant supply of kitchen waste from nearby houses. A sample of mud was collected on a day in the late autumn, and treated as follows.

(ii) Isolation

This was based upon the classical technique of Winogradsky (1887) for the detection and isolation of photosynthetic bacteria, and upon the method described by van Niel (1944) for enrichment cultures and pure culture isolation of Athiorhodaceae.

The mud sample was incorporated into the mixture given in appendix 2 and poured into a glass cylinder which was placed

in the light incubator (see chapter 2) at 28 - 31° for 9 days. Inside the wall of the cylinder there appeared diffuse areas of red-brown colour which were more predominant on the side facing the light source. About 1 ml. of mud suspension was withdrawn from a region of dense colour and added to a glass-stoppered bottle filled to the brim with enrichment culture medium (appendix 2). After 2 days in the light incubator at 28 - 31°, there was a heavy, red-brown bacterial growth which showed a strong, red fluorescence under ultra-violet light, indicating the probable presence of porphyrin.

Shake cultures were then set up in soft glass tubes containing enrichment culture medium solidified with 1% (w/v) agar, using a serial dilution technique to obtain discreet colonies. After light incubation for 4 days, a red-brown type of colony was seen to be preponderant and appeared to excrete a red pigment, probably porphyrin, into the surrounding agar. Thus a single colony of this type was picked out, after breaking the tube, This shake culture procedure was repeated twice, giving a total of three single-colony isolations.

The final such isolate was transferred to a stab culture in enrichment culture medium solidified with 1.5% (w/v) agar. A microscopic examination of both wet and stained preparations from this culture gave no indication that more than one morphological type was present. Later, Professor C. B. van Niel (private communication) was of the opinion that the culture was pure after microscopic examination and study of a culture in a Petri dish which had been incubated aerobically in the light, giving only uniform, pink colonies. A similar uniformity was observed in cultures in liquid or solid media incubated anaerobically in the light or aerobically in the dark.

(iii) General properties and identification

The morphology of Rps. capsulatus (Oxford strain) was examined by means of wet preparations and Gram stains.

Organisms derived from liquid or solid media were non-motile, Gram-negative rods, between 0.5 and 0.8 μ in width and not less than 1 μ in length; long forms were much morefrequent in the older or more alkaline cultures. Occasional short chains, consisting of two to five rods, were seen in young cultures. No spores were seen in a preparation stained with carbol fushin and methylene blue. The production of slimy masses of bacteria difficult to suspend uniformly was observed in cultures of pH greater than about 8.

The colour of cultures grown in the light was either brown, peach-coloured, red or purple, depending upon the constituents of the growth medium, the age of the culture and the access of air during growth. Such colour variation is probably due to variation in the relative proportions of the different carotenoids (van Niel, 1944; van Niel, Goodwin & Sissins, 1956) The pigments detected by spectroscopic examination of cultures or extracts of Rps. capsulatus (Oxford strain) were bacteriochlorophyll, carotenoids, cytochrome, magnesium protoporphyrin, and free porphyrins.

The organism was able to grow in either light or dark, but air was required for growth in the dark. Either organic compounds or hydrogen were required as major growth substrates; neither sulphide nor thiosulphate would support growth. Small amounts of thiamin, nicotinic acid and biotin were also essential for growth. Details of these and other nutritional properties are given in chapter 2.

These properties are in accordance with the definitions of the Athiorhodaceae on the basis of nutrition and physiology, and of the genus Rhodopseudomonas on the basis of morphology, as summarised by van Niel (1944). There was a single discrepancy, however, since motility, a property included in the definition of Rhodopseudomonas, was never observed in cultures of Rps. capsulatus (Oxford strain).

Diagnostic aharacters of the individual species of Rhodopseudomonas are compared with the corresponding properties

TABLE 1

DIAGNOSTIC CHARACTERS OF RHODOPSEUDOMONAS SPECIES COMPARED WITH THOSE OF Rps. capsulatus (OXFORD STRAIN)

A. Keys for the Identification of Rhodopseudomonas species (van Niel, 1944)

I. (1) Rod-shaped in all media:
   (a) Short rods, sometimes curved; no mucus produced; red to dark red-brown : palustris
   (b) Slender rods; usually much slime; pale brown to peach coloured : gelatinosa.
   (2) More or less spherical below pH 7 :
   (a) Rod-shaped above pH 7; zig-zag chains produced : capsulatus
   (b) Spherical above pH 7; mostly single, no chains : spheroides

II. (1) Gelatin liquefied : gelatinosa
   (2) Gelatin not liquefied:
   (a) No mucus formed; colour the same in anaerobic as in aerobic cultures : palustris
   (b) Mucus formed above pH 8; brown in anaerobic, red in aerobic cultures:
   (i) Develop on 0.2% propionate; much mucus above pH8: capsulatus
   (ii) Do not develop on 0.2% propionate; slime evident above pH 7: spheroides.

III. (1) Thiosulphate used : palustris
   (2) Thiosulphate not used:
   (a) 0.2% propionate used : capsulatus
   (b) propionate not used:
   (i) Mannitol and sorbose used: spheroides

(ii) Mannitol and sorbose not used : gelatinosa

B. The Vitamin Requirements of Rhodopseudomonas species (Hutner,1946)

(1) palustris, all stains : p - aminobenzoic acid.
(2) capsulatus, some strains: thiamin, nicotinic acid and biotin; other stains thiamin only.
(3) gelatinosa, all strains: thiamin and biotin.
(4) spheroides, all strains: thiamin, nicotinic acid and biotin.

C. Corresponding Observations for Rps. capsulatus (Oxford strain)

(1) Very short rods in young cultures around pH 7, longer at higher pH.
(2) Occasional chains seen, not in zig-zag form.
(3) Mucus produced in cultures of pH greater than about 8.
(4) Gelatin not liquefied.
(5) Brown in anaerobic, red in aerobic cultures.
(6) Growth obtained on 0.2% propionate.
(7) Growth not obtained on thiosulphate.
(8) Growth obtained on sorbose but only very slight growth on mannitol.
(9) Thiamin, nicotinic acid and biotin all essential for growth.

(Details of growth tets given in chapter 2)

TABLE 2

A COMPARISON BETWEEN A TYPICAL Rps. capsulatus AND THE OXFORD STRAIN

(properties of Rps. capsulatus according to van Niel, 1944, and Hutner, 1946).

Similarities

(1) Both form very short rods below, and longer rods above pH 7.

(2) Both form mucus above pH 8.

(3) Both are brown in anaerobic, red in aerobic cultures.

(4) Air inhibits the growth of neither.

(5) The optimum temperature for the growth of both is below 30°.

(6) Both grow on propionate, glucose, fructose, alanine and glutamate.

(7) Neither grows on ethanol, mannitol, sorbitol, leucine or thiosulphate.

(8) Thiamin, nicotinic acid and biotin are essential for the growth of the Oxford strain, and some of the strains of Rps. capsulatus investigated by Hutner (1946)

Differences

(1) The zig-zag chains characteristic of Rps. capsulatus were never observed in cultures of the Oxford strain; occasional short, straight chains were seen.

(2) Rps. capsulatus typically fails to grow on mannose or glycerol; the Oxford strain grew in the light on mannose as rapidly as on glucose or other hexoses, and grew slowly on glycerol.

(details of growth tests given in chapter 2)

This comparison is consistent in most respects with an identification of the organism as Rhodopseudomonas capsulatus. Hence a list of similiarities and differences between the Oxford strain and a typical Rps capsulatus is given in table 2. The most important difference is the failure of the Oxford strain to produce the zig-zag chains typical of Rps. capsulatus; this was considered to be outweighed by the many similarities, since the occasional short, straight chains that were observed showed that there was some tendency towards chain formation. Thus the Oxford strain was assigned to the species Rhodopseudomonas capsulatus van Niel.

(iv) Stock cultures

After some observations on the nutrition of Rps. capsulatus (see chapter 2), the stock culture medium was changed from that used in enrichment cultures to medium A (appendix 3), supplemented with Difco yeast extract (0.2% w/v) and Difco agar (1.5% w/v). Stab cultures were subcultured monthly and incubated in the light incubator at 28 - 31° for 30 hr; they were stored at 0 - 4°.

B. Rhodopseudomonas palustris

The strain of this species used was the laboratory strain, obtained from Dr. J. R. Elsden and derived originally from the collection of Professor C. B. van Niel. It was believed to require pantothenate for growth and known to produce porphyrin in cultures.

The stock culture medium, decided upon after some nutrition tests, was medium B (appendix 4), supplemented with Difco yeast extract (0.2% w/v) and Difco agar (1.5% w/v). Stab cultures were subcultured monthly and incubated in the light incubator at 28 - 31° for 3 days; they were stored at 0 - 4°.

C. Rhodopseudomonas spheroides

The strain used was the same as that employed by Dr. June Lascelles, in this laboratory, in her studies on porphyrin and bacteriochlorophyll synthesis; (Lascelles, 1956 a); it had been obtained from the same sources as those of Rps. palustris. Stab cultures on medium C (appendix 5), supplemented with Difco yeast extract (0.2% w/v) and Difco agar (1.5% w/v) were kindly provided by Dr. Lascelles. The organism had been subcultured monthly, incubated in the light at 32 - 34° for 40 hr. and stored at 0 - 4°.

CHAPTER 2. GROWTH EXPERIMENTS WITH Rps. capsulatus and Rps. palustris

Most of the results described in this chapter have been obtained in growth tests with Rps. capsulatus (Oxford strain); these were done to work out favourable conditions for its growth, to help in its identification (see chapter 1), and to obtain knowledge necessary for the design or interpretation of experiments with suspensions. Experiments with Rps. palustris are mentioned for comparative purposes, with special reference to the effects of iron in the two organisms.

A. Procedures for Growth Tests

(i) Chemicals and glassware.

All chemicals used in growth media and suspension mixtures, and in the course of their preparation, were of analytical reagent grade when obtainable; otherwise, standard reagent grade chemicals were used.

Glassware was cleaned with hot chromic acid, rinsed 6 - 8 times with tap water and twice with de-ionised water. When necessary, glassware was sterilised by maintaining at 140 - 160° for 1½ to 2 hr. The 6 x ½ in. tubes used for growth tests and suspension experiments were previously plugged with white, absorbent cotton-wool; grey, non-absorbent cotton-wool was used for the 6 x ⅝ in. tubes for stock cultures and inoculum development.

(ii) Inoculum development

The inocula for growth experiments with Rps. capsulatus were derived from liquid cultures in medium A (appendix 3), supplemented with 0.2% (w/v) Difco yeast extract, in 6 x ⅝ in. tubes incubated for 24 hr. in the light at 28 - 31°; the liquid cultures were in turn derived from stab cultures in the same medium, further supplemented with 1.5% (w/v) agar,

incubated for 30 hr. in the light at 28 - 31°. Such stab cultures were derived from the stock culture at the end of each month, and subcultured weekly.

The procedure for Rps. palustris was the same except that medium B. (appendix 4) was used instead of medium A, and both broth and stab cultures were incubated for 3 days. Incubation vessels for growth experiments were sown with 1 µg. organisms (dry weight) per ml. of culture fluid.

(iii) Conditions of incubation.

All incubations in the light in both growth and suspension experiments were done in a specially-designed light incubator; incubation vessels were placed 6 - 9 in. from incandescent tungsten filament lamps (15 and 25 watt), which were spaced around and along a vertical column down the centre of the incubator. The temperature inside a 6 x ½ in. tube containing water was kept between 28 and 31° by manual control of air vents and of the speed of a fan. Occasionally, a substantial overnight fall ~~temperature~~ in the ambient temperature caused the incubator temperature to fall below this limit, but never below 25°.

The incubation vessels used in growth and suspension experiments in the light were 6 x ½ in Pyrex tubes containing 5 ml. fluid, unless stated otherwise. By the use of such narrow tubes, the access of light to dense cultures and suspensions was promoted and the access of air was minimised.

Aerobic cultures (10 ml. volume) were grown in tubes shaped like an inverted T (Monod, Cohen-Bazire & Cohn, 1951), which were immersed in a water bath at 30° and shaken (36 oscillations /min.) by means of a rocking device (van Heynigen & Gladstone, 1953). Such cultures were exposed to laboratory daylight; when rigorous exclusion of light was desired the tubes were completely encased in tinfoil.

(iv) Measurement of growth.

Growth was measured turbidimetrically with an EEL photoelectric colorimeter (Evans Electroselenium Co. Ltd., Harlow, Essex), using 6 x ½ in. tubes so that the course of growth of light-grown cultures could be measured directly in the incubation vessels. A neutral density light filter was used, and the EEL reading was adjusted to zero with uninoculated medium. The EEL reading was proportional to the dry weight of the organisms up to a reading of 40, which was given by cultures containing 1.0 mg. dry wt. bacteria / ml. (Rps. capsulatus), or 1.1 mg. dry wt. /ml. (Rps. palustris). Since the EEL reading for a culture using a green light filter was no more than 10% greater than with a red light filter, no correction was made for the small variation in colour of the organisms which was found under different conditions of growth in the light.

All measurements of growth described below were average values from duplicated cultures.

B. Growth Experiments with Rps. capsulatus.

The conditions of inoculation, incubation and measurement of growth were as described in section A of this chapter, unless stated otherwise.

(i) Growth in the chemically defined medium.

Apart from the addition of acid-hydrolysed casein, and an increase in the amount of nicotinic acid, medium A (appendix 3) was identical with that used by Dr. June Lascelles in this laboratory for the growth of Rps. spheroides. Medium A was found to support satisfactory growth of Rps. capsulatus; about 5 mg. dry wt. of bacteria /ml. of culture were obtained after 3 days incubation in the light. The pH of the medium (7.8) was optimum for growth; the optimum temperature was between 28 and 31°.

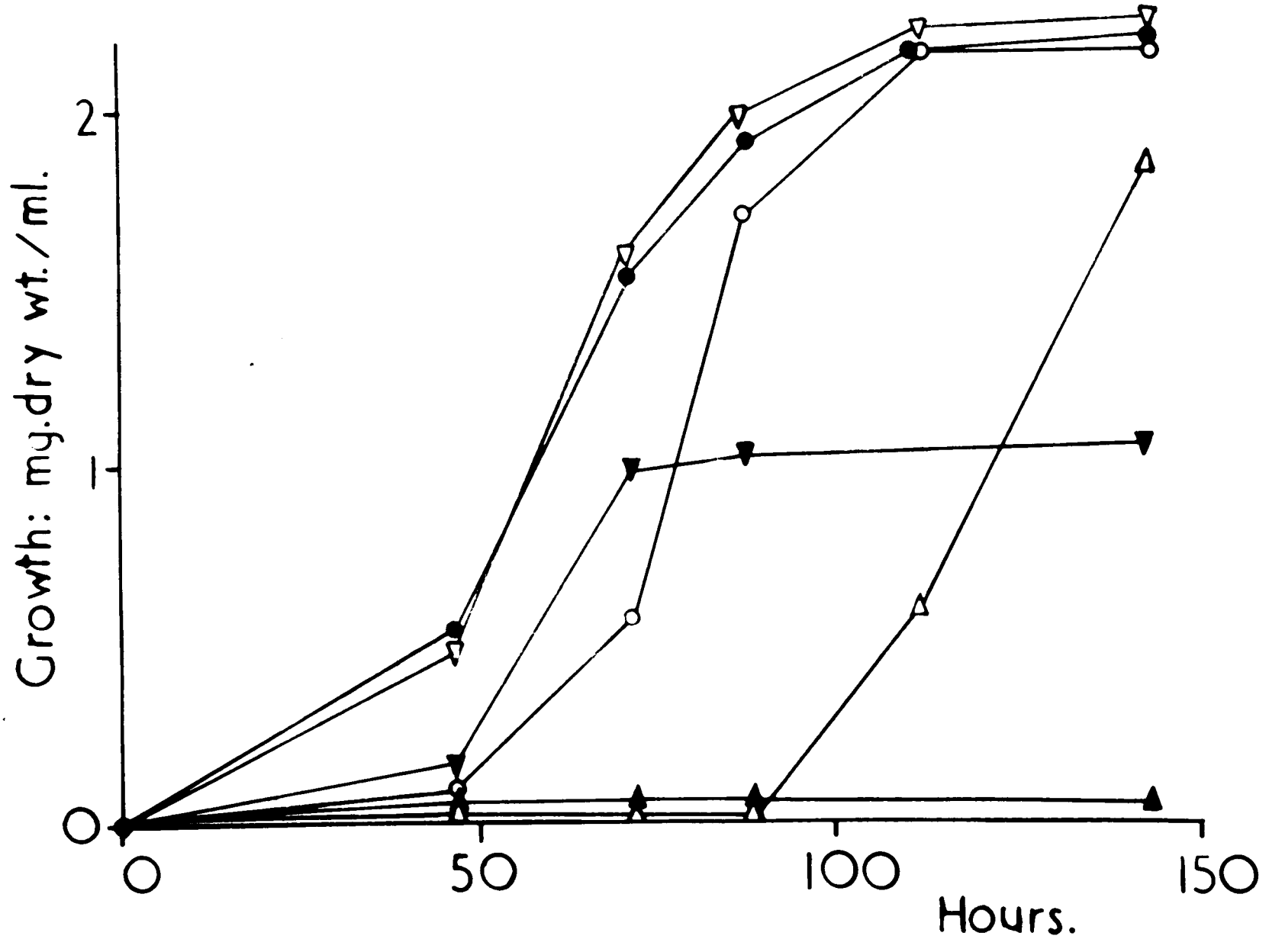
The effects of varying the constituents of the medium are described below.

FIGURE 6.

THE TIME-COURSE OF GROWTH OF R.s. capsulatus WITH METHIONINE AND COBALAMIN

Cultures were grown in medium D (appendix 6), with additions as follows: ▲—▲ nil; ▼—▼ $(NH_4)_2 SO_4$ (0.002M); Δ—Δ $(NH_4)_2 SO_4$ (0.006 M); ▽—▽ $(NH_4)_2 SO_4$ (0.006M); with anaerobic incubation; ●—● $(NH_4)_2 SO_4$ (0.006M) + DL-methionine (10^{-6} M); ○—○ $(NH_4)_2SO_4$ (0.006M) + cobalamin (2×10^{-10} M).

The conditions of incubation were as given on p. 47 (semi-aerobic incubation in the light), unless otherwise stated.



(ii) Nitrogen sources

Growth of Rps. capsulatus was never observed in the absence of fixed nitrogen or with nitrate or nitrite as its sole source. Ammonium could serve as the sole source of fixed nitrogen (apart from small amounts of added nicotinic acid, thiamin and biotin), but there was a delay of 3 - 4 days before detectable growth commenced (fig. 6). This delay could be reduced to 1 - 2 days (i.e. the same as that obtained under the most favourable conditions of growth on the standard growth medium A (appendix 3)) in any one of the following ways, (fig. 6):

(a) incubation under an atmosphere of hydrogen or nitrogen in Thunberg tubes in the light,

(b) reduction of the concentration of ammonium to one-third of that used in the standard growth medium, thereby reducing the final growth yield to about half of that normally obtained,

(c) addition of DL-methionine at 10^{-6} M,

(d) addition of cobalamin (vitamin B_{12}) at 2×10^{-10} M, though in this case there was a slightly longer delay than with methionine or anaerobiosis.

On the other hand, if cultures were incubated aerobically in the dark, the delay before growth could be detected was about

6 days and the amounts of methionine or cobalamin required to restore the delay to that observed in the standard growth medium A (1 - 2 days) were 2×10^{-4} M and 2×10^{-9} M, respectively.

To summarise the situation, growth of Rps. capsulatus was considerably delayed if both air and a moderate concentration of an ammonium salt were present. This toxic effect was completely abolished by a low concentration of methionine, and almost abolished by a very low concentration of cobalamin. The concentrations of these compounds necessary were greater if the supply of air was increased.

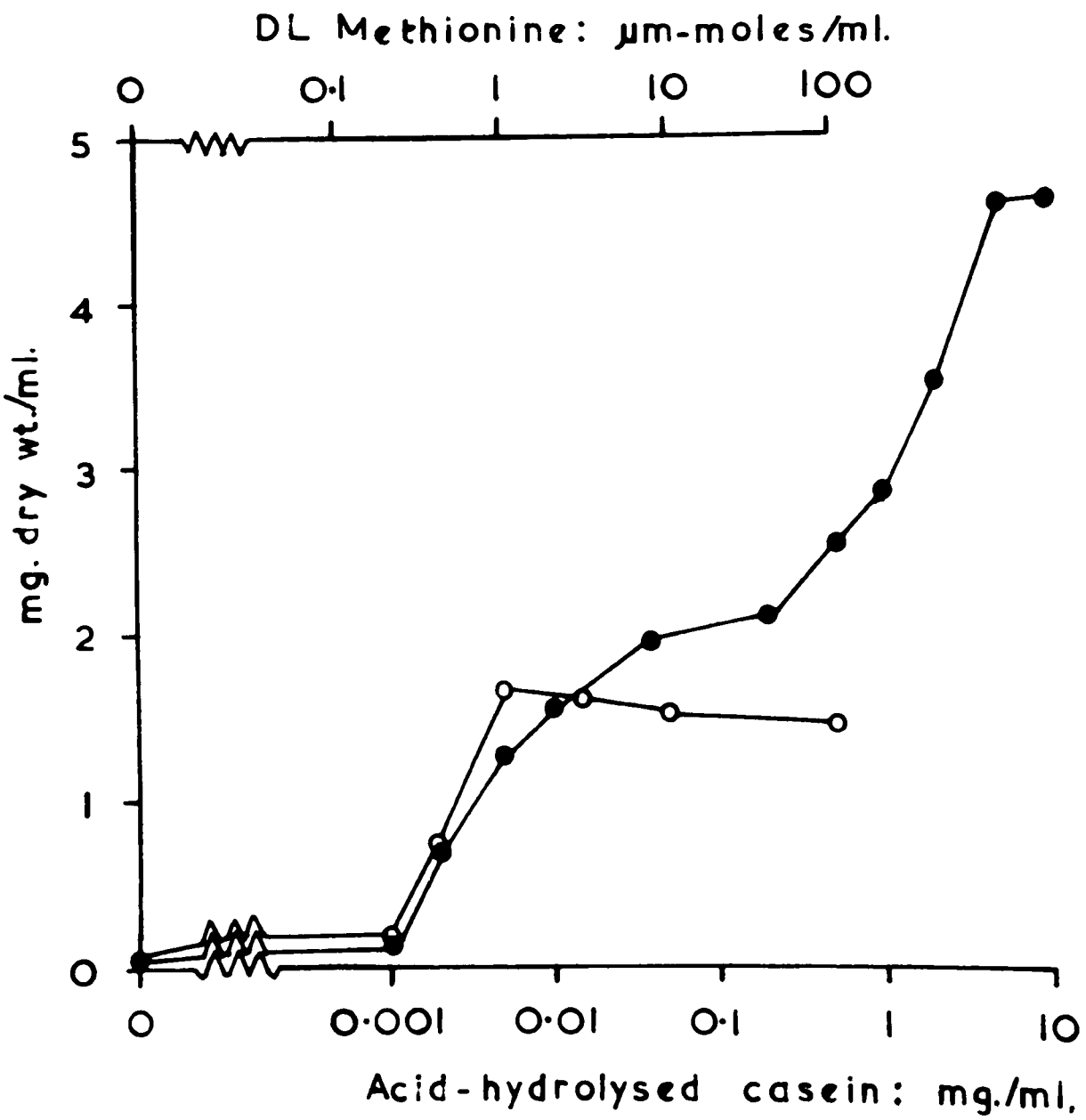
In an attempt to replace methionine and cobalamin in these effects, the following compounds were tried separately, each at three different concentrations: glycine, DL-serine, sodium formate, δ-aminolaevulic acid, L-cysteine, glutathione, DL-aspartic acid, DL-asparagine, L-glutamine, DL-alanine, DL-leucine, L-arginine, DL-tyrosine, DL-tryptophan, DL-phenylalanine, biotin, p-aminobenzoic acid, and sodium sulphide. Furthermore, the carbon source of the medium (medium D, appendix 6) was changed from fumarate to succinate, malate, glucose or pyruvate, and the concentrations of magnesium, calcium, iron and phosphate ions, and of biotin, were varied. None of these modifications caused a reduction in the delay before growth commenced in the presence of 0.006 M ammonium sulphate and air. On the other hand, sodium L-glutamate (British Drug Houses, reagent grade), which formed part of the standard growth medium A (appendix 3), did replace methionine and cobalamin completely if used at a relatively high concentration (2×10^{-4} M). However, since a purer sample (sodium L-glutamate derived from L-glutamic acid; British Drug Houses amino acids) had to be added at a concentration of 2×10^{-2} M before the same response was observed, it was inferred that these effects were probably due to impurities of methionine and/or cobalamin; L-glutamic acid has been reported to be a notable source of growth factors (Hutner, 1946).

FIGURE 7

THE GROWTH OF Rps. capsulatus WITH ACID-HYDROLYSED CASEIN AND METHIONINE

Cultures were grown in the light in medium D (appendix 6), with the addition of 0.0006 M $(NH_4)_2 SO_4$. Conditions of incubation were as given on p.47 ; the time of incubation was 90 hr.

Additions: O—O DL-methionine; ●—● acid-hyrolysed casein.



Acid-hydrolysed casein (appendix 8) would also replace methionine and cobalamin in these tests (fig.7). A 0.0002% (w/v) solution of acid-hydrolysed casein supported about the same growth response as did 4×10^{-7} M DL-methionine. Calculations from the composition of casein given by Olcott (1951) indicated that this concentration of acid-hydrolysed casein would be expected to contain 5×10^{-7} M L-methionine, so that an adequate explanation for the replacement is provided.

When acid-hydrolysed casein was added to growth media in much higher concentration (0.1% (w/v) and upwards), growth was always much heavier than in its absence. No combination of carbon sources or amino acids which was tested allowed a maximum growth of more than 3.5 mg. dry wt /ml., in the light; the addition of 0.5% (w/v) acid-hydrolysed casein to media containing sugars or carboxylic acids allowed yields of 5 - 8 mg. dry wt./ml. The corresponding yields from cultures grown aerobically in the dark were 0.8 and 1.7 mg. dry wt./ml., respectively. No attempt was made to devise a balanced mixture of amino acids to replace these higher concentrations of acid-hydrolysed casein.

(iii) Carbon sources

Various sugars, carboxylic acids, alcohols and amino acids were tested singly at two different concentrations for their ability to act as growth substrates for Rps. capsulatus in the presence of 10^{-6} M DL-methionine, inorganic salts and vitamins. Growth was measured after four days incubation in the light. Heavy, rapid growth was supported by glucose, fructose, mannose, sorbose, galactose, xylose, sucrose, maltose, pyruvate, lactate, acetate, propionate, butyrate, succinate, fumarate, malate, aspartate, asparagine, or by an atmosphere of hydrogen containing 5% (v/v) carbon dioxide; slow or weak growth was supported by glycerol, glutamate or alanine. No growth was observed with lactose, gluconate, mannitol, sorbitol, isopropanol, ethanol, methanol, formate, malonate, glutarate, stearate, citrate, glycine, leucine, nitrite, sulphide or thiosulphate, with or without the addition of carbon dioxide to the atmosphere.

(iv) Vitamins

Growth of Rps. capsulatus was never observed in the absence of either thiamin, nicotinic acid or biotin. The minimum concentrations required to support optimal growth were 10^{-6} M, 4×10^{-5} M and 2×10^{-8} M, respectively.

No stimulation of growth was produced by adding to cultures riboflavin, pantotnenic acid, β-alanine, pyridoxin, p-aminobenzoic acid, adenine, guanine, uracil, or thymine, each at two different concentrations. The effect of cobalamin in reducing the delay before growth commenced is described in paragraph (ii), above.

C. Growth Experiments with Rps. palustris

(i) Growth in the chemically defined medium

After preliminary experiments, medium B (appendix 4) was used as the standard growth medium for Rps. palustris. After 4 days growth in the light, 1.6 - 2.0 mg. dry wt./ml. was the yield of organism usually obtained. The pH of the medium (6.8) was optimum for growth; the optimum temperature was between 30 and 33°.

(ii) Nitrogen sources

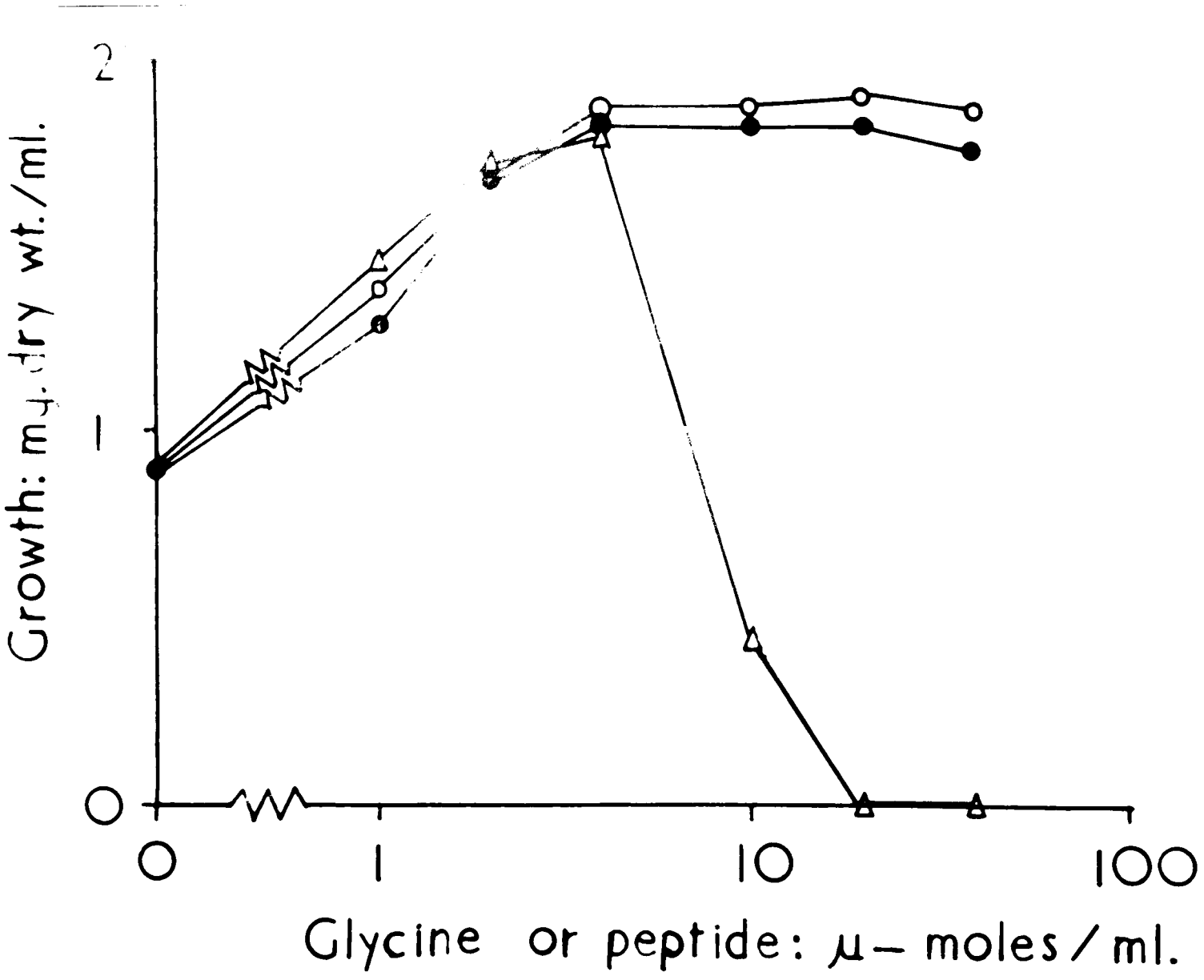
Rps. palustris was never observed to grow in the absence of fixed nitrogen. In contrast to the findings with Rps. capsulatus (see section B (ii) of this chapter), growth was not delayed when ammonium was the sole source of fixed nitrogen, though the final yield of cultures was doubled when glutamate, glycine or acid-hydrolysed casein (appendix 8) was added as supplementary source of nitrogen. Acid-hydrolysed casein did not support the very heavy growth which was a noteworthy feature of its effect on Rps. capsulatus. In cultures of Rps. palustris it could be completely replaced by 2×10^{-3} M glycine, which stimulated growth with or without the addition of 2×10^{-2} M glutamate. Glycine added in concentrations greater than 4×10^{-3} M strongly inhibited growth; no such inhibition was produced by glycyl leucine or leucyl glycine (fig. 8)

FIGURE 8

THE GROWTH OF Rps. palustris WITH GLYCINE AND THE TWO GLYCINE-LEUCINE DIPEPTIDES

Cultures were grown in the light in medium B (appendix 4), with the omission of glycine. Conditions of incubation were as given on p. 47 ; the duration of incubation was 98 hr.

Additions: Δ—Δ glycine; O—O glycyl leucine; ●—● leucyl glycine.



(iii) Carbon sources

The following compounds supported the growth of cultures of Rps. palustris in the light, when tested separately at 0.02M in medium B (appendix 4) with lactate and glutamate omitted: Pyruvate, lactate, malate, fumarate, succinate, acetate, glycerol and glutamate.

(iv) Vitamins

Growth of Rps. palustris could be obtained in a medium containing only mineral salts and lactate derived from redistilled lactic acid. However, 10^{-6} M pantothenic acid caused an acceleration of the growth of cultures, and was incorporated into the standard growth medium. No effect of p-aminobenzoic acid was observed; Hutner (1946) found a requirement for this vitamin to be characteristic of Rps. palustris.

D. The Effect of Iron Citrate on Growth and the Production of Porphyrins and Bacteriochlorophyll in Cultures of Rps. capsulatus and Rps. palustris.

In experiments with cultures and suspensions of Athiorhodaceae, iron was added in the form of iron citrate, prepared as in appendix 7. It was presumed that the citrate ions, by the formation of a complex salt, would help to keep in solution the large concentrations of iron needed for optimum growth and synthesis of bacteriocholophyll. No different effects were observed when ferrous sulphate was used. The state of oxidation of the iron citrate was unkown; the solution darkened on keeping, probably due to oxidation of the freshly-prepared ferrous solution. No attempt was made to produce a greater degree of iron deficiency in cultures than could be achieved merely by withholding iron citrate from media.

Porphyrins and bacteriochlorophyll in cultures of Rps.capsulatus and Rps. palustris were identified and estimated by the methods used with suspensions of Rps. capsulatus, described in chapter 3.

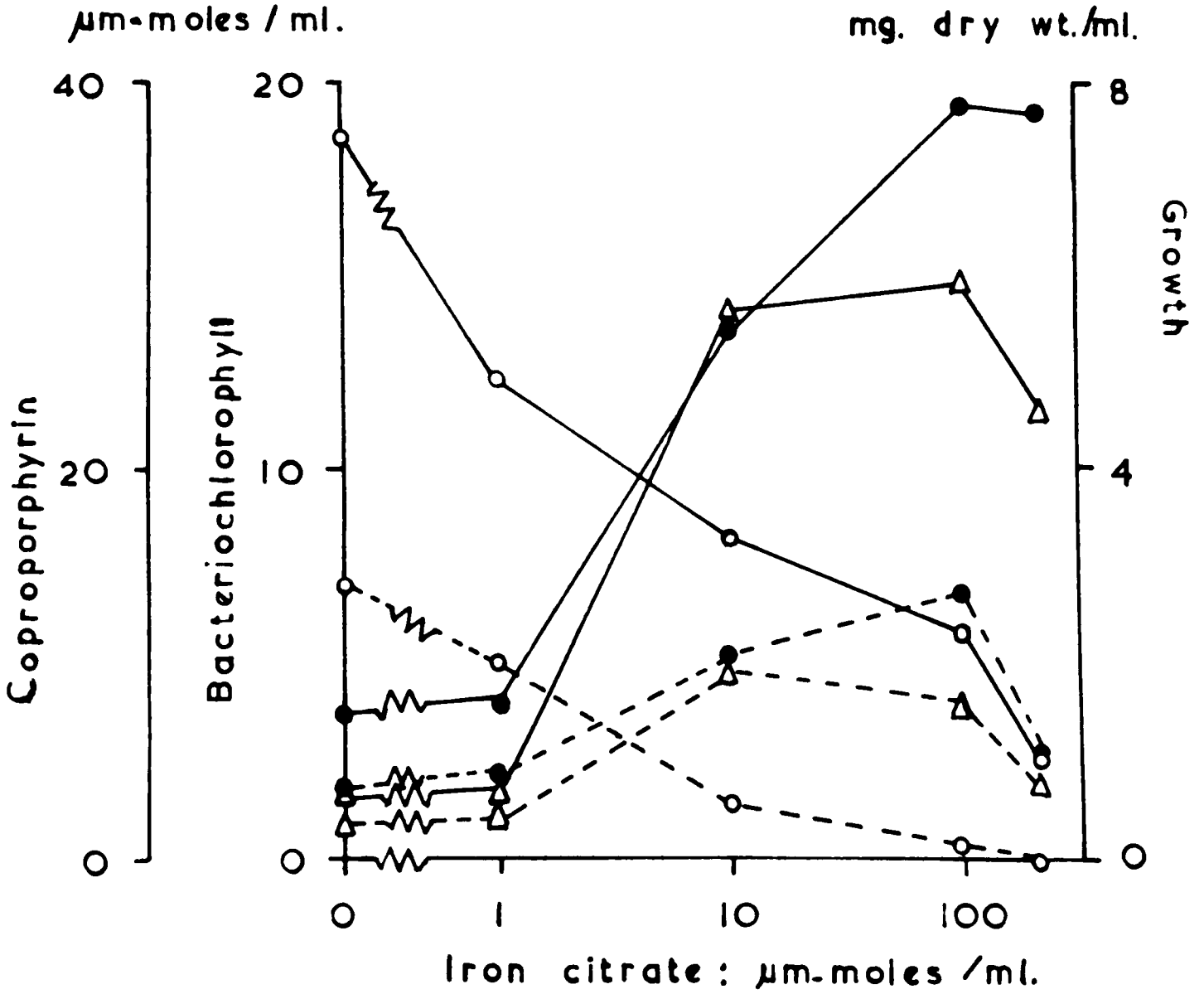
Both organisms produced more coproporphyrin III and less

FIGURE 9

THE EFFECTS OF IRON ON GROWTH AND THE FORMATION OF COPROPORPHYRIN AND BACTERIOCHLOROPHYLL IN CULTURES OF Rps. capsulatus AND Rps. palustris

Rps. capsulatus was grown in medium A (appendix 3) and Rps. palustris in medium B (appendix 4), each with iron citrate varied as indicated; conditions of incubation were as given on (in the light) p.47A; the duration of incubation was 90 hr. for Rps. capsulatus (solid line) and 116 hr. for Rps. palustris (broken line).

Δ—Δ growth; O—O coproporphyrin; ●—● bacteriochlorophyll.



bacteriochlorophyll in light-grown cultures when their growth was limited by using media without added iron citrate (fig. 9)

The addition of iron to a culture promoted the synthesis of both a greater total amount of bacteriochlorophyll and a greater amount per unit dry weight of organism. With both organisms, the increased production of bacteriochlorophyll due to the addition of iron was less than the associated decreased production of coproporphyrin. The effects of iron are similar to those reported with cultures of Rps. spheroides (Lascelles, 1955).

There were two noteworthy differences between cultures of Rps. capsulatus and Rps. palustris in their productions of porphyrins and bacteriochlorophyll:

(i) At any given concentration of iron, Rps. capsulatus produced larger amounts of both coproporphyrin and bacteriochlorophyll, as well as heavier growth, than did Rps. palustris. The much greater production of coproporphyrin by Rps. capsulatus than by Rps. palustris when moderate or large amounts of iron were added was especially evident (fig. 9).

(ii) Cultures of Rps. capsulatus produced magnesium protoporphyrin (see chapter 3), never observed in cultures of Rps. palustris. Examination of cultures of Rps.capsulatus grown in medium A (appendix 3), supplemented with 0.02%(v/v) of Tween 80 (see p. 84) showed that 24-hour cultures had produced 30 - 50 µm-moles magnesium protoporphyrin /ml. but no detectable coproporphyrin; 48 - hour cultures contained 40 - 60 µm-moles of coproporphyrin /ml., but no more magnesium protoporphyrin than in 24 - hour cultures. This sequence of appearance is discussed on p. 82 , in relation to the effects of iron in bacterial suspensions.

Chapter 3. THE IDENTIFICATION and ESTIMATION OF PORPHYRINS, MAGNESIUM PROTOPORPHYRIN AND BACTERIOCHLOROPHYLL

The porphyrin derivatives detected and measured in cultures and suspensions of Rps. capsulatus were porphyrins, porphyrin precursors, magnesium protoporphyrin, and bacteriochlorophyll. The porphyrin precursors will be described later (chapter 5). Cytochrome was also detected in cultures, but was not further investigated.

A. Porphyrins

Porphyrins (and their precursors; see chapter 5) produced by Rps. capsulatus during incubation passed out into the culture or suspension fluids; the porphyrin which could be extracted by acetone from the washed bacteria, after an experiment leading to active synthesis of porphyrin, was less than 0.5% of that found in the suspension fluids. Thus the supernatant fluid obtained by centrifuging the bacteria was used for all assessments of porphyrin production.

(i) Detection

Detection of porphyrins in small concentrations, both in the supernatant fluid and in chromatographic work, was greatly facilitated by their strong, pink fluorescence in ultra-violet light. Greater concentrations allowed inspection of the absorption bands of the supernatant fluids with a hand spectroscope.

(ii) Identification

Measurement of the wavelengths of the visible absorption bands of the supernatant fluids from bacterial suspensions after incubation was done roughly with a hand spectroscope, or more accurately with a Hartridge reversion spectroscope. For inspection of the shape and position of the absorption curves in both visible and ultra-violet regions of the spectrum, a Unicam SP 600 spectro-photometer was used.

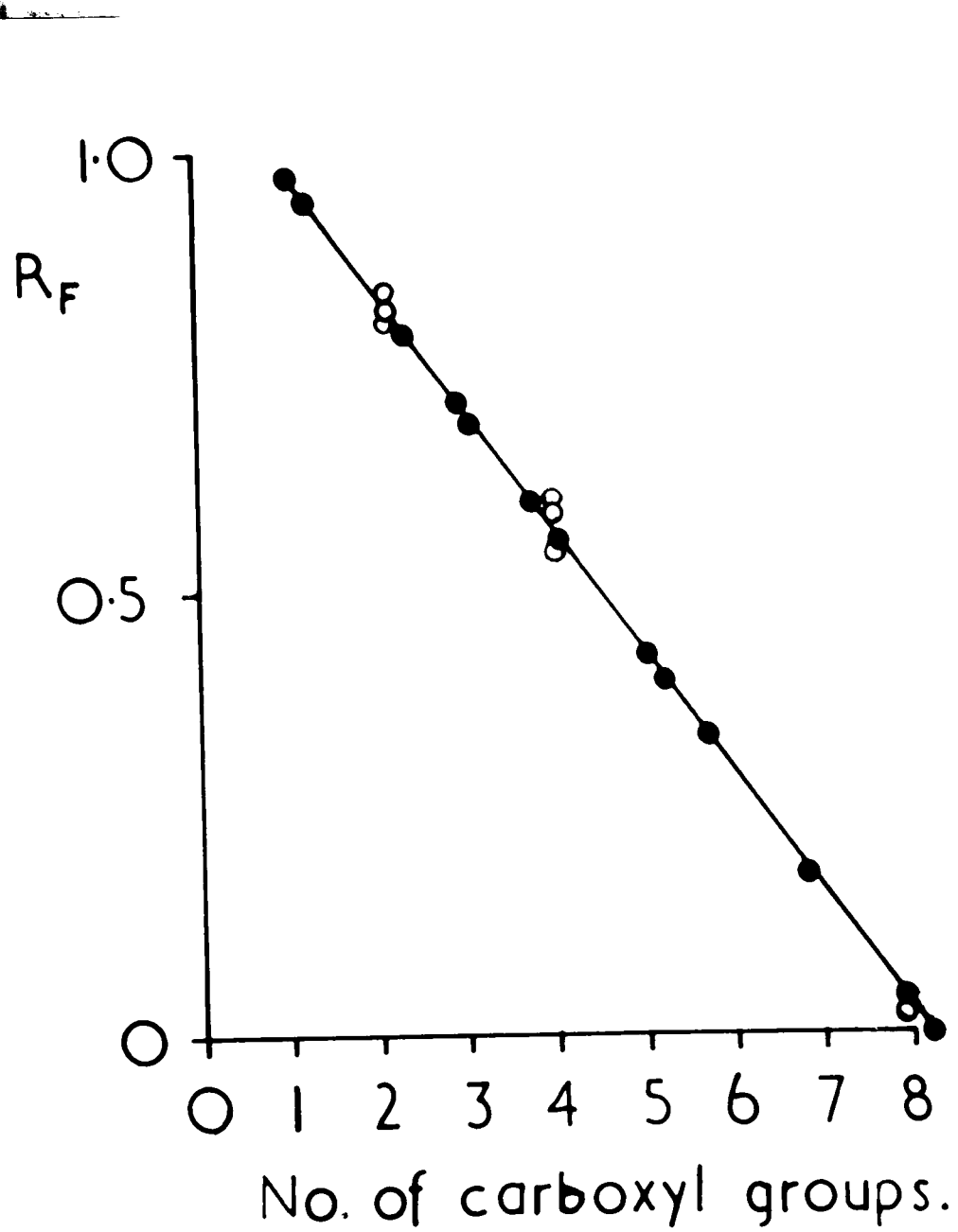
However, the absorption spectrum may properly be used only for a rough identification of the major constituent of a porphyrin mixture, since a minor constituent may remain undetected by this means. Furthermore, copro- and haematoporphyrins are indistinguishable by their absorption spectra in visible and ultra-violet light, as are the isomers of the individual porphyrins (e.g. coproporphyrins I and III), (see Lemberg & Legge, 1949; Vanotti, 1954).

Hence the porphyrins present in the supernatant fluids were further identified by paper chromatography. For the separation of porphyrins with different numbers of carboxyl groups, the method of Eriksen (1953) was used, in which there is an approximate straight-line relationship between the numbers of carboxyl groups of the porphyrin molecules and their R_F values. For rough, exploratory work with supernatant fluids containing large amounts of porphyrin, these fluids were applied directly to the paper. Otherwise they were brought to pH 1 with HCl and the porphyrins absorbed onto Filter-Cel (Johns-Manville Co. Ltd.) and eluted with $2\underline{N}$ - NH_4 OH, thereby eliminating substances interfering with the separation on paper. The solutions, obtained in this way, of ammonium salts of porphyrins were applied to a 20 cm. square of Whatman No. 1 chromatography paper so that the applied spots were no more than 0.5 cm. in diameter. The chromatogram was developed by the ascending method with a mixture of 2, 6-lutidine (5 vols.) and water (3 vols.), in an atmosphere containing ammonia derived from a concentrated solution in a small beaker within the chromatography tank. The paper was dried at room temperature, and the individual spots detected by their pink fluorescence in ultra-violet light.

The R_F -values of markers of uroporphyrin, coproporphyrin, and haematoporphyrin (kindly provided by Mr. Jure Lancelles) were plotted against the numbers of carboxyl groups in their molecules to give three points forming a straight line. From this were

Figure 10 THE RELATIONSHIP BETWEEN THE RF-VALUES OF MARKER PORPHYRINS, THE NUMBERS OF CARBOXYL GROUPS IN THEIR MOLECULES, AND THE RF-VALUES OF PORPHYRINS FROM SUSPENSIONS OF Rps. capsulatus

Suspensions were incubated with mixture I (table 4), under the standard conditions (p.74). Porphyrins were extracted from the supernatant fluids by the method on p. 60 and separated on paper chromatograms on different occasions by the method of Eriksen (1953). The RF -values of the marker porphyrins (uroporphyrin, coproporphyrin and haematoporphyrin) are indicated by O , giving the straight line graph. The Rf values of the porphyrins from Rps. capsulatus are indicated ● , and plotted to coincide with the straight line.



derived the Rf values to be expected of porphyrins with 1 to 8 carboxyl groups: these values were found to be close to those obtained from chromatograms of mixtures of porphyrins from various experiments with Rps. capsulatus (see fig.10), so that the number of carboxyl groups of each component of such mixtures could be determined.

When it was wished to identify the isomers of coproporphyrin, the paper-chromatography method of Chu, Green & Chu (1951) was used. The mixed porphyrins, absorbed from the supernatant fluids of bacterial suspensions onto Filter-Cel, as above, were esterified by standing overnight at 0° with methanol previously saturated with dry HCl. The mixed esters were extracted with washed chloroform, which was then washed with water, dried by filtering through filter paper, and applied to a 15 cm. square of Whatman No. 1. chromatography paper. The chromatogram was developed with a mixture of kerosene (4 vols.) and chloroform (2.6 vols.), dried, and then developed again in the same direction with a mixture of kerosene (5 vols.) and n-propanol (1 vol.), so that the second solvent front reached the same position as the first. The paper was dried at room temperature, and the porphyrins detected by their fluerescence in ultra-violet light

Examination for coproporphyrins I and III was by reference to markers of the methyl esters of these porphyrins, kindly provided by Professor C. Himington.

(iii) Estimation

Paper chromatograms, developed by the method of Eriksen, showed that porphyrin mixtures derived from the supernatant fluids of cultures and suspensions of Rps. capsulatus contained only small amounts of porphyrin with 1, 3, 5, 6 or 7 carboxyl groups, but larger amounts of uroporphyrin, coproporphyrin and a "dicarboxylic porphyrin"; such a mixture presented difficulties of estimation. The "dicarboxylic porphyrin" was

later identified as a mixture of magnesium protoporphyrin and the protoporphyrin probably arising from its decomposition, and appeared only under certain conditions (see chapter 4); when magnesium protoporphyrin was present, as judged by inspection with a hand spectroscope, the method of estimation of porphyrins was modified to that given in section B. of this chapter. Otherwise, attentions could be confined to copro- and uro- porphyrins.

A rough estimate of the proportion of uroporphyrin present was made by bringing the supernatant fluids to pH 4 with glacial acetic acid and extracting twice with ethyl acetate to remove coproporphyrin. HCl was added to the aqueous layer to a concentration of 1.4 N and the optical density at 406 mμ (the Soret maximum) measured. The concentration of uroporphyrin was calculated from the extinction coefficient reported by Bimingtom & Sveinsson (1950).

Since the ratio of uroporphyrin to coproporphyrin was usually less than 1 to 15, the coproporphyrin content was estimated directly and used as an approximation to the true total content of free porphyrin; any value reported as "coproporphyrin", in subsequent chapters, has been estimated in this way. HCl was added to the suitably diluted supernatant fluid to a concentration of 1.4 N, and the mixture kept in the dark for at least 4 hours to allow the oxidation of any porphyrin precursor to porphyrin to go to completion. The optical density at 403 mμ (the Soret maximum) was measured; the extinction coefficient of coproporphyrin was that of Jope & O'Brien (1945).

A Unicam SP 600 spectrophotometer was used for all measurements of optical densities in this work, except when wavelengths of less than 350 mμ were examined, when the SP 500 type was used.

B. Magnesium Protoporphyrin

Magnesium protoporphyrin was detected by Granick (1948 b) in cultures of an induced mutant strain of Chlorella unable to synthesise chlorophyll, but there are no reports of its occurrence in other organisms. Consequently, especial care was taken over the identification of this compound in Rps. capsulatus.

Magnesium protoporphyrin passed from the bacteria into the culture or suspension fluids, though not so readily as did coproporphyrin; repeated washing of the bacteria with water, after an experiment leading to active synthesis of magnesium protoporphyrin, removed successively smaller amounts of this compound until none could be detected in the water used for the fifth washing. However, the total amount thus removed by washing was less than 5% of that found in the original culture fluid; hence the supernatant fluids obtained after centrifuging the bacteria were used in all assessments of the production of magnesium protoporphyrin.

(i) Detection

Detection of magnesium protoporphyrin was facilitated by the characteristic shade of orange-pink of its fluorescence in ultra-violet light, and by its two strong absorption bands in visible light (Hill, 1925; Granick & Gilder, 1947; Granick 1948 b). The band at 590 mμ is well-placed for detection in the presence of a large excess of coproporphyrin.

(ii) Extraction

Magnesium protoporphyrin was extracted from cultures grown for 24 hr. in medium A (appendix 3) supplemented with Tween 80 (see p. 84), as described on p. 74 . The method was based upon the solubility properties reported by Granick (1948 b); the main difficulties were in the avoidance of a pH less than 5, (the magnesium complex being split by weak acid), and in the prevention of emulsification with ether due to the presence of Tween 80. All ether used was previously washed, as described in appendix 9, to remove peroxides. The separating funnel was

kept in the dark as much as possible during the extraction, since the splitting of the complex was promoted by light.

The centrifuged culture fluid (300 ml., containing 12 μ - moles of magnesium protoporphyrin and no detectable amount of other porphyrins) was brought to pH 5.5 - 6.0 with 0.8 ml. glacial acetic acid, and shaken with a mixture of 200 ml. ethanol and 200 ml. ether. The top layer was then shaken with its own volume of 30% (v/v) ethanol in water, thereby being reduced in volume, and the new top layer in turn shaken with its own volume of 30% ethanol. The process was repeated a further two times so that the top layer was concentrated to about 10 ml. This solution was evaporated with a stream of nitrogen in the dark, yielding a thick, clear, cherry-red oil which could not be dried further. Attempts to make a purer preparation, by re-extraction into alkali, failed since the mixture always emulsified unless alcohol and ether were added; this, and the oily nature of the preparation were thought to be due to the presence of Tween 80. The oil contained about 10 μ -moles of magnesium protoporphyrin, indicating a yield of about 80%. Its absorption spectrum (see fig. 11) gave no indication of any substance, other than magnesium protoporphyrin, absorbing light between 300 and 1000 mμ.

(iii) Identification

A portion of the oily preparation made as above was dissolved in 0.02 N - KOH in 50% (v/v) ethanol, with about 5% (v/v) of ether added to disperse the emulsion. The absorption spectrum is given in fig. 11; there was good agreement in the positions and relative heights of the peaks with the spectrum reported by Granick (1948 b) for syntnetic magnesium protoporphyrin (see table 3)

A further portion of the oily preparation was dissolved in ether, and shaken with a little 4.2 N - HCl to break up the magnesium complex. The HCl was neutralised with a saturated solution of sodium acetate, and the ether layer washed with

Figure 11 THE SPECTRUM OF MAGNESIUM PROTOPORPHYRIN PRODUCED BY Rps. capsulatus

A preparation made as described on p. 65 was dissolved in 0.02 N - KOH in 50% (v/v) ethanol containing 5% (v/v) of ether. The solution contained 47 µm -moles magnesium protoporphyrin /ml. (curve II), and was diluted 1 in 12 with the same solvent mixture to obtain curve I.

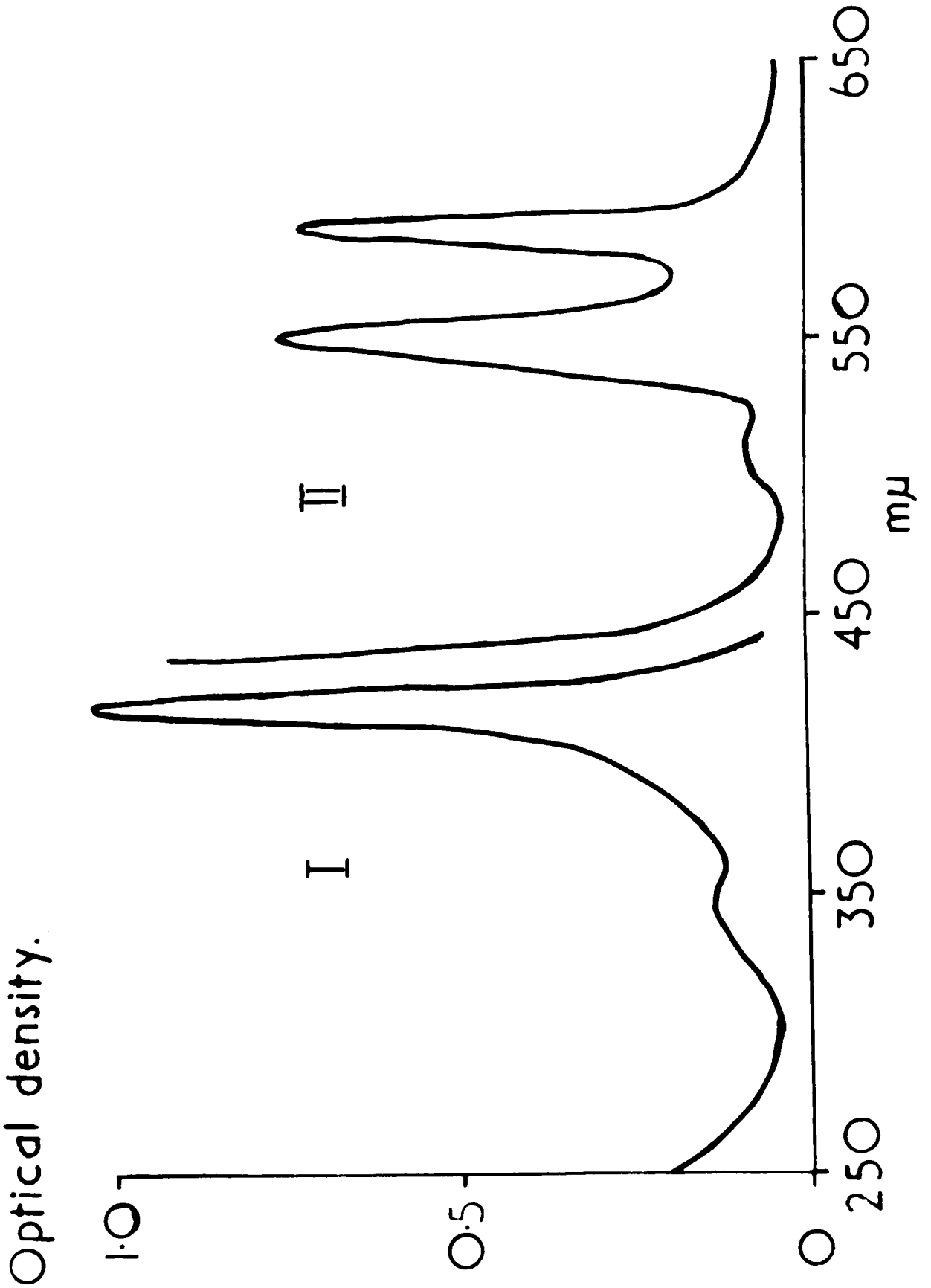


TABLE 3

ABSORPTION MAXIMA of MAGNESIUM PROTOPORPHYRIN

A preparation extracted from culture fluid of Rps. capsulatus compared with the spectrum reported by Granick (1948 b) for the synthetic compound.

Synthetic Mg. Protoporphyrin		Mg. Protoporphyrin from Rps. capsulatus	
Solvent: 0.02N-KOH in 50%(v/v) ethanol		0.02N-KOH in 50% (v/v) ethanol, containing about 5% (v/v) of ether	
Wavelength of Maximum (mu)	Ratio of Optical Density to that at 589 mu	Wavelength of Maximum (mu)	Ratio of Optical Density to that at 590 mu
589	1.00	590	1.00
551	1.07	551	1.08
510	0.15	510	0.20
418	15.5	418	15.6
340	1.21	342	1.66

water and shaken with 0.1N and then 1.4 N - HCl, alcohol being added when necessary to disperse the emulsion. The 0.1 N - HCl fraction contained no porphyrin detectable by fluerescence in ultra-violet light, nor did the ether after repeated extraction with 1.4 N - HCl; all the porphyrin was transferred to 1.4 N - HCl. The wavelengths of the visible absorption bands of this solution, measured with a Hartridge reversion spectroscope, were as follows, (those of proto-porphyrin obtained from L. Light & Co. Ltd., dissolved in 1.4 N - HCl, are given in parentheses): 601 (601), 578 (579), 556 (556) mμ. After making each solution alkaline with 10 N - NaOH, the corresponding wavelengths were: 642 (642), 590 (591), 540 (540) mμ. Thus all of the porphyrin arising from acid treatment of the oily preparation had solubility and light absorption properties of protoporphyrin. Paper chromatography by the method of Eriksen (1953) confirmed that the only porphyrin present in an ethereal solution of the acid-treated preparation contained two carboxyl groups, since the single fluorescent spot obtained had the same R_F as the haematoporphyrin marker.

Finally, the metal atom of the complex was identified. 3 ml. of a concentrated ethereal solution of the oily preparation were shaken in a narrow test-tube with 0.1 ml 2N -HCl and then neutralised with 0.1 ml. 2N - NaOH. The aqueous bubble was withdrawn with a Pasteur pipette, leaving the protoporphyrin in the ether. A control extraction was made by shaking 3 ml. of the ethereal solution with a previously neutralised mixture of 0.1 ml. 2N - HCl and 0.1 ml 2N - NaOH so that the complex was not split. 1 drop of each extract was added to 2 drops of a 0.01% (w/v) solution of quinalizarin (1, 2, 5, 8 - tetra-hydroxy anthraquinone) in ethanol, on a white tile (Granick, 1948 b). After about 2 min., blue granules appeared in the test compared with a violet colour and no granules in the control, indicating the presence of either magnesium, lanthanum, beryllium,

or some of the rare earth elements (see Fiegl, 1937); it was assumed that magnesium was responsible.

(iv) Estimation

In cultures and suspensions of Rps. capsulatus containing added iron salts (see chapters 2 and 4), coproporphyrin and magnesium protoporphyrin appeared together in the supernatant fluids. For routine work it was found possible to estimate the concentrations of each of these compounds individually, without preliminary extraction from the supernatant fluids, by taking advantage of their high concentration and of great differences in their absorption spectra.

Since the extinction coefficients of magnesium protoporphyrin in 0.02 N -KOH in 50% (v/v) ethanol were known (Granick, 1948b), the supernatant fluids were diluted with this solvent mixture, centrifuged if necessary to remove any precipitated salts, and the optical densities measured at the Soret peaks: 416 mμ for magnesium protoporphyrin and 394 mμ for coproporphyrin. The optical density at each of these wavelengths contained a minor component contributed by the compound whose major absorption peak was at the other wavelength; the correction needed to calculate the optical density due to the major component only, at each wavelength, was derived as follows (OD = optical density) :

Where the OD's of a pure compound A at wavelengths 1 and 2 are A and nA, respectively, and the OD's of a pure compound B at wavelengths 2 and 1 are B and mB respectively, and where the OD's of a mixture of A and B at wavelengths 1 and 2 are D_1 and D_2, respectively,

then $A + mB = D_1$ ... ... (i)

and $B + nA = D_2$ ... ... (ii)

$$\therefore B = D_2 - nA$$

Substituting in (i), $A + m(D_2 - nA) = D_1$

rearranging, $$A = \frac{D_1 - mD_2}{}$$

Similarly, $$B = \frac{D_2 - nD_1}{1 - nm}$$

Hence the corrected optical densities (A and B) for the pure components of a mixture can be calculated if the factors n and m can be found by reference to the spectra of the pure components. In the present investigation, they were derived from the absorption spectra in 0.02 N - KOH in 50% (v/v) ethanol of authentic samples of magnesium protoporphyrin (prepared as described on p. 65), and of coproporphyrin (obtained from Dr. June Lascelles). The correction formulae could therefore be applied as follows:

Corrected OD_{418} (due to magnesium protoporphyrin only)

$$= \frac{OD_{418} - 0.17 . OD_{394}}{0.96}$$

Corrected OD_{394} (due to coproporphyrin only)

$$= \frac{OD_{394} - 0.26 . OD_{418}}{0.96}$$

The molar extinction coefficient of coproporphyrin at 394 mμ, in the solvent mixture used in this estimation, was found to be 2.5×10^5, by comparison with a similar sample diluted with 1.4 N - HCl for which the extinction coefficients are known (Jope & O'Brien, 1945). The molar extinction coefficient of magnesium protoporphyrin at 418 mμ in the solvent mixture used in the estimation is 2.7×10^5 (Granick, 1948 b). The concentration of each component of the porphyrin mixture could thus be calculated from the observed optical densities at the two wavelengths.

The validity of this method was checked by mixing the authentic samples of magnesium protoporphyrin and coproporphyrin in various proportions, and plotting the corrected optical densities against the composition of the mixtures as shown in fig. 12.

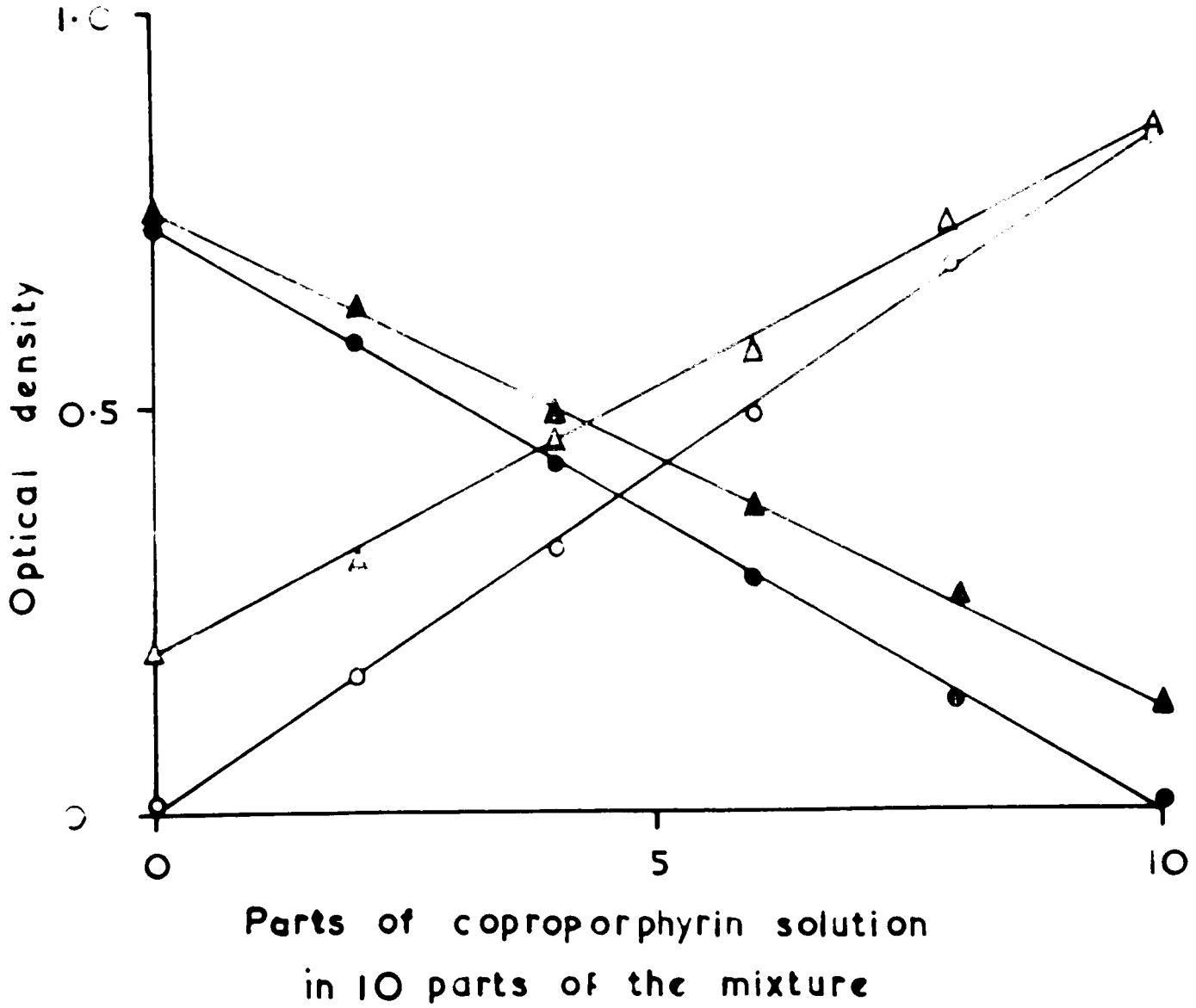
FIGURE 12

THE OPTICAL DENSITIES OF MIXTURES OF COPROPORPHYRIN AND MAGNESIUM PROTOPORPHYRIN

Solutions (in 0.02 N - KOH in 50% (v/v) ethanol) of coproporphyrin (3.4 µm-moles /ml.) and magnesium protoporphyrin (3.2 µm-moles / ml.) were mixed in the proportions shown (v/v). The optical densities (OD) at 394 mµ and 418 mµ were measured, and corrected as described on p. 70 .

△—△ observed OD at 394 mµ; ▲—▲ observed OD at 418 mµ

○—○ corrected OD at 394 mµ; ●—● corrected OD at 418 mµ



The corrected optical densities yielded straight lines passing through the origins. When coproporphyrin or magnesium protoporphyrin wasadded to supernatant fluids from suspension experiments with Rps. capsulatus, and the mixture analysed by the method described above, the recoveries obtained were always between 97 and 99%. The inaccuracy of the method was large when the proportion of one constituent of the mixture was very low; in practice, no estimation was recorded if it gave a concentration of less than 5% of that of the other constituent.

C. Bacteriochlorophyll

In contrast to the porphyrins and magnesium protoporphyrin produced by Rps. capsulatus, bacteriochlorophyll remained within the bacteria during growth or suspension experiments; it was thus extracted by methanol for identification and estimation.

(i) Identification

The absorption bands of the whole bacteria, of the methanol extract, and of the pheophytin produced on treatment of the extract with acid (see chapter 6), were at wavelengths agreeing with those reported for bacteriochlorophyll and bacteriopheophytin (French, 1940; van Niel, 1944; Weigl, 1953; Holt & Jacobs, 1954). Moreover, solubility and chromatographic properties of the pheophytin (see chapter 6) were similar to those of bacteriopheophytin (van Niel & Arnold, 1938; Griffiths & Stanier, 1956).

(ii) Estimation

The method was similar to that of Lascelles (1956a), which was itself based upon that of van Niel & Arnold (1938). 5 ml. of culture or suspension were centrifuged thoroughly, and bacteriochlorophyll extracted from the packed bacteria with 5 ml. methanol. The extract was added to a mixture of 0.75 ml. CCl_4 and 0.05 ml. 7N -HCl, stood in the dark for at least 10 min. to allow complete conversion to bacteriopheophytin, and the

optical density measured at the peak at 746 mμ. The molar extinction coefficient at this wavelength is 3.8 x 10^4 (van Niel & Arnold, 1938; Lascelles, 1956 a). This figure was confirmed by comparing the absorption spectrum of a pure sample of bacteriopheophytin, prepared as in chapter 6 , and dissolved in CCl_4 containing 6% (v/v) 7N - HCl, with the data of van Niel and Arnold.

In suspension experiments, a considerable amount of bacteriochlorophyll was introduced with the bacteria at the beginning of the experiments (see fig. 13); this was measured in a control sample of bacteria and deducted from the amount measured after incubation, to give the amount synthesized.

CHAPTER 4

THE SYNTHESIS OF PORPHYRINS, MAGNESIUM PROTOPORPHYRIN AND BACTERIOCHLOROPHYLL BY SUSPENSIONS OF Rps. capsulatus

By varying the conditions of incubation of the bacterial suspensions, it was possible to promote the synthesis of either coproporphyrin, magnesium protoporphyrin or bacteriochlorophyll. However, the growing and harvesting of the bacteria destined for suspensions was the same in each case.

A. Preparation and Incubation of Suspensions

(i) Growth and harvesting of the bacteria

The method of inoculum development for suspension experiments was the same as that described on p.46 , as applied to growth experiments. The liquid cultures thus obtained in test tubes could be stored at 0 - 4° for up to 5 days, without apparent loss of vigour.

8 oz. medical flat bottles plugged with cotton wool were each filled to the shoulder with 200 ml. medium A (appendix 3), with iron citrate omitted so as to produce bacteria deficient in iron. Each bottle was inoculated with 1 ml. (4 - 5 mg. dry wt. of bacteria) of the liquid, inoculum-development culture, and incubated in the light incubator described on p.47 for 20 - 24 hr.; the growth was then about 1.5 mg. dry wt./ml. and examination of the culture fluid with a hand spectroscope showed the presence of magnesium protoporphyrin and traces of a free porphyrin (probably coproporphyrin).

The cultures were centrifuged at 10,000 revolutions per minute for 10 minutes (3,000 revolutions per minute was inadequate for precipitating Rps. capsulatus), and the bacteria washed by resuspending in an amount of 1% (w/v)NaCl equivalent to half the original volume of culture and recentrifuging. The washed bacteria were suspended in

water to a concentration of 8 - 10 mg. dry wt. /ml; this suspension was diluted 1 in 10 with the appropriate incubation mixture to form the final suspensions.

(ii) Incubation of suspensions

The choice of incubation mixture depended upon the desired main end-product of synthesis; the optimal production of coproporphyrin was in mixture I, of magnesium protoporphyrin in mixture II, and of bacteriochlorophyll in mixture III (table 4). The final volume of suspensions was 5 ml., incubated in 6 x ½ in. Pyrex tubes in the light incubator as described for growth experiments on p. 47 . The duration of incubation was 22 - 26 hr.; at the end of this period the rate of formation of all three measured products was decreasing (fig. 13). Suspensions were then centrifuged, and the bacteria and supernatant fluids analysed as described in chapter 3. All critical measurements of synthesis were done with duplicated suspensions.

B. Synthesis under the Standard Conditions.

The time courses of the formation of coproporphyrin, magnesium protoporphyrin and bacteriochlorophyll, each in the appropriate incubation mixture, are given in fig. 13. The synthesis of bacteriochlorophyll differed from those of the other two products in its delay of 1 - 2 hr. before beginning and its complete cessation after only 15 - 20 hr. of incubation.

(i) Coproporphyrin

During the first year after the isolation of the Oxford strain of Rps. capsulatus, the largest concentration of coproporphyrin formed in suspensions was about 150 µm - moles/ml, which is about the same as that produced by Rps. spheroides under similar conditions (Lascelles, 1956 a). However, during the next two years the synthetic power of Rps.capsulatus apparently increased, until concentrations of coproporphyrin of up to 310 µm -moles /ml. (0.02% w/v) were found in supernatant

Figure 13 TIME COURSE OF SYNTHESIS OF COPROPORPHYRIN, MAGNESIUM PROTOPORPHYRIN AND BACTERIOCHLOROPHYLL IN SUSPENSIONS OF Rps. capsulatus

Conditions of incubation were standard, as described on p. 74 . The synthesis of coproporphyrin (O—O) was followed in mixture I, of magnesium protoporphyrin (Δ—Δ) in mixture II and of bacteriochlorophyll (●—●) in mixture III, (table 4).

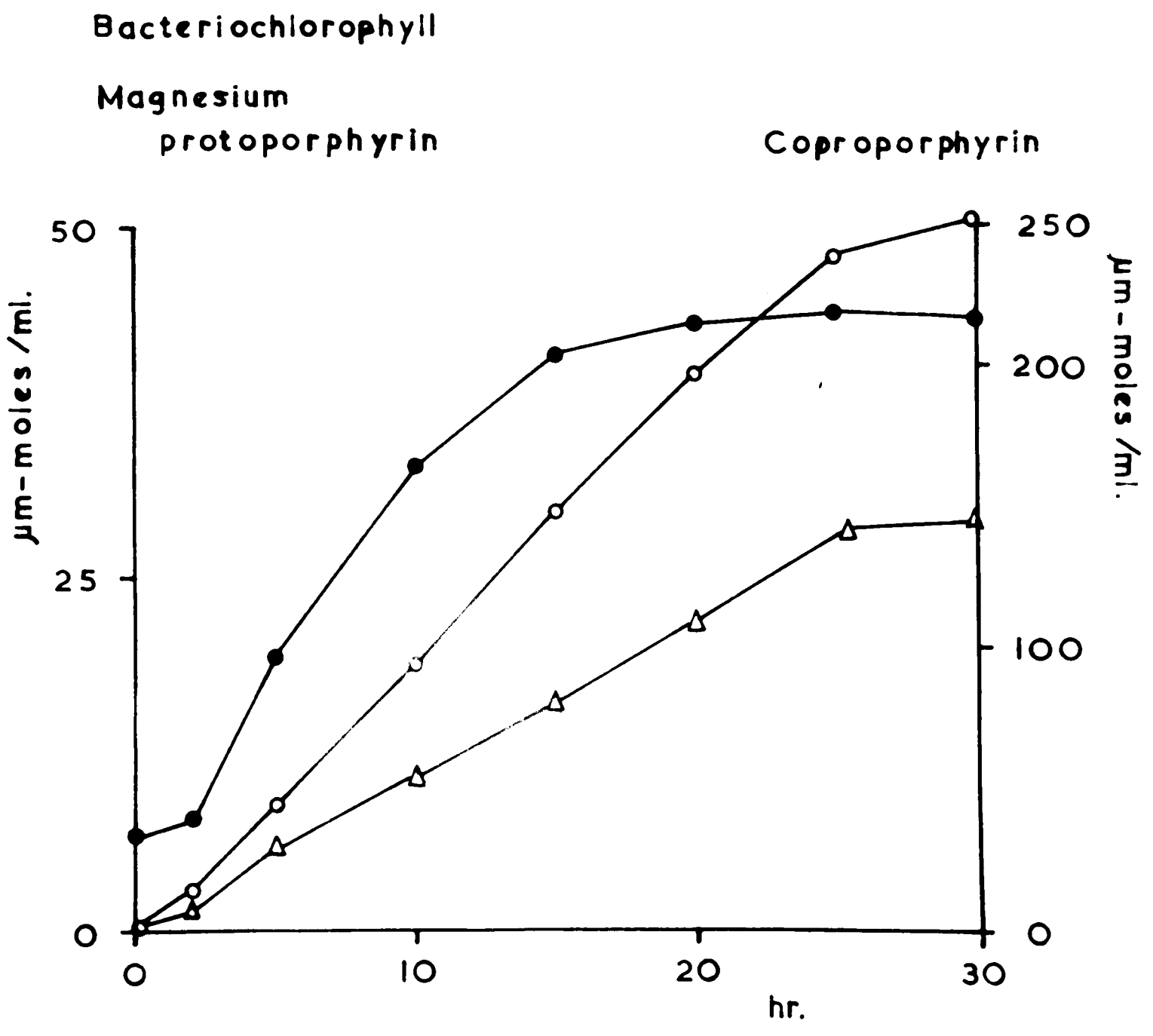


TABLE 4

INCUBATION MIXTURES FOR SUSPENSIONS OF Rps. capsulatus

All three mixtures contained the following compounds at the indicated concentrations:

Glycine	10^{-2} M
Sodium succinate	4×10^{-2} M
Nicotinic acid	4×10^{-5} M
$(NH_4)_2 HPO_4$	4×10^{-3} M
$Mg SO_4$	4×10^{-3} M
$Mn SO_4$	5×10^{-6} M
$Ca Cl_2$	4×10^{-4} M

In addition, the individual mixtures were completed as follows:

Mixture I	(for coproporphyrin):	
	DL - Methi nine	2×10^{-3} M
	Potassium phosphate buffer, pH 7.0	2×10^{-2} M
Mixture II	(for magnesium protophorphyrin):	
	DL - Methionine	5×10^{-4} M
	Iron citrate (appendix 7)	2×10^{-5} M
	Tween 80 (Honeywill & Stein, Ltd. London, S.W.1.)	0.2% (v/v)
	Potassium phosphate buffer, pH 6.6	2×10^{-2} M
Mixture III	(for bacteriochlorophyll)	
	Iron citrate (appendix 7)	2×10^{-4} M
	Potassium phosphate buffer, pH 6.6	2×10^{-2} M

fluids. In an attempt to decide whether this change was due to changes in the organism after repeated subculture under laboratory conditions or to some unappreciated variation in the conditions of incubation of suspensions, the re-isolation of the organism was attempted, using the same techniques with mud from the same source as desribed in chapter 1. However, none of the cultures of photosynthetic bacteria which were now isolated were of Rps. capsulatus, and the question was left open.

Paper chromatography of extracts from supernatant fluids indicated that a small proportion of porphyrins with 1, 2, 3, 5, 7 and 8 (and possibly 6) carboxyl groups in their molecules accompanied coproporphyrin (see fig. 10 and chapter 3). The uroporphyrin amounted to 2 - 6% of the coproporphyrin formed by suspensions after the usual duration of incubation (22 - 26 hr.), but on two occasions when incubation was prolonged to 40 hr., this proportion was increased to 10 - 15%. The other porphyrins detected on paper chromatograms amounted to probably less than 2% of the total porphyrin, according to a rough estimation by comparing the intensity of fluorescence of the spots on chromatograms under ultra-violet light with that of known amounts of haematoporphyrin. Free porphyrins other than coproporphyrin were not further investigated.

When extracts from supernatant fluids from suspension experiments were analysed by thepaper chromatography of Chu, Green & Chu (1951) (see chapter 3), no isomer of coproporphyrin other than coproporphyrin III was detected.

(ii) Magnesium protoporphyrin and bacteriochlorophyll

In general, the amounts of these compounds produced were less than that of coproporphyrin, when each product was estimated after incubation of suspensions in its appropriate mixture (fig. 13). In the presence of a concentration of

added iron citrate greater than 5×10^{-5} M, less coproporphyrin than bacteriochlorophyll was synthesised, but the only conditions under which more magnesium protoporphyrin than coproporphyrin was formed were those in cultures growing in medium A (appendix 3) with 0.02% (v/v) of added Tween 80 (see p. 84).

Growth of the bacteria always occurred in suspension experiments; dry weight doubled in mixture I, and increased fivefold in mixtures II or III, the difference being probably due to the iron citrate contained in mixtures II and III. In this respect, the methods used in the present study differed from the classical technique of washed-cell suspensions (see Woods, 1953), in which growth is limited by withholding necessary substrates. In the present experiments, when iron citrate was added the incubation mixture was in fact a good medium for growth; the amount of bacteriochlorophyll per unit dry weight of bacteria increased by only 30 - 50% during incubation in mixture III

C. The Effects of Varying the Conditions of Incubation

(i) General

The composition of the incubation mixtures used for Rps. capsulatus (table 4) was derived from that designed by Lascelles (1956 a) for the study of porphyrin synthesis by Rps. spheroides; the various modifications made are as follows:

(a) α -oxoglutarate was replaced by succinate, since the former compound innibited porphyrin formation in suspensions of Rps. capsulatus. Mixtures of succinate with fumarate, malate, lactate, pyruvate, or glutamate yielded no more porphyrin than when succinate was used alone at 4×10^{-2} M; succinate was presumably acting efficiently as hydrogen donor for photosynthesis, as well as carbon source for porphyrin synthesis.

TABLE 5 THE EFFECTS OF OMITTING COMPONENTS OF THE INCUBATION MIXTURES ON THE SYNTHESIS OF COPROPORPHYRIN, MAGNESIUM PROTOPORPHYRIN AND BACTERIOCHLOROPHYLL BY SUSPENSIONS OF Rps. capsulatus

Coproporphyrin, magnesium protoporphyrin and bacteriochlorophyll were estimated after incubation of suspensions with mixtures I, II and III, respectively, under the standard conditions described on p. 74 . The composition of mixtures is in table 4.

Omissions	Coproporphyrin		Magnesium Protoporphyrin		Bacteriochlorophyll Synthesized	
	µm.moles/ml.	% reduction	µm-moles/ml.	% reduction	µm.moles/ml.	% reduction
None	245	0	31	0	59	0
Glycine	64	74	8	74	14	64
Succinate	0	100	0	100	2	95
Nicotinic acid	132	46	28	10	31	21
DL-Methionine	140	44	19	39	-	-
$(NH_4)_2HPO_4$	53	78	10	68	9	65
$Mg\ SO_4$	4	98	0	100	1	97
$Mn\ SO_4$	24	90	5	84	5	87
$Ca\ Cl_2$	207	15	30	3	32	18
Potassium phosphate buffer	116	53	15	51	10	61
Iron citrate	-	-	0	100	4	84
Tween 80	-	-	2	94	-	-

(b) Nicotinic acid stimulated porphyrin production (table 5), despite the fact that it was added to the medium in which the bacteria were grown; the optimal concentration of nicotinic acid for growth of Rps. capsulatus was five times that necessary for Rps. spheroides (see pa.54). The addition of thiamin (3×10^{-6} M) or biotin (4×10^{-8} M) to suspensions of Rps. capsulatus had no effect on porphyrin production.

(c) The concentration of magnesium sulphate was increased five-fold, and that of phosphate buffer trebled, each modification producing a 10 - 20% increase in porphyrin synthesis.

(d) 2×10^{-3} M DL -methionine was added (see below)

The reduction in synthesis caused by omitting individual components of the incubation mixtures I, II and III is shown in table 5. The percentage reductions in coproporphyrin synthesis are similar to those with Rps. spheroides, (Lascelles, 1956 a), except that the omission of glycine produced only 74% reduction in synthesis, as compared with 95% with Rps.spheroides; this suggests that Rps. capsulatus is better able to use ammonium as a source of nitrogen for porphyrin production.

The effects of omitting components of the incubation mixtures on the synthesis of magnesium protoporphyrin and bacteriochlorophyll (table 5) were in general similar to the results for coproporphyrin; any noteworthy differences are described below.

(ii) Iron citrate

The reasons for the use of this compound of iron have already been mentioned (p. 56). The effects of its addition to suspensions of Rps.capsulatus were similar to those observed with cultures of Rps. capsulatus and Rps. palustris (pp.56-58; fig.9); the appearance of coproporphyrin in the incubation medium was strongly inhibited, and the formation of

magnesium protoporphyrin and bacteriochlorophyll much increased (table 5 and fig. 14).

The effect of different concentrations of iron was studied with incubation mixture II, since it was the only one of the three mixtures in which vigorous synthesis of any of the three products could be promoted, (by changing the amount of added iron). The results of this experiment, illustrated in fig. 14, were as follows:

(a) As the concentration of iron was increased, the formation of coproporphyrin and bacteriochlorophyll behaved as mentioned above; the increase in the amount of bacteriochlorophyll appearing was only about one-fifth of the reduction in the amount of coproporphyrin.

(b) The amount of iron needed for magnesium protoporphyrin synthesis was intermediate between that for bacteriochlorophyll synthesis and any needed for coproporphyrin synthesis (probably provided by impurities in the growth and suspension media, since no special precautions were taken to remove iron).

The observation that magnesium protoporphyrin is formed in cultures before coproporphyrin, reported on p. 58 , is also in accord with the idea that iron is needed for magnesium protoporphyrin synthesis; the amount of iron available to each organism in a culture is presumably greater at the early and middle stages of growth than towards the end (when coproporphyrin appeared), (see Discussion)

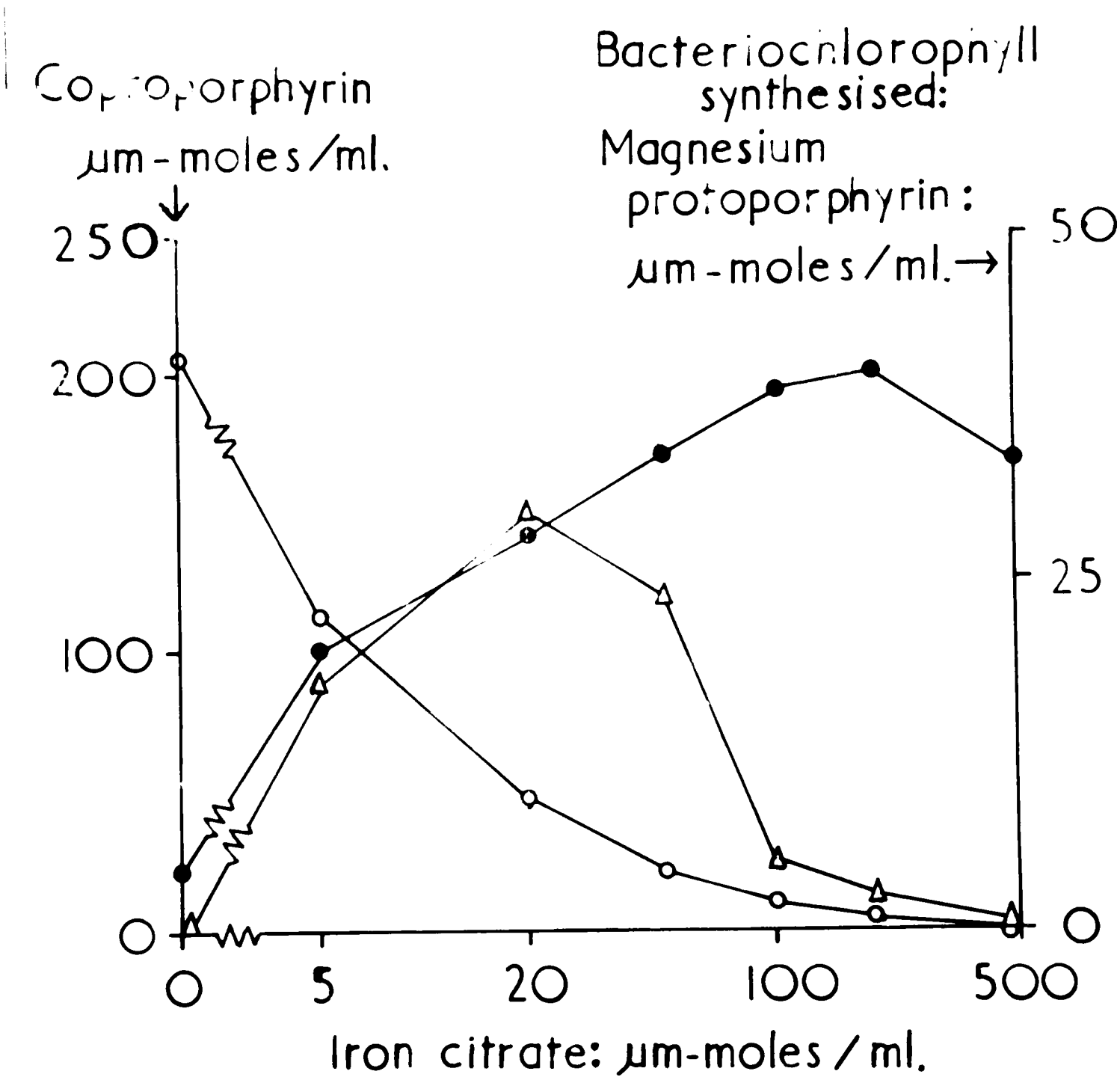
(iii) Methionine

The effect of different concentrations of DL-methionine was also studied with incubation mixture II (fig. 15). In this experiment, the presence of added iron in the mixture inhibited the production of coproporphyrin, but a similar percentage stimulation by 2×10^{-3} M methionine was observed in mixture I (table 5), which contains no added iron.

Figure 14

THE EFFECT OF IRON ON THE FORMATION OF COPROPORPHYRIN, MAGNESIUM PROTOPORPHYRIN AND BACTERIOCHLOROPHYLL IN SUSPENSIONS OF Rps.capsulatus

Suspensions were incubated under the standard conditions (p. 74) in mixture II (table 4), with the content of iron citrate varied as shown; (O—O coproporphyrin; Δ—Δ magnesium protoporphyrin; ●—● bacteriochlorophyll)



The effect of methionine on the production of magnesium protoporphyrin was similar to the effect of iron, in that an intermediate concentration promoted optimal synthesis; on the other hand, the formation of bacteriochlorophyll was inhibited and that of coproporphyrin stimulated by adding methionine. In fact, the picture was that which would have been expected if methionine had been in some way reducing the effective concentration of iron available at the sites of bacteriochlorophyll and porphyrin synthesis. However, this possibility was made unlikely by the similar effects of methionine at widely different concentrations of added iron. These observations are mentioned in the Discussion, in relation to the effect of small concentrations of methionine on the growth of the organism (chapter 2)

(iv) Tween 80

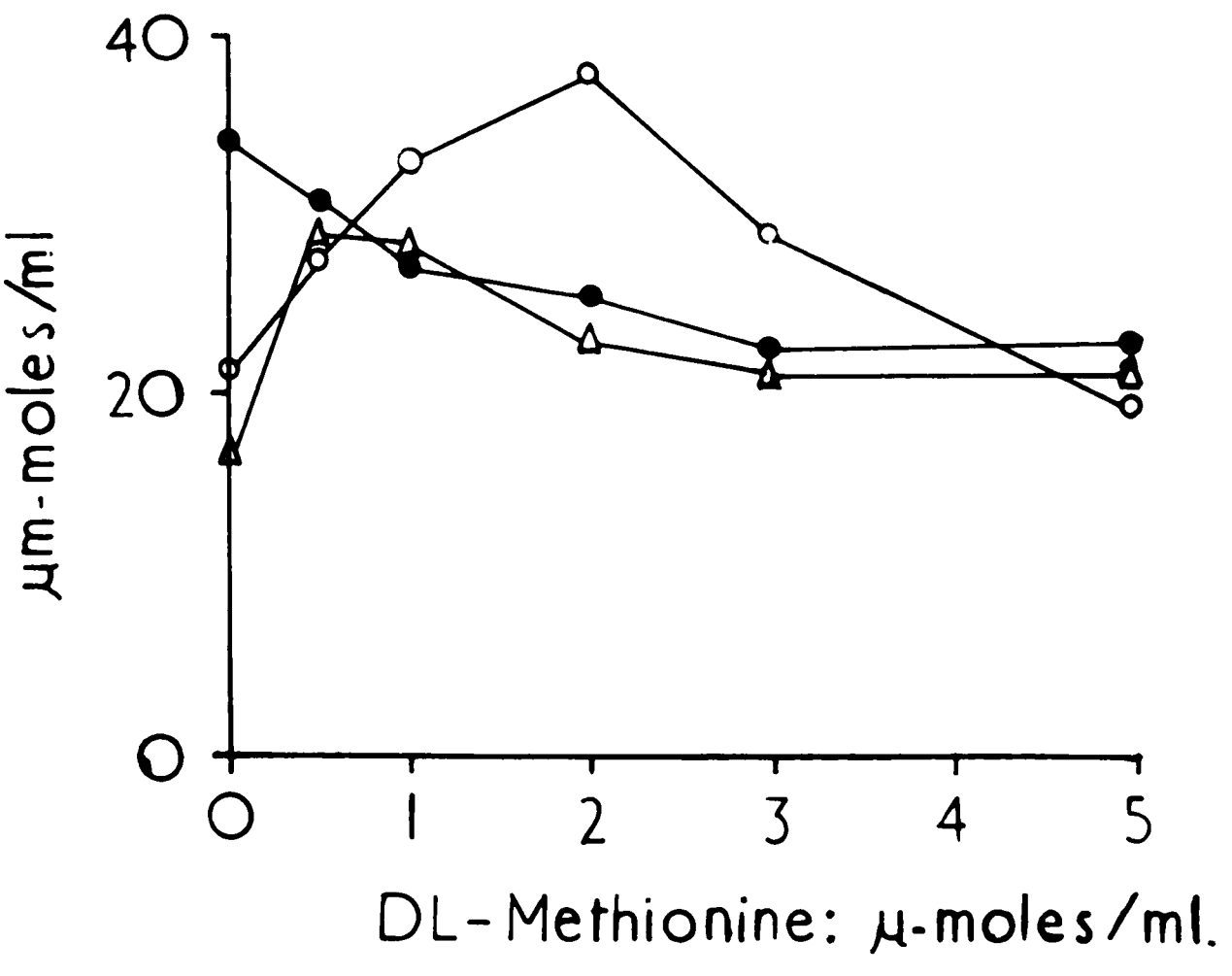
This anionic detergent, obtained from Honeywill & Stein, Ltd., London, S.W.1., was an essential component of any incubation mixture or culture medium in which a concentration of magnesium protoporphyrin greater than 2 - 4 µm-moles /ml. was to be formed (see table 5). Its effect on the production of coproporphyrin (in suspensions incubated with mixture I) varied from a 5% inhibition to a 20% stimulation, on different occasions; growth and bacteriochlorophyll production were little affected (investigated under the appropriate conditions). The above results were gained using 0.2% (v/v) Tween 80; concentrations larger than 1% (v/v) inhibited growth in cultures and the formation of all three products measured in suspensions.

It was thought possible that Tween 80 was acting at the surface of the bacterium by increasing the permeability towards magnesium protoporphyrin. However, the small proportion of magnesium protoporphyrin remaining with the bacteria during centrifugation, after incubation of cultures or suspensions, (see p. 64) was no greater when the appearance of the compound in the supernatant fluid was reduced by decreasing the amount of Tween 80 added.

Figure 15

THE EFFECT OF METHIONINE ON THE FORMATION OF COPROPORPHYRIN, MAGNESIUM PROTOPORPHYRIN AND BACTERIOCHLOROPHYLL IN SUSPENSIONS OF Rps. capsulatus.

Suspensions were incubated under the standard conditions (p. 74) in mixture II (table 4), with the content of DL -methionine varied as shown; (O—O coproporphyrin; Δ—Δ magnesium protoporphyrin; ●—● bacteriochlorophyll).



D. Experiments to Determine Whether Magnesium Protoporphyrin is Converted to Bacteriochlorophyll by Rps. capsulatus

For these experiments, magnesium protoporphyrin was extracted from a culture of Rps. capsulatus grown with Tween 80, as described on pp. 64-65. The clear, cherry-red extract, containing 15 μ -moles of magnesium protoporphyrin, was diluted with 5 ml. of water and evaporated to about 1 ml. by bubbling a stream of nitrogen; this was repeated twice to remove all traces of ethanol and ether. The solution was finally brought to a volume of 5 ml. with water and stored at -20° until required for the experiments described below. Examination with a hand spectroscope before and after such storage showed the presence of only traces of free protoporphyrin. The persistence of foam on the surface of the solution suggested that Tween 80 was present.

Suspensions of Rps. capsulatus were prepared and incubated in the usual way (p. 74) with mixture III (table 4). No greater production of bacteriochlorophyll was observed with 10, 40 or 100 μm-moles of added magnesium protoporphyrin /ml. than with none. The same result was obtained when the concentration of added iron was varied from nil to 5×10^{-4} M, when 10^{-3} M DL-methionine was added, when 0.02% Tween 80 was added, when ammonium was omitted, when glycine was omitted, when ammonium and glycine were omitted, or when incubation was carried out in the dark in 100 ml. conical flasks containing 5 ml. of suspension.

Suspensions were then prepared from bacteria which had been twice frozen (-20°) and thawed when in the packed state after centrifuging. In this case, no bacteriochlorophyll was produced on incubating with mixture III, whether magnesium protoporphyrin had been added or not.

No conclusions about the conversion of magnesium protoporphyrin to bacteriochlorophyll could be drawn from these experiments, since magnesium protoporphyrin may be unable to penetrate to the site of bacteriochlorophyll synthesis in the organism (see Discussion).

CHAPTER 5

THE PRODUCTION OF COPROPORPHYRINOGEN BY SUSPENSIONS OF Rps. capsulatus

A. The Effect of Aneorobic Incubation of Suspensions

During the investigations of coproporphyrin synthesis by suspensions of Rps. capsulatus, described in the previous chapter, it was noticed that the supernatant fluids, (from suspensions incubated in the usual way with mixture I; see p. 74 and table 4), were paler in colour immediately after centrifuging than when they had been standing in open tubes for half an hour. Similarly, supernatant fluids diluted with hydrochloric acid for coproporphyrin estimation (chapter 3) contained less coproporphyrin immediately after dilution than when the mixtures were stood in open tubes for a few minutes. Since the number of bacteria contaminating supernatant fluids after centrifuging was probably negligible, it was assumed that a colourless chemical precursor of coproporphyrin was present; further investigation went as follows:

(i) Incubation in Thunberg tubes under hydrogen

Suspensions were incubated in atmospheres of either air or hydrogen, each at three different levels of added iron so as to produce supernatant fluids containing different amounts of porphyrinogenic substance (fig. 16).

Anaerobic incubation had little effect on the total amount of coproporphyrin eventually appearing, but up to 95% of this was not measurable by light absorption at 403 mμ (the standard method for coproporphyrin, see chapter 3) immediately after centrifuging the suspensions.

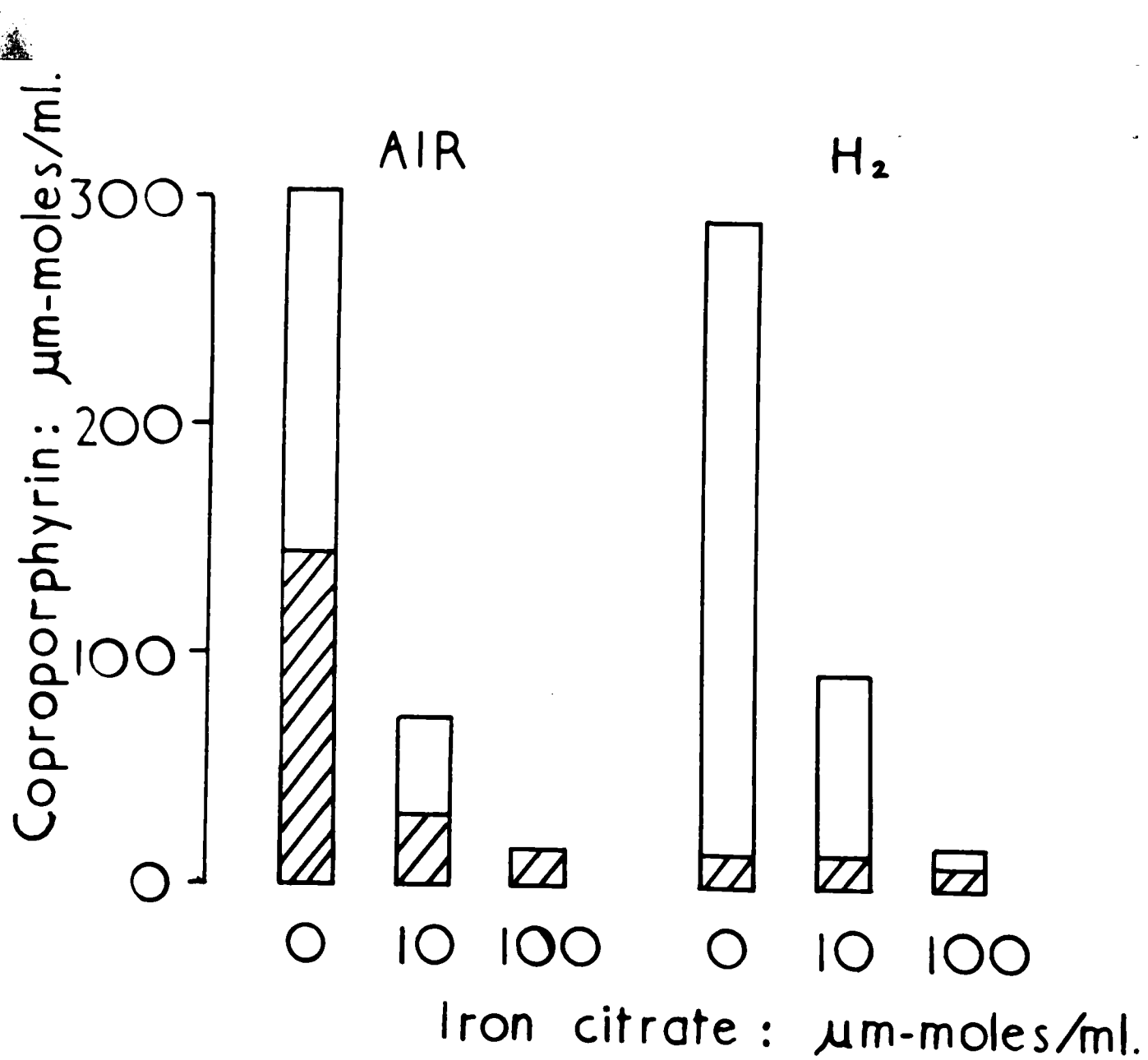
(ii) The standard procedure for anaerobic incubation on a larger scale

For further study, it seemed necessary to devise a means of obtaining larger amounts of supernatant fluid, from suspensions incubated anaerobically, than was convenient in Thunberg tubes. 2-oz. medical flat Bottles were filled to

FIGURE 16

THE APPEARANCE OF COPROPORPHYRIN IN SUPERNATANT FLUIDS FROM SUSPENSIONS OF Rps. capsulatus INCUBATED IN AIR OR HYDROGEN

Suspensions were incubated under standard conditions (p. 74) with mixture I (table 4), with iron citrate added as shown. Suspensions under hydrogen were in Thunberg tubes. **Shaded areas**: coproporphyrin measured in samples withdrawn immediately after centrifuging suspensions; **white areas**: coproporphyrin measured after supernatant fluids were stood in open tubes in the dark for 24 hr.



the brim (about 60 ml.) with a suspension of Kps. capsulatus prepared in the standard way (p. 74) in mixture I (table 4); the screw-caps were applied with just enough slackness to allow the release of any gas pressure from the bottles during incubation. The 2-oz. bottles were used because it was supposed that their small width would allow a high average intensity of illumination in suspensions; nevertheless it was found that 40-watt lamps promoted the production of more porphyrin than did 25-watt lamps. The duration of incubation was 22 - 24 hr.

B. The Effects of Oxidising and Reducing Agents on Supernatant Fluids from Anaerobically Incubated Suspensions

(i) Changes taking place in air

Supernatant fluids from suspensions incubated as described on pp.87-89 were a pale peach colour immediately after centrifuging; the addition of alkali changed the colour to orange, and acidification produced a rose colour which gradually changed in air to the deep pink normally found in an acidified solution of coproporphyrin. It was evident that some coloured compound other than coproporphyrin was present, and examinations of the acidified supernatant fluid with a hand spectroscope revealed an intense, sharp absorption band at 500 mμ. Subsequent spectrophotometric surveys (see fig. 18) showed that this peak was at precisely 500 mμ in a 1.4 N - HCl solution.

When such acid supernatant fluids were examined with a hand spectroscope repeatedly over a period of 3 - 4 hours, it was seen that the band at 500 mμ increased and then decreased in intensity as the group of three bands from 548 mμ to 590 mμ (due to coproporphyrin) was steadily intensifying. It therefore seemed probable that one of the following sequences of reaction was taking place, (where x is a colourless precursor of porphyrin and y is the compound absorbing at 500 mμ):

(a) x ⟶ y ⟶ porphyrin, or:

(b) x ⟶ porphyrin
↓
y ⟶ (rapid disappearance)

A third possibility was that compound y was unconnected with either x or porphyrin (except that both x and y appeared during anaerobic incubations of suspensions).

Some evidence on these possibilities was gained as follows.

(ii) The effects of other oxidising agents

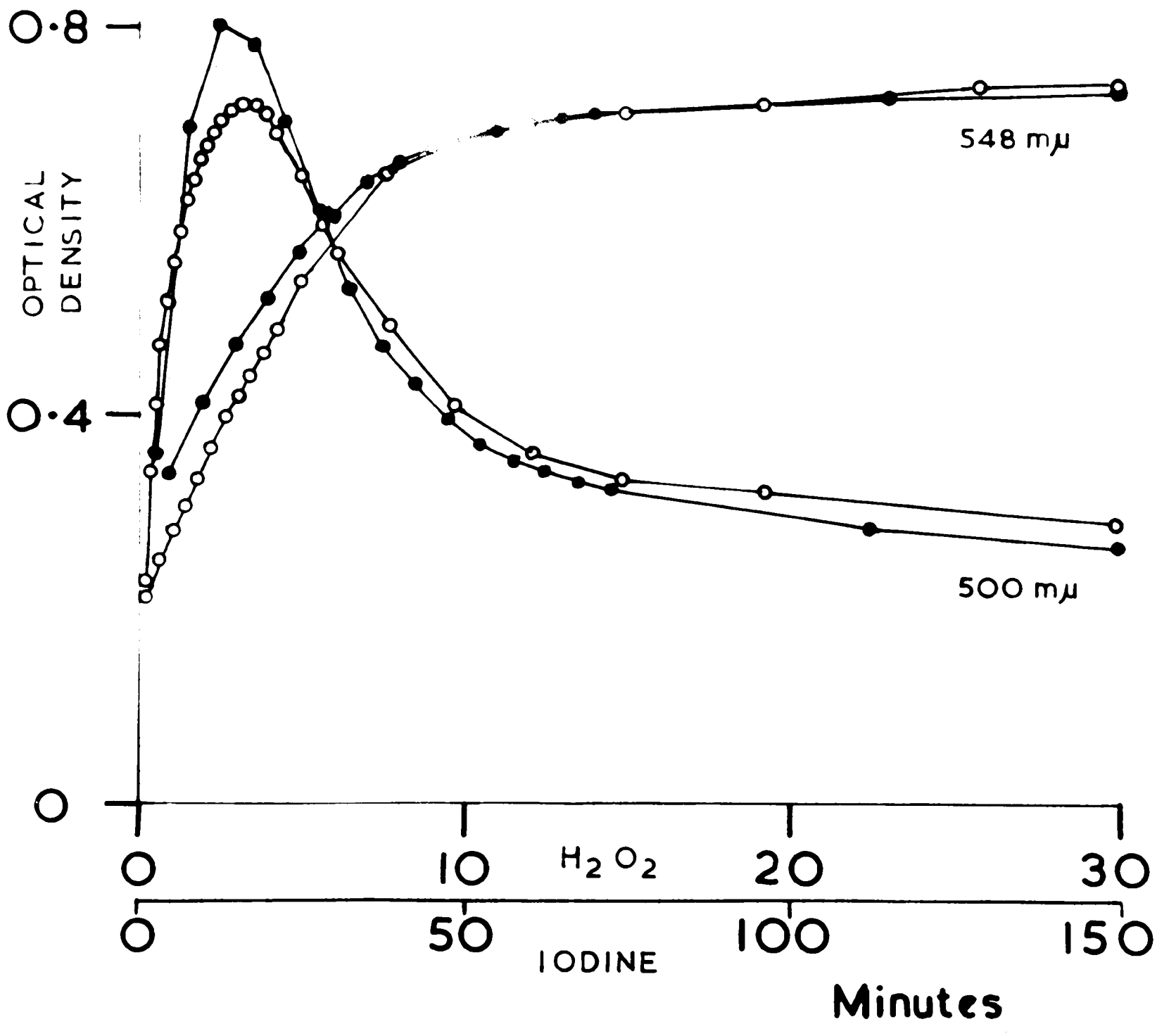
Supernatant fluids from suspensions incubated as on pp.87-89 were diluted with freshly prepared mixtures of hydrochloric acid with either hydrogen peroxide or iodine as soon as possible after centrifuging, and rapidly placed in the spectrophotometer; the frequent readings of optical density are shown in fig. 17.

With either oxidising agent, the rise and fall in optical density at 500 mμ and the rise at 548 mμ (which is the strongest absorption maximum of coproporphyrin in the visible region of the spectrum; see fig. 18) were more rapid than with air alone. When the time saales of the graphs were arranged as in fig. 17, so that one minute with H_2O_2 was equivalent to five minutes with I_2 (at the concentrations of these reagents used), the curves could almost be superimposed on each other. Moreover, after the absorption at 500 mμ had begun to fall, the rate of fall at 500 mμ ~~had begun to fall, the rate~~ was proportional to the rate of rise at 548 mμ; over any increment of time up to the end of measurments the fall in optical density at 500 mμ was 2.1 times the rise at 548 mμ. This same ratio was calculated when concentrations of iodine or hydrogen peroxide 10 or 4 times greater or less than those in the above experiment were used, though at these other concentrations the sharp rise and fall of the absorption at

FIGURE 17

CHANGES IN OPTICAL DENSITY AT 500 mμ AND 548 mμ AFTER ADDING OXIDISING AGENTS TO SUPERNATANT FLUIDS FROM SUSPENSIONS OF Rps. capsulatus

Suspensions were incubated as on pp.87-89; HCl and either H_2O_2 or I_2 were added at zero time so that supernatant fluids were diluted 1 in 5 and the other concentrations were: HCl 1.4 N, H_2O_2 0.2 vol., I_2 10^{-4} M. Measurements of optical density were made at 548 mμ and 500 mμ; O—O with iodine, ●—● with H_2O_2



500 mμ was usually less than one-fifth of that recorded in fig. 17, in magnitude. The same ratio of 2·1 was also found in the slow changes taking place in air, and even when reducing agents were added (see below).

These findings were taken as evidence that sequence (a) in the above list was operating, that is compound y is intermediate between compound x and porphyrin (see Discussion). If this is indeed so, and if it is further assumed that the only changes in the concentrations of compound y and porphyrin are due to the unimolecular reaction y $\longrightarrow$ porphyrin, then the molar extinction coefficient of compound y at 500 mμ is about 2·1 times that of coproporphyrin at 548 mμ (1.7×10^4; Jope & O'Brien, 1945)

$\therefore E_{\text{mol. of compound y at 500 m}\mu} \simeq 3.7 \times 10^4$

(iii) The effects of reducing agents

Ascorbic acid or cysteine decreased the rate of formation of coproporphyrin when they were added to supernatant fluids and the mixtures stood in open tubes in the dark (table 6). The final amount of coproporphyrin appearing was little affected.

From the experiments so far described, the evidence suggests that compound x, a colourless (or very weakly coloured) precursor of porphyrin, forms porphyrin via compound y (absorbing at 500 mμ in acid solution), and that both transformations are oxidations. Other compounds may have participated in the process, but were not detected

C. The Extraction of Coproporphyrin Precursors

Before any further study was made of the properties of the porphyrin precursors excreted by Rps. capsulatus, their extraction and purification was attempted. A preliminary attempt to use organic solvents to extract the precursors from supernatant fluids of suspensions was abandoned when such solvents were found to accelerate the rate of conversion of precursors to porphyrin. Next, the following methods were tried.

Table 6

THE EFFECT OF REDUCING AGENTS ON THE APPEARANCE OF COPROPORPHYRIN IN SUPERNATANT FLUIDS FROM SUSPENSIONS OF Rps. capsulatus.

Suspensions were incubated as on pp87-89; fresh supernatant fluids were diluted 1 in 5 at zero time with a mixture of reducing agent and HCl to a final concentration of 1.4 N - HCl.

Additions	Final conc(mg/ml)	µm-moles coproporphyrin /ml. measured after: ½ min.	40 min.	10 hr.
Nil	-	4	30	56
Ascorbic acid	1	5	24	54
	10	4	20	53
Cysteine-HCl	1	4	23	55
	10	5	17	52

(i) Ion-exchange chromatography

The strong anion-exchange resin, Dowex - 1 (Dow Chemical Co.) (200 - 400 mesh) was washed several times with 2 N - Na OH and 2N - HCl alternately. It was poured as a slurry into a glass column (100 mm x 8 mm) bounded at its lower end with a disc of sintered glass. The resin column was washed with 10 ml. 2 N NaOH and then 100 ml. H_2O; 1 ml. of fresh supernatant fluid from a suspension of Rps. capsulatus (incubated as on pp.87-89) was then applied to the top, and washed with 10 ml. H_2O. An orange band, rapidly changing colour to dark red-brown, was held in the top centimetre of the column. This band was not eluted by 2 N-acetic acid, but N-HCl or N-monochloracetic acid removed most of the pigment from the column; no precursors could be detected in the eluate, but only porphyrin (recovery 95%). 5 N - HCl eluted the remaining pigment from the column; in this only diffuse, unidentified absorption bands were seen. It was concluded that precursors were oxidised to porphyrin within the first minute after applying to the column. The rapid darkening of the column was uninfluenced by adding ascorbic acid, cysteine or sodium thioglycollate (each at 2×10^{-2} M) to the supernatant fluid before application or to the water used for washing the column.

In an attempt to introduce milder conditions, a column of magnesium oxide was prepared and some fresh supernatant fluid applied; again the top of the column rapidly darkened and no precursors could be eluted.

(ii) Precipitation by heavy metals

Next, an attempt was made to follow the method which Cookson & Rimington (1954) used to extract porphobilinogen (see Introduction) from urine. A solution of mercuric acetate was mixed with some supernatant fluid freshly prepared from a suspension of Rps. capsulatus. All pigment and precursor material was thereby removed from solution, and the washed

precipitate could be dissolved by HCl to yield a solution mainly of porphyrin. No porphyrin or precursor was released by the action of H_2S on the washed precipitate. Similar results were obtained when lead or zinc salts were used instead of mercuric. It seemed that here again, the treatment applied had accelerated the oxidation of precursors to porphyrin. Thus the organic solvent method was finally chosen for further study since it offered conditions in which precursors were more stable than with the methods tried above.

(iii) Extraction with organic solvents

The results of preliminary trials with ether are shown in table 7. It was interesting that the stability to oxidation of the precursors in the ether extract was markedly incluenced by the concentration of HCl in the aqueous layer. Of the other organic solvents tried, ethyl acetate extracted precursors from 0.25 N HCl more completely than did ether, but the extracts in ethyl acetate were the less stable. Chloroform extracted precursors only partially from all the concentrations of HCl tried; n-butanol extracted the precursors completely but very rapid conversion to porphyrin was observed. Hence ether was the chosen solvent and 0.25 N was the chosen concentration of HCl, a combination held to give the best compromise between completeness of extraction and stability of extract.

(iv) The standard method adopted

(a) Three 2-oz. bottles were filled with a suspension of Rps. capsulatus and incubated, as on pp.87-89. Before centrifuging the suspension (180 - 200 ml.), 4 ml. of 20% (w/v) ascorbic acid was added. The supernatant fluid was returned to 2-oz. bottles (filled to the brim and screw-capped), and chilled at 0 - 4°.

(b) 10 ml. 5 N - HCl was added to the chilled supernatant fluid, and the mixture shaken separately with 150 ml. and then 100 ml. of peroxide-free ether (appendix 9) in a separating funnel. The ether layers were washed in turn

TABLE 7

THE EXTRACTION OF PORPHYRIN PRECURSORS FROM SUPERNATANT FLUIDS BY ETHER

Supernatant fluids were from suspensions of Rps. capsulatus incubated as on pp.87-89, ~~with the additions shown~~. Ether was freed from peroxides by the method in appendix 9, and shaken in separating funnels with the aqueous mixtures. When no increase in concentration of porphyrin could be detected in the aqueous layer when this was separated and stood in air, the degree of extraction of precursors was judged "complete". The stability of precursors in the ethereal extract was judged from the rate of increase in its fluorescence under ultra-violet light and the rate of darkening of its initial pale orange colour.

Additions and final conc.	Degree of extraction	Stability of extract
Nil	None	-
NaOH (0.1 N)	None	-
Acetic acid (2N)	None	-
HCl (0.05 N)	Partial	Extracts of increasing stability. ↓
HCl (0.1 N)	Complete	
HCl (0.25 N)	Almost complete	
HCl (1 N)	Partial	

with a single quantity of 50 ml. 0.25 N -HCl and then combined.

(c) The combined ethereal extract was shaken with 20 ml. 0.5% (w/v) ascorbic acid, and 40% (w/v) NaOH added (usually 1 - 2 ml.) until all of the yellow colour was in the aqueous layer.

(d) The aqueous layer was rapidly separated and neutralised with 5 N - HCl until the colour changed from yellow to red and then a precipitate began to appear (pH about 6). The solution was freed from ether by bubbling through it a stream of nitrogen, and finally freeze-dried. The dried preparation was stored at -20° in the dark under nitrogen.

All manipulations were done as far as possible in the dark, because light accelerated the oxidation of precursors. The final preparation contained more precursors and less porphyrin if efficiency of extraction and final yield wase sacrificed to allow a greater speed of operation, especially over the period from the extraction into ether to the neutralisation of the concentrated solution.

The losses of porphyrin precursors at each stage of a typical extraction process, and the final yield obtained, are shown in table 8. On different occasions, the final yield varied between 55% and 78%. The aqueous extract before freeze-drying was about six times as concentrated as the original supernatant fluid with respect to porphyrin precursors.

The quality of the final, dried preparation is shown in table 9. The optical density at 500 mμ increased and then decreased on exposure of the acid solution to air, whilst that at 548 mμ was rising; this sequence was the same as that found in the original supernatant fluid (see fig. 17), and it was concluded that the dried preparation contained some of the colourless precursor of porphyrin (compound x). However, it contained greater proportions of the compound y (absorbing at 500 mμ) and

TABLE 8

AN ANALYSIS OF THE EFFICIENCY OF EXTRACTION OF COPROPORPHYRIN PRECURSORS

Suspensions of Rps. capsulatus were incubated and porphyrin precursors extracted from the supernatant fluid as on pp. 95-97. Samples for analysis were acidified with HCl to 1.4 N and stood in air for 5 hr. to allow conversion to porphyrin to go to completion.

Samples taken from:	Total vol. (ml.)	Coproporphyrin * Concentration (µm-moles/ml.)	Coproporphyrin * Total Amount (µ-moles)	% of amount in original supernatant fluid
Original supernatant fluid:	180	271	49	-
Extracted supernatant fluid :	190	42	8	16
The washing with 0.25 N -HCl :	50	20	1	2
Final preparation before freeze-drying:	22	1590	35	71

* Including precursors estimated as coproporphyrin.

N.B. No porphyrin or precursors were detected in the ether after it had been extracted by aqueous alkali.

TABLE 9

AN ESTIMATE OF THE PROPORTION OF PRECURSORS TO PORPHYRIN IN A DRIED PREPARATION

The precursor preparation was the same as that in table 8. A few grains of the freeze-dried preparation were dissolved in one drop of 0.1 N - NaOH and the solution immediately diluted (zero time) with 5 ml. 1.4 N - HCl; the optical densities at 500 mμ and 548 mμ were measured at the times shown.

	Optical densities		
Wavelength	½ min.	30 min.	5 hr.
500 mμ	0.140	0.199	0.060
548 mμ	0.026	0.044	0.176

coproporphyrin than did the original supernatant fluid (compare fig. 17 and table 9).

The dried preparation of precursors contained about 5% (w/w) of porphyrin plus precursors, as measured by its coproporphyrin content when an acidified solution was allowed to oxidise in air. The amount of sodium hydroxide added during the extraction process was sufficient to account for the remaining 95% as sodium chloride. Not more than 5% (w/w) of ascorbic acid was also presumed to be present.

In one experiment, a more concentrated preparation was made by continuing to add HCl after neutralisation of the alkaline extract (see standard method, stage (d)), so that most of the pigment was precipitated leaving a straw-coloured supernatant fluid. The suspension was centrifuged and the precipitate freeze-dried. This preparation contained more than 90% of porphyrin plus precursors (measured as coproporphyrin), but the mixture contained a greater proportion of porphyrin than that prepared by the standard procedure.

D. The Properties of the Extracted Coproporphyrin-Precursors

In this section, as in section B. above, the colourless precursor (that is, without any detected absorption bands in the visible region of the spectrum) will be called compound x, and that absorbing at 500 mμ will be called compound y.

(i) Spectral properties

The peak of compound y at 500 mμ (in acid solution) is shown in fig. 18. The two peaks and the shoulder between 548 mμ and 590 mμ are those of coproporphyrin, whose expected absorption curve if no compound y were present was derived from the spectrum of pure coproporphyrin and plotted as the crossed line. An estimate of the molar extinction coefficient of compound y at 500 mμ has already been made. (p. 92).

The spectrum of the precursor preparation in ether is in fig. 19. The absorption peaks of such a solution were

FIGURE 18

THE ABSORPTION SPECTRUM IN ACID OF COPROPORPHYRIN PRECURSOR FROM Rps. capsulatus AND OF A PREPARATION OF COPROPORPHYRINOGEN

The precursor preparation was made by the standard method (pp. 95-97), and a little dissolved in 0.2 ml. 0.1 N- NaOH. The coproporphyrinogen was made by chemical reduction of coproporphyrin, as described on pp.105-106. Each solution was immediately diluted with HCl to a concentration of 1.4 N - HCl, and the spectra surveyed as rapidly as possible. Solid line: precursor preparation; broken line: coproporphyrinogen preparation; crossed line: the expected absorption curve of pure coproporphyrin in the region of 500 mμ.

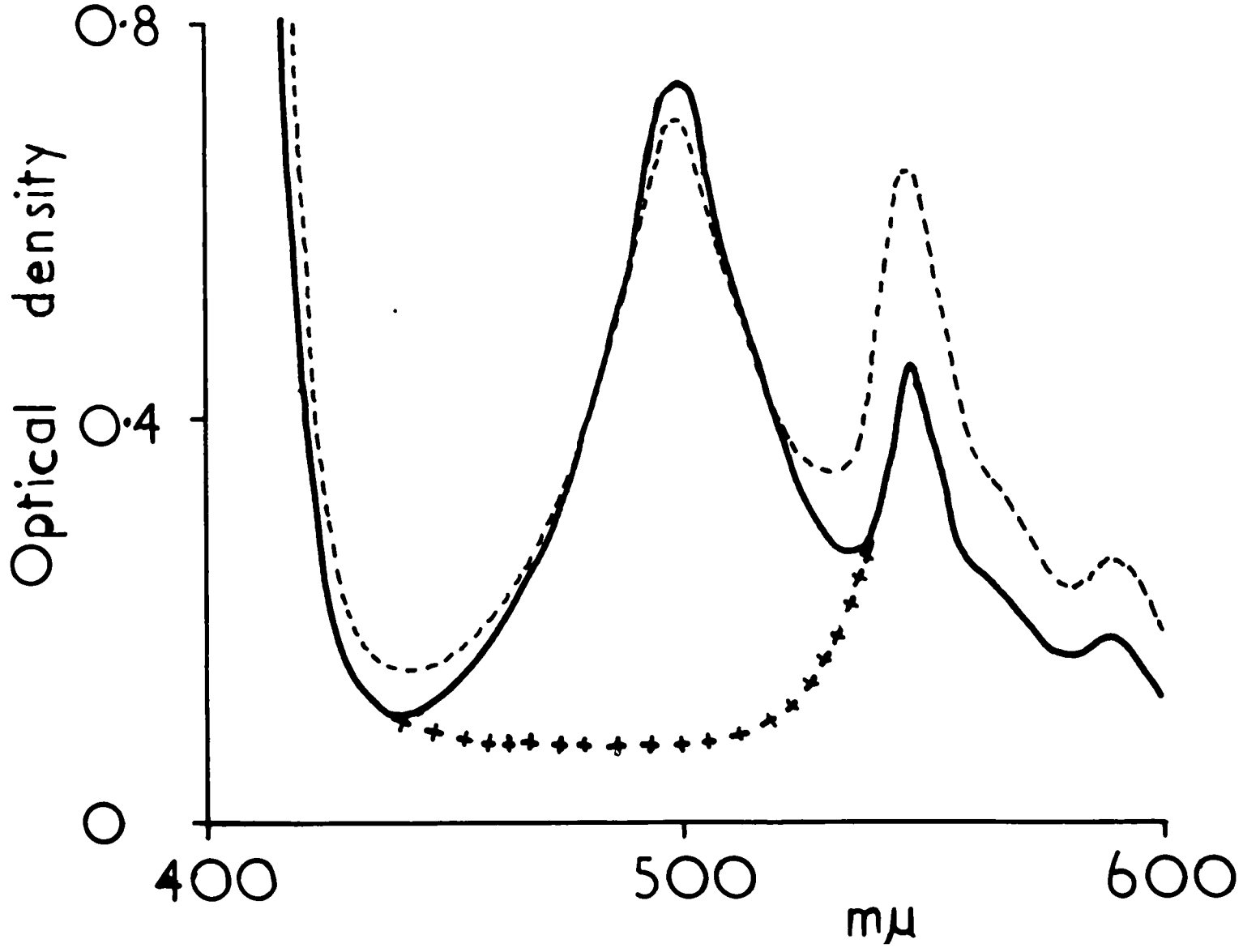
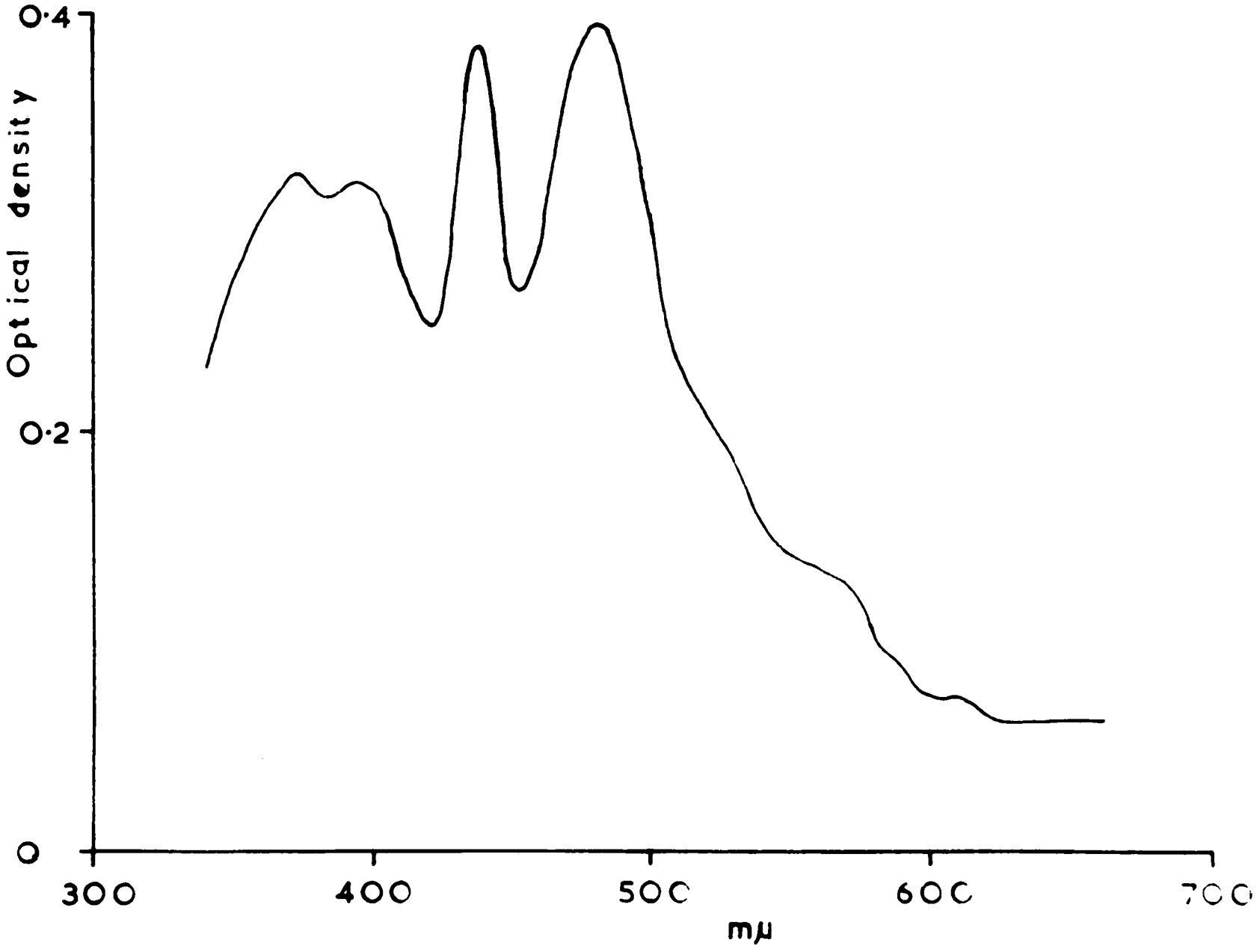


FIGURE 19

THE ABSORPTION SPECTRUM OF A PREPARATION OF COPROPORPHYRIN PRECURSORS DISSOLVED IN ETHER

The precursor preparation was made by the standard method (pp.95-97), and a little dissolved in peroxide-free ether (appendix 9). The spectrum was surveyed immediately, and as rapidly as possible.



rapidly changing in relative height, so that the curve is accurate only in respect of the wavelengths of the peaks. The peak at 395 mμ and the "shoulders" at wavelengths between 500 mμ and 620 mμ were probably due to coproporphyrin. The peaks at 372 mμ and 481 mμ may have been due to compound Y, since peaks in similar positions were observed in alkaline solutions of precursor preparations which had very strong absorption at 500 mμ when acidified (see Dissussion). The sharp peak at 437 mμ was seen only in organic solvents and could not be detected after standing a solution in air for 30 min. However, in such solutions, the peaks at 395 mμ and 481 mμ had developed so strongly that it was not possible to decide whether the peak at 437 mμ had disappeared or was merely obscured. If it had disappeared, it may have been due to an intermediate between compounds x and y.

No fluorescence under ultra-violet light could be detected in ethereal solutions of precursors from which all porphyrin had just been extracted with 0.25 N - HCl.

(ii) Solubility properties

Some of these have been already described. Those summarised below were observed with a dried preparation prepared in the standard way (pp.95-97). consisting mainly of compounds x and y together with a little porphyrin.

(a) Insoluble in petroleum ether (boiling point 40 - 60°). Sparingly soluble in $CHCl_3$. Moderately soluble in ether, ethyl acetate and dilute HCl, but it was difficult to dissolve the dry powder in HCl. Very soluble in ethanol, methanol, acetone and aqueous alkali (and very rapidly oxidised in these solvents).

(b) The pH of minimum solubility in water was determined by adding different amounts of 0.1N -HCl to a solution of precursors in 0.1N - NaOH, and estimating the amount of precipitation visually at different pH. Precipitation increased gradually from none at pH 4.8 to a maximum at

pH 3.6 - 3.8 and then the precipitate redissolved at lower pH until it was all in solution at pH 2.5.

(iii) Chemical tests

Since the porphyrin precursors may be similar to bile pigments (see Discussion), three of the tests used for these compounds (see Gray, 1953), were applied to an aqueous preparation of precursors made in the standard way (pp95-97).

(a) Ehrlich's reagent. To 2 ml. of the precursor preparation were added 2 ml. of 2% (w/v) p-dimethylamino-benzaldehyde in 5 N - HCl, and then 4 ml. of saturated, aqueous sodium acetate. The control contained 2 ml. 5 N - HCl without the aldehyde reagent. No differences in colour or absorption bands observed by hand spectroscope could be detected.

(b) Zinc acetate. A 10% (w/v) ethanolic solution of zinc acetate was added to an equal volume of the precursor preparation. No green fluorescence was seen, but only a diffuse absorption spectrum in the hand spectroscope which may have been due to a zinc coproporphyrin complex, because coproporphyrin appeared immediately on acidification with 0.1N - HCl.

(c) Ferrous sulphate. To 50 ml. of the aqueous solution of precursors were added 25 ml. of freshly prepared ferrous sulphate solution (20%,w/v) and 25 ml. of 10% (w/v) NaOH. The mixture was stood in the dark one hour and a sample filtered; the filtrate gave no indication of a complex with Erlich's reagent when treated as in (a) above. However, an examination of the changes in intensity of the absorption bands at 500 mμ and 548 mμ, after acidification of another sample of the mixture of ferrous sulphate, alkali and precursor, showed that there was more compound x and less compound y or porphyrin than in a control sample which had been incubated without ferrous sulphate. Thus ferrous

sulphate was behaving like other reducing agents (see above) in protecting the precursors from oxidation.

Hence the porphyrin precursors gave negative results, with tests commonly used for bile pigments (see discussion)

(iv) The persistence of the absorption band at 500 mμ

In acid solutions of precursors in which the production of porphyrin had been carried to completion by standing them in the dark in open tubes for 5 hr., a persistent band at 500 mμ was observed with the hand spectroscope. This persistence was also found when oxidation was accelerated by iodine or hydrogen peroxide (see fig. 17) or retarded by ascorbic acid or cysteine. It may have been due to a compound similar to that whose absorption at 500 mμ decreased when porphyrin was appearing, but degraded in some way so that it was unable to be further oxidised.

(v) Types of porphyrin produced

Precursor preparations, made as described on pp95-97, were allowed to stand in air in acid solution until no more porphyrin appeared, and the porphyrin analysed by the paper chromatographic methods of Eriksen (1953) and Chu, Green & Chu (1951), as described in Chapter 3. Apart from coproporphyrin, the only porphyrin detected on papers developed by the Eriksen method had probably 3 carboxyl groups; this was present only in traces, and not at all in some experiments. All of the coproporphyrin occupied the same position as the marker of coproporphyrin III, on papers developed by the method of Chu, Green & Chu.

(vi) A comparison with a chemically prepared sample of Coproporphyrinogen

Coproporphyrinogen was prepared by a method similar to that used for mesoporphyrinogen by Fischer, Bartholomäus & Röse (1913). 3 mg. of coproporphyrin was dissolved in 6 ml. 0.1N -NaOH and diluted with 18 ml. H_2O. 10g. of 3% (w/w) sodium amalgam was

added to the mixture in a thick-walled test tube, which was then shielded from the light with a cylinder of black paper and shaken on a machine for 3 hr. At the end of this period, the solution was colourless, though an orange colour rapidly appeared when it was exposed to light.

The solution was decanted from the mercury and acidified to 0.25 N - HCl, and the coproporphyrinogen extracted by the method normally used for supernatant fluids from suspensions of Rps. capsulatus (pp.95-97), with the quantities of ether and alkali scaled down in proportion to the small volume of solution to be extracted. Nothing was observed during the course of extraction to indicate that the preparation of coproporphyrinogen was behaving differently from the porphyrin precursors from Rps. capsulatus.

The neutralised aqueous extract of coproporphyrinogen was then examined for the various properties described below, using the same methods as applied to precursor preparations and described above. No differences between coproporphyrinogen and precursors from Rps. capsulatus were detected in the following properties:

(a) The production of an absorption band at 500 mμ which increased and then decreased in intensity as the concentration of coproporphyrin in the solution was increasing.

(b) The acceleration of these processes by light, alkali, iodine or hydrogen peroxide, and their retardation by ascorbic acid or cysteine.

(c) The position and shape of the absorption peak at 500 mμ in 1.4 N - HCl (see fig. 18).

(d) The absorption peaks at 372 mμ, 437 mμ and 481 mμ in a freshly-prepared ethereal solution.

(e) The relative solubilities of a dried sample in different organic and aqueous solvents, and the pH of minimum solubility in water.

(f) The negative reactions with the bile-pigment tests with Ehrlich's reagent, zinc acetate and ferrous sulphate plus Ehrlich's reagent.
(g) The persistence of an absorption band at 500 mμ after the formation of porphyrin in acid solutions exposed to air had ceased.

It was concluded that the colourless precursor of coproporphyrin produced by Rps. capsulatus was coproporphyrinogen.

CHAPTER 6

THE INCUBATION OF COPROPORPHYRIN WITH SUSPENSIONS OF Rps. capsulatus AND Rps. spheroides; THE INCORPORATION OF 2^{-14} C GLYCINE INTO COPROPORPHYRINOGEN AND BACTERIOCHLOROPHYLL BY SUSPENSIONS OF Rps. capsulatus

The final stage of this study was to discover whether coproporphyrinogen could be converted to bacteriochlorophyll by suspensions of Rps. capsulatus. Part of the work required the production of ^{14}C - labelled coproporphyrinogen; the incorporation of 2 - ^{14}C glycine by Rps. capsulatus into this product and bacteriochlorophyll were measured at the same time.

A. The Incubation of Coproporphyrinogen with Suspensions of Rps. capsulatus and Rps. spheroides

Suspensions of Rps. capsulatus were prepared by the standard method given in chapter 4. Suspensions of Rps. spheroides were prepared by the method of Lascelles (1956a), which is similar to that for Rps. capsulatus except that the growth medium contains no acid hydrolysed casein (medium C; appendix 5).

Coproporphyrinogen was extracted from supernatant fluids from suspensions of Rps. capsulatus, using the standard methods for incubation and extraction described in the previous chapter, except that the neutralised aqueous extract was not freeze-dried, but stored at -20° in Thunberg tubes under nitrogen in strict darkness. The final concentration of porphyrin plus porphyrin precursors (measured as total coproporphyrin after acidified samples had been allowed to stand in air for 5 hr.)incubated with bacterial suspensions in these experiments was 80 - 120 µm-moles /ml. At this level, the concentration of sodium chloride derived from the coproporphyrinogen preparation (see chapter 5) was about 2×10^{-3} M

Suspensions of Rps. capsulatus were incubated with such coproporphyrinogen preparations in mixtures II or III (table 4), for 24 hr. under the standard conditions (light, semi-aerobic)

described in chapter 4. No greater synthesis of either magnesium protoporphyrin or bacteriochlorophyll was detected than in controls containing none of the coproporphyrinogen preparation. The same result was obtained when iron citrate was omitted, when either glycine, succinate or ammonium was omitted, when the duration of incubation was 6 hr. or 40 hr., or when the bacteria destined for suspensions were harvested after growth for 16 hr. or 40 hr. instead of the standard 20 - 24 hr.

Suspensions of Rps. spheroides, incubated in the light with mixture I of Lascelles (1956a) and supplemented with 4×10^{-5} M iron citrate, similarly synthesised no more bacteriochlorophyll with added coproporphyrinogen preparation than without.

However, slight synthesis of bacteriochlorophyll or magnesium protoporphyrin from the added coproporphyrinogen might have remained undetected in the above experiments. Moreoever, active incorporation of added coproporphyrinogen might have occurred without increased synthesis of bacteriochlorophyll or magnesium protoporphyrin. The experiments with ^{14}C - labelled compounds next described were designed to check these possibilites.

B. The Biosynthesis of ^{14}C - Labelled Coproporphyrinogen and Bacteriochlorophyll.

No work with magnesium protoporphyrin was done in these experiments with ^{14}C - labelled compounds because of the difficulty of its purification to the high degree necessary in work with radioactive tracers (see chapter 3).

(i) The extraction and purification of bacteriopheophytin

Before the incorporation of ^{14}C into bacteriochlorophyll could be studied, the extraction and purification of the compound to be assayed for radioactivity were necessary undertakings. Bacteriopheophytin was selected for this purpose since it is more stable than bacteriochlorophyll, and may easily be obtained by removing the magnesium atom

of bacteriochlorophyll with dilute mineral acid. The methods finally adopted were based on solubility properties reported by van Niel & Arnold (1938) and the chromatographic technique of Griffiths & Stanier (1956).

The following amounts are for 60 ml. of suspension of Rps. capsulatus incubated with mixture III (table 4) in a 2-oz. medical flat bottle under the standard conditions described in chapter 4. The suspension was centrifuged at 12,000 revolutions /min. for 20 min. to obtain well packed bacteria, which were extracted with 25 ml. of methanol previously dried by distilling with calcium oxide. The deep green extract was acidified with 0.2 ml. 10N - HCl which caused an immediate turbidity due to bacteriopheophytin, which is only sparingly soluble in acid methanol. Estimation of the bacteriopheophytin (see chapter 3) precipitated and in solution at this stage showed that only 8% of the total remained in solution. The extract was stood in the dark for an hour to allow the precipitate to flocculate, and then centrifuged; the precipitate was twice washed with dry methanol and dried by a stream of nitrogen. The absorption spectrum of a solution of this precipitate in carbon tetrachloride showed peaks probably due to carotenoids; the height of the neighbouring peak at 530 mμ due to bacteriopheophytin was more than twenty times greater.

Dry magnesium oxide and Super-Cel were mixed in the ratio 1 : 2 (w/w) and heated at 110° for 2 hr. to ensure dryness. The mixture was suspended in petroleum ether (boiling point 40 - 60°) and poured into a glass column (15 cm. x 1 cm.) with a sintered-glass disc at its lower end. The crude bacteriopheophytin preparation was dissolved in 0.5 ml. acetone and 25 ml. petroleum ether added. The solution was applied to the top of the chromatography column, causing all pigment to be adsorbed in a narrow band at the top.

Elution of bacteriopheophytin was begun with successive volumes of 3 ml. of acetone /petroleum ether mixtures, first 4%, then 8%, then 12% (all v/v). Such stepwise increases in concentration of acetone produced a more concentrated eluate than when a single large increase was used. The band was finally eluted with 30 ml. of 20% acetone in petroleum ether and the portion of eluate containing the pigment was evaporated with a stream of nitrogen, in the dark. The absorption spectrum of this fraction is given in fig. 20. The position and relative heights of the main peaks (362 mμ, 390 mμ, 530 mμ and 755 mμ) were close to the values given for bacteriopheophytin by Weigl (1953), except that the peak at 755 mμ is relatively higher in carbon tetrachloride than in the solvents used by Weigl. The subsidiary peaks and shoulders were always of the same relative heights in bacteriopheophytin preparations from different chromatography runs, and when a preparation was re-chromatographed. It was concluded that the bacteriopheophytin was chromatographically and spectrometrically pure.

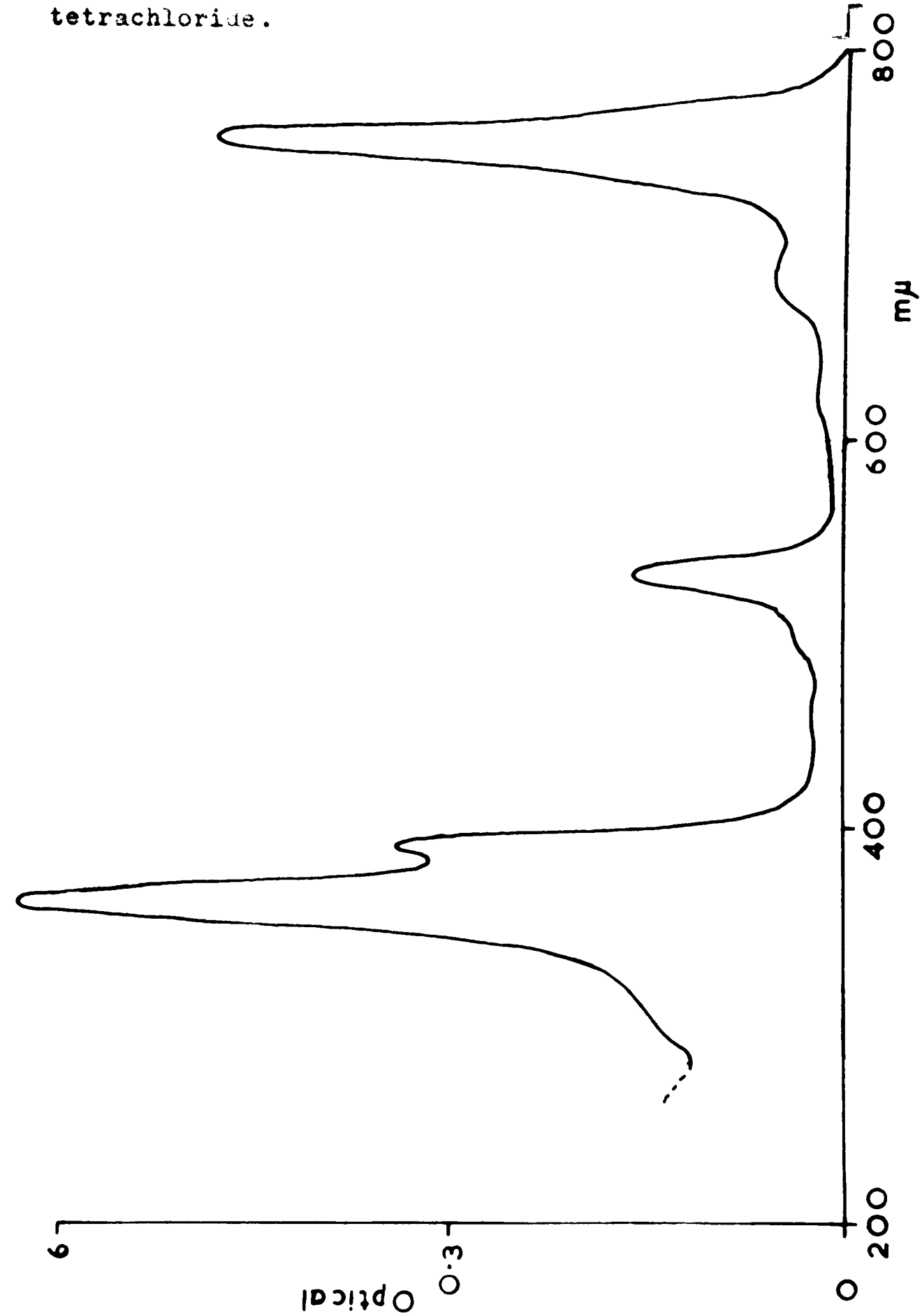
(ii) Incubation with ^{14}C -glycine.

Suspensions of Rps. capsulatus were prepared and incubated by the standard method for the production of coproporphyrinogen described in the previous chapter, (60 ml. of suspension in 2-oz. medical flat bottles), using mixture I for coproporphyrinogen and the same technique for bacteriochlorophyll except that mixture III (table 4) was used. To each 60 ml. bottle before incubation was added 0.6 ml. of a solution of 2 -^{14}C glycine containing 1.6×10^{-3} M glycine and 5 μc/ml. of radioactivity. At the end of incubation (20 - 24 hr.) the supernatant fluids or packed bacteria were extracted and purified for coproporphyrinogen or bacteriopheophytin, respectively, by the standard methods described above. Dried preparations of each product were stored at -20°

FIGURE 20

THE ABSORPTION SPECTRUM OF
BACTERIOPHEOPHYTIN FROM Rps. capsulatus

The preparation was extracted and chromatographed as described in the text. The solvent was carbon tetrachloride.



(iii) Assay of radioactivity.

Samples of coproporphyrinogen, bacteriopheophytin, and the original 2 $^{-14}$C glycine added to suspensions, were applied to aluminium planchettes by a technique to be described below; planchettes were set in a fixed and reproducible position in an E.R.D. square lead castle with their upper surfaces (bearing the radioactive preparation) at a distance of 2 mm. from the mica end-window of a Geiger-Muller counting tube (type EHM 2S, General Electric Company). The applied-voltage characteristic of this tube was found to be essentially horizontal between 1425 volts and 1500 volts; the potential applied during use was 1450 volts. Impulses from the counting tube were counted with a Panax type 100 C scaler with the resolving time pre-set at 800 µsec. Since none of the samples prepared gave more than 1000 c.p.m., the correction for coincident disintegrations was negligible and was not applied. The reliability of the equipment was frequently tested with a standard sample of ^{14}C - labelled, polymethyl methacrylate plastic sheet. Counting rates were not recorded unless between 600 and 620 c.p.m. were measured with this sample. The usual background count was 8.0 - 9.5 c.p.m. For each sample and the background, sufficient counts were made to bring the standard deviations from the mean counting rates down to less than 3%.

After trials of various ways of applying samples of coproporphyrinogen and bacteriopheophytin to planchettes, the following methods were adopted:

(a) Bacteriopheophytin

The dried preparations after chromatographic purification were dissolved in about 0.2 ml. carbon tetrachloride. A circle enclosing an area of 0.7 sq. cm. was lightly marked in the centre of each planchette with a cork-borer, and 0.05 - 0.1 ml. of the solution, containing 15 - 80 µm-moles of bacteriopheophytin, applied within the marked area with

a fine-tipped pipette. The solution was dried with a stream of nitrogen directed through a Pasteur pipette during the application, so that an even spread could be achieved within the marked area. After assay of radio-activity, each planchette was folded into a test tube containing 5 or 10 ml. of carbon tetrachloride, and the amount of bacteriopheophytin estimated from the optical density at 755 mμ.

(b) Coproporphyrinogen

A similar method was used for this product, except that the amounts on the planchettes were estimated indirectly with aliquots taken from the applied solution. This was because the solvent used in the estimation of the coproporphyrin was 1.4 N - HCl, which effectively dissolved the planchettes as well as the coproporphryin. The aqueous solution applied contained about 1 μ-mole coproporphyrin plus precursors (estimated as coproporphyrin) per ml.; measured volumes of 0.01 ml - 0.04 ml. were applied with a micro-pipette to each planchette.

The results obtained with these plating methods are illustrated in fig. 21. The straight-line relationship between μm-moles of product on the planchettes and the counting rates per μm-mole indicated that results were not unduly influenced by any unevenness of spreading on planchettes. With bacteriopheophytin, the line was horizontal, indicating that self-absorption of radiation by the samples was negligible, that is to say they were all "infinitely thin". With the coproporphyrinogen preparation, the inclined straight line showed that some self-absorption was taking place, probably due to the high content of sodium chloride (see previous chapter). The specific counting rate of an "infinitely thin" sample was thus obtained by extrapolating the graph to zero amount of sample.

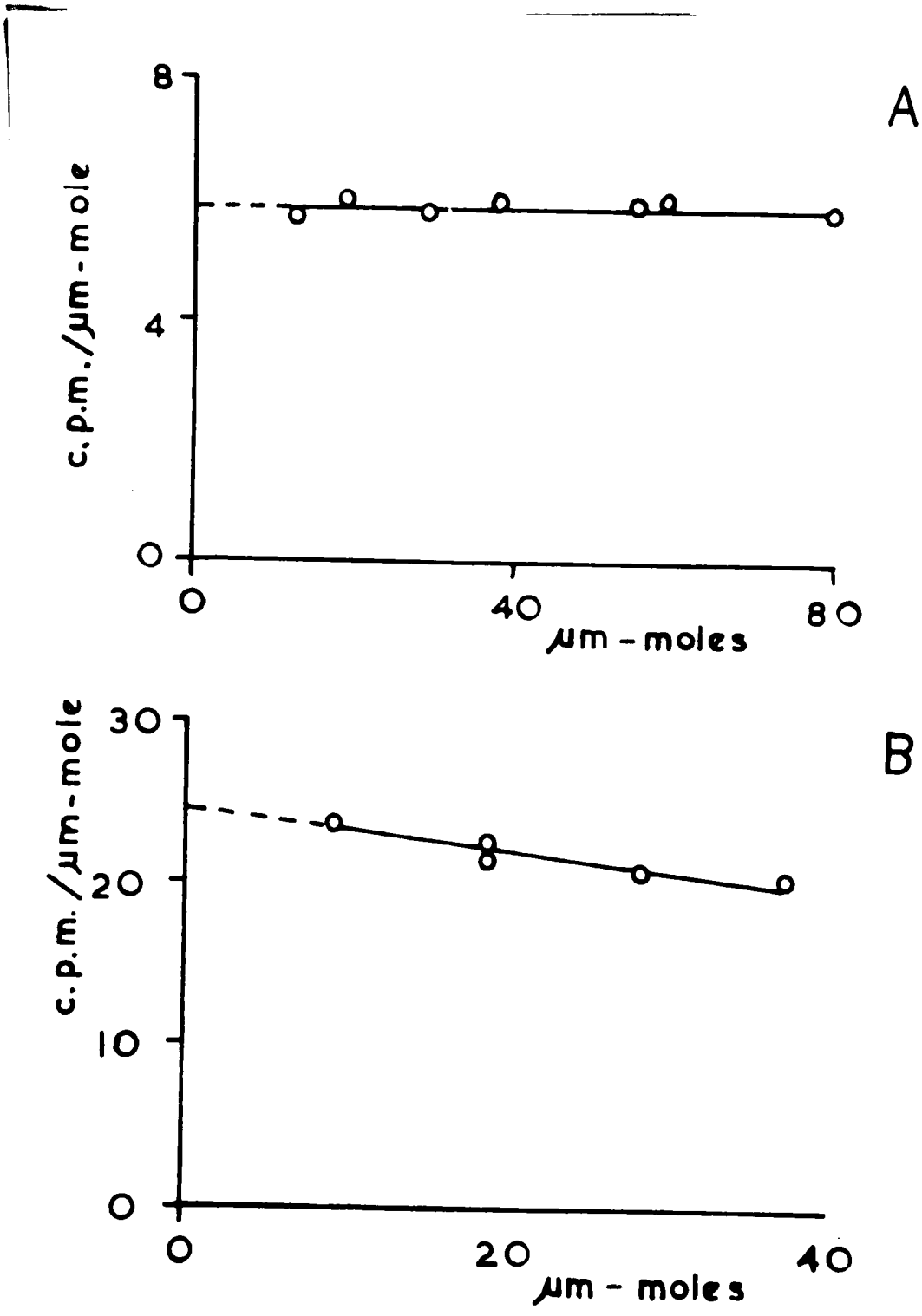
Finally, the counting rate per μm-mole of the original

FIGURE 21

SELF-ABSORPTION CURVES FOR PREPARATIONS OF BACTERIOPHEOPHYTIN AND COPROPORPHYRINOGEN

Preparations were made and applied to planchettes as described in the text. The amount of material on each planchette is plotted against the specific counting rate obtained. Coproporphyrinogen was probably oxidised to coproporphyrin on the surface of planchettes, and was determined as such.

A : bacteriopheophytin; B : coproporphyrinogen.



sample of 2-^{14}C glycine was obtained by applying measured volumes of a suitably diluted solution to planchettes in the same way as for coproporphyrinogen; no self-absorption was detected.

From these data, the number of β-carbon atoms of the added glycine incorporated into one molecule of coproporphyrin or bacteriopheophytin was calculated, by dividing the molar counting rate for the product by the molar counting rate for the substrate. Results are shown in table 10.

The incorporation of the β-carbon atom of added glycine into bacteriopheophytin was very active in suspensions of Rps. capsulatus, though it must be remembered that the phytol group of bacteriopheophytin contains 20 of the 55 carbon atoms in the molecule, and hence no conclusions could be made about the incorporation into the porphyrin moiety. No decrease in the molar counting rate was produced by re-chromatography of the bacteriopheophytin (see above), indicating that the samples were pure.

On the other hand, the re-extraction (by the standard method) of the coproporphyrinogen preparation did reduce the molar counting rate of coproporphyrin derived from it, suggesting that a radioactive compound other than those estimated as coproporphyrin was present in the first extract, and perhaps also in the second extract. Thus the incorporation figures for coproporphyrin in table 10 may be too high. There was not enough material for a third extraction, and the experiment was not repeated.

C. The Incubation of ^{14}C -Labelled Coproporphyrinogen with Suspensions of Rps. capsulatus

Experiments with unlabelled coproporphyrinogen described in section A of this chapter were repeated, using two preparations labelled ^{14}C, one singly extracted and the other re-extracted from the first, as discussed in section B. The methods of incubation of the suspensions of Rps. capsulatus were the same

TABLE 10

THE INCORPORATION OF 2-^{14}C GLYCINE INTO COPROPORPHYRIN AND BACTERIOPHEOPHYTIN, AND OF ^{14}C - COPROPORPHYRINOGEN INTO BACTERIOPHEOPHYTIN BY SUSPENSIONS OF Rps. capsulatus

The methods for preparing and incubating suspensions, and for estimating radioactivity of the products are given in chapter 6.

Experiment No.	Treatment of product or variation in incubation	Molar c.p.m.of product. / Molar c.p.m. of substrate
A.	**Glycine ⟶ Bacteriopheophytin**	
1	Standard method	8.2
2	Standard method	7.2
3	Standard method	6.8
	Sample re-chromatographed	7.0
B.	**Glycine ⟶ Coproporphyrin**	
4	Standard method	12.5
	Sample re-extracted	11.5
C.	**Coproporphyrinogen ⟶ Bacteriopheophytin**	
5	Incubation with singly-extracted coprophyrinogen	(0.0016)
6	Incubation with doubly-extracted coproporphyrinogen	(0.0005)

as before; the incubation medium was mixture III (table 4), with coproporphyrinogen added in the same way as before.

The counting rate for the bacteriopheophytin extracted from the bacteria after incubation was only 2.5 c.p.m. and 1.2 c.p.m. above the background rate, in the two experiments (see table 10); the standard error of the estimation of such low counting rates was high, and no significance was attached to the figures, especially when it was considered that neither preparation of coproporphyrinogen was known to be free from contaminating radioactivity (see section B. of this chapter).

However, it could be concluded that less than 0.05% of the bacteriochlorophyll synthesised in these experiments was derived from the added coproporphyrinogen. The possibility that pyrrole compounds cannot enter the organism is considered in the Discussion.

CHAPTER 7

DISCUSSION OF RESULTS

A. Coproporphyrinogen

A review of the studies of Fischer and his co-workers on the subject of porphyrinogens is given by Fischer & Stern (1940); two pieces of evidence are given on their structure:

(a) Six hydrogen atoms are quantitively taken up for each molecule of porphyrin reduced. This means that four methene bridges and the two pyrrolenine groups (see fig. 1) have been hydrogenated, so that porphyrinogens are at the same level of oxidation as a monopyrrole such as PBG.

(b) Molecular weight estimations suggest that four pyrrole groups are still present (Fischer & Zerweck, 1924).

There is no evidence as to whether the ring structure of porphyrin is preserved or broken up to a linear tetrapyrrole; the fragmentation and resynthesis of the tetrapyrrole structure, leading to different porphyrins, has been reported (Fischer, Bartholomäus & Röse, 1913; Fischer & Rothhaas, 1930). Lemberg & Legge (1949) state that porphyrinogens may give rise to some "urobilinoid" substances on re-oxidation, but Fischer & Stern (1940, p. 420) describe the re-oxidation as a quantitative process which can proceed in air or by the action of oxidising agents. In the present work, the persistent absorption band at 500 mμ after oxidation of the biologically-synthesised coproporphyrinogen to coproporphyrin has ceased (p. 105) is in agreement with Lemberg & Legge on this point.

Properties of coproporphyrinogen which have been reported include its extreme sensitivity to light, its oxidation by iodine, its negative reaction with Erlich's reagent, its greatersolubility in acetone, methanol and ethanol than in water, ether, chloroform and petroleum ether (Fischer, Bartholomäus & Röse, 1913; Mauzerall & Granick, 1958). The properties given in Chapter 5 confirm these reports, and supplement them with more detailed observations, including evidence that the compound excreted by Rps. capsulatus is the same as the chemically prepared

coproporphyrinogen.

Compounds absorbing at 500 mμ have been reported as intermediates in the formation of uroporphyrin I, uroporphyrin III and coproporphyrin III from biologically formed precursors or from porphyrinogens, (Bogorad, 1955 a, b; Mauzerall & Granick, 1958), but no evidence has been presented to show that they are true intermediates and not merely formed by some side reaction and then degraded to something other than porphyrin. In the present work, the following of the time course of the increase and decrease of the absorption band at 500 mμ (fig. 17), and the discovery that the ratio between decrease in optical density at 500 mμ and increase at 548 mμ (due to coproporphyrin) is 2.1 under a variety of conditions, provide fairly good evidence that the reactions:

coproporphyrinogen ⟶ compound absorbing ⟶ coproporphyrin at 500 mμ.

do in fact take place. This numerical relationship incidentally provides an approximate estimate of the molecular extinction coefficient, at 500 mμ in 1.4 N - HCl, of the intermediate (p. 92)

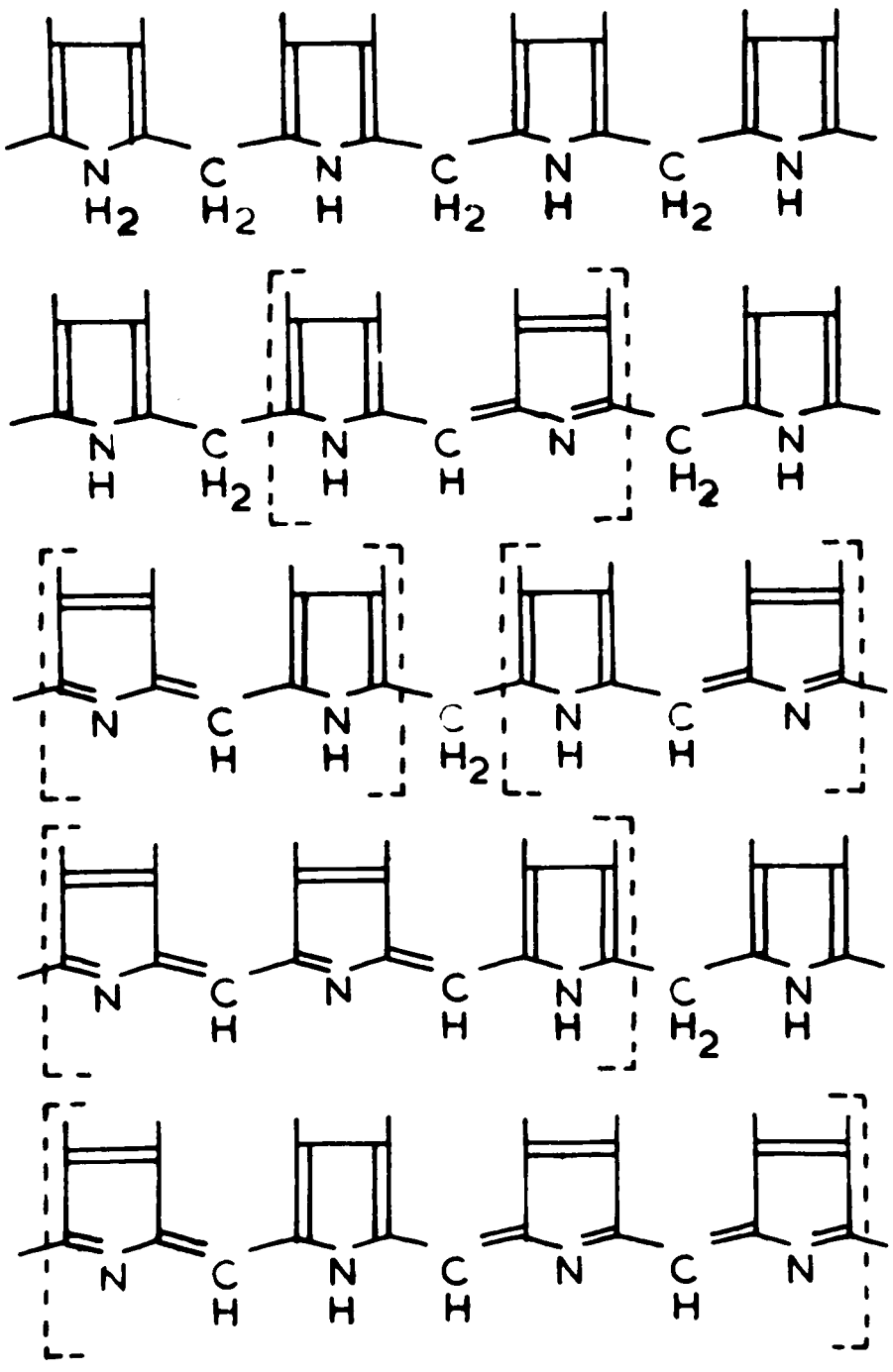
The structure of the compound absorbing at 500 mμ may best be considered on the analogy of the known open-chain tetrapyrroles. The bile pigments, the best known representatives of this group, form an oxidation-reduction series (fig. 22) from the colourless, completely reduced bilanes (also called tetrapyrryl methanes), through bilenes and bilidienes to the fully oxidised bilitrienes. Six hydrogen atoms are removed during this process, as when porphyrinogens are oxidised to porphyrins. Furthermore, mesobilirubinogen (a bilane) is converted on standing solutions in air to urobilin (a bilene b) and then to mesobiliviolin and mesobilirhodin (isomeric bilidienes); further oxidation requires stronger agents than air (see Gray, 1953).

Thus it may be valid to draw analogies between the absorption spectra of the bilane series and those observed for the coproporphyrinogen series. The main visible absorption bands are at

FIGURE 22

SKELETON FORMULAE ILLUSTRATING THE OXIDATION-REDUCTION SERIES OF OPEN-CHAIN TETRAPYRROLES

The nomenclature is that of Lemberg & Legge (1949).
The brackets enclose resonating groups of atoms.



greater wavelengths in the more oxidised bile pigments, or more strictly, when more pyrrole groups are conjugated together to form a single resonating group of atoms (see fig.22) Thus in the visible regions, bilanes do not absorb, bilenes and bilidienes a, c have bands between 450 mμ and 500 mμ, and bilidienes a, b and bilitrienes have bands above 550 mμ. For example, meso-urobilin, a bilene b, is yellow in neutral solution, with bands at 330 mμ and 450 mμ, and orange in acid, with bands at 375 mμ and 495 mμ (Granick & Gilder, 1947)

Thus the oxidation product of coproporphyrinogen, absorbing at 500 mμ in acid (with suggestions of a peak at 380 mμ, obscured by the Soret band of porphyrin), and probably 372 mμ and 481 mμ in ether, may be similar to a bilene, that is a tetrapyrrole containing one dipyrryl methene group and with two hydrogen atoms less than in coproporphyrinogen. The molar extinction coefficient of meso-urobilin at 495 mμ in acid is 50,100 (see Granick & Gilder, 1947), which is of the same order of magnitude as the estimate made for the peak at 500 mμ in the present work (p. 92). This makes this intermediate unlikely to be a bilidiene a, c, which would be expected to have a molar extinction coefficient about double the above figure. In any case, a bilidiene a, c type of structure would be impossible in a cyclic tetrapyrrole.

The possible cyclic structure of porphyrinogens may be responsible for the negative results of the tests for bile pigments which were applied in the present study.

B. Magnesium protoporphyrin

The present work provides the first report of this compound in bacteria (see Introduction). No evidence was found to show that it is an intermediate on the pathway of bacteriochlorophyll synthesis.

The finding that the concentration of added iron citrate for optimal synthesis of magnesium protoporphyrin by suspensions of Rps. capsulatus is intermediate between that for coproporphyrin

and that for bacteriochlorophyll suggests that iron may be required by the reactions as follows:

Glycine + Succinyl CoA ⟶ coproporphyrinogen $\xrightarrow{Fe}$ magnesium protoporphyrin $\xrightarrow{Fe}$ bacteriochlorophyll

(assuming that coproporphyrinogen and magnesium protoporphyrin are intermediates).

The formation of coproporphyrin is inhibited by the addition of iron citrate to suspensions of Rps. capsulatus but not so strongly as with suspensions of Rps. palustris or Rps. spheroides. The appearance of magnesium protoporphyrin in growth and suspension media with Rps. capsulatus, but not with Rps. spheroides (Lascelles, private communication) or Rps.palustris, may be due to this more active porphyrin synthesis by Rps.capsulatus in the presence of moderate amounts of iron, thereby giving rise to magnesium protoporphyrin as well as coproporphyrin. On the other hand, suspensions of Rps. spheroides form free protoporphyrin and haem from δ-aminolaevulic acid under conditions in which no bacteriochlorophyll is synthesised (Lascelles, 1956 a; see Introdution). Rps. capsulatus may produce magnesium protoporphyrin rather than the free protoporphyrin or haem because conditions of incubation used in the present work were suitable for bacteriochlorophyll synthesis (incubation with glycine and succinate in the light); thus a critical step in bacteriochlorophyll synthesis may be the insertion of magnesium into the porphyrin ring.

C. Miscellaneous conclusions

(a) The failure to observe the utilisation of added coproporphyrinogen or magnesium protoporphyrin by suspensions of Rps. capsulatus does not necessarily mean that they are not utilised within the organism. Such compounds may not penetrate from the external medium to the sites of formation of magnesium

protoporphyrin and bacteriochlorophyll in the organism. Thus no porphyrins are formed by Chlorella from the monopyrrole, porphobilinogen, unless the cell wall has been modified by freezing and thawing (Bogorad & Granick, 1953 a). Similarly, porphobilinogen is converted to porphyrin by haemolysates but not by whole erythrocytes (Dresel, 1955).

(b) The effect of methionine and cobalamin on the growth of Rps. capsulatus (chapter 2) is interesting but not easy to explain. Ammonium delayed the growth of cultures when air was present and this delay was prevented by a very small concentration of added methionine or by cobalamin at a concentration five thousand times smaller. Cobalamin is known to mediate in the synthesis of methionine (Helleiner & Woods, 1956), but it cannot be decided from the present results whether methionine bears the primary function in the relief of ammonium toxicity and added cobalamin is stimulating its synthesis, or whether cobalamin bears the primary function and is spared for this purpose by adding methionine. Added methionine also increased the synthesis of coproporphyrin and magnesium protoporphyrin by suspensions of Rps. capsulatus, though in amounts 500 - 2000 times greater than that found effective in the growth experiments above. The effect of cobalamin was not tried in these suspensions.

(c) The inhibition of the growth of Rps. palustris by concentrations of added glycine greater than 4×10^{-3} M but not by its peptides with leucine was very striking (chapter 2). Van Niel (1954) mentions that this inhibition by glycine is a common feature of Athiorhodaceae. Instances have been reported where peptides are more active for the growth of micro-organisms than are the free amino acids, explained by cross-inhibitions between amino acids (but not with their peptides) or by the presence of enzymes destroying amino acids but not their peptides (Gale, 1945; Kihara & Snell, 1952; Kihara, Klatt & Snell, 1952). The situation is a little ~~difficult~~different with Rps. palustris, however, because the amino acid in

quantities inhibits growth, but in smaller concentrations supports growth no less actively than do the peptides.

ACKNOWLEDGEMENTS

It is a pleasure to thank Professor D. D. Woods, F.R.S. for his supervision of this work and for his most valuable instruction and guidance in the general principles of biochemistry and microbiology. Thanks are also due to Dr. June Lascelles whose instruction on the devious ways of photosynthetic bacteria and porphyrins laid the foundation necessary before this study could begin.

APPENDIX 1

Basal salts for the growth of Athiorhodaceae

(Lascelles, 1956a)

The following salts were dissolved in 500 ml. H_2O:

KH_2PO_4	500 mg.
K_2HPO_4	500 mg.
$(NH_4)_2\ HPO_4$	800 mg.
$Mg\ SO_4 .\ 7H_2O$	200 mg.
$CaCl_2$	40 mg.

The solution was stored at 0 - 4°.

APPENDIX 2

Media used in the Isolation of Rps. capsulatus.

(a) Mud Incubation Mixture for Winogradsky Column

The following mixture was made up to 100 ml. final volume:

Basal salts (appendix 1)	50 ml.
Cellulose powder	5 g.
Mud	25 ml.

(b) Enrichment Culture Medium

The following mixture was made up to 100 ml. final volume:

Basal salts (appendix 1)	50 ml.
Iron citrate solution (appendix 7), 4×10^{-3} M	1 ml.
Sodium DL-lactate solution, 1 M	2 ml.
Sodium L-glutamate monohydrate	370 mg.
Glycine	15 mg.
Calcium pantothenate	100 µg.
Thiamin hydrochloride	100 µg.
Biotin	1 µg.
Nicotinic acid	100 µg.

The pH was adjusted to 6.8 with N-NaOH. The medium was sterilised by autoclaving at 10 lb./sq.in. for 10 min. immediately before use.

APPENDIX 3

Medium A : The Growth Medium for Rps.capsulatus

The following mixture was made up to 1 litre final volume:

Basal salts (appendix 1)	500 ml.
Iron citrate solution (appendix 7) 4×10^{-3} M	10 ml
DL-Malic acid	2.7 g
Sodium L-glutamate monohydrate	3.7 g.
Acid hydrolysed casein (appendix 8)	20 ml.
Thiamin hydrochloride	1 mg.
Nicotinic acid	5 mg.
Biotin	10 µg.

The pH was adjusted to 6.8 with 10 N - NaOH. The medium was sterilised by autoclaving at 10 lb./sq.in. for 10 min.

APPENDIX 4

Medium B : The Growth Medium for Rps. palustris

The following mixture was made up to 1 litre final volume:

Basal salts (appendix 1)	500 ml.
Iron citrate solution (appendix 7) 4×10^{-3} M	10 ml.
DL-Lactic acid	1.4 ml.
Sodium L-glutamate monohydrate	3.7 g.
Glycine	150 mg.
Calcium pantothenate	1 mg.

The pH was adjusted to 6.8 with 10 N - NaOH. The medium was sterilised by autoclaving at 10 lb./sq. in. for 10 min.

APPENDIX 5

Medium C : The Growth Medium for Rps. spheroides (Lascelles, 1956a)

The following mixture was made up to 1 litre final volume:

Basal salts (appendix 1)	500 ml.
DL - Malic acid	2.7 g.
Sodium L-glutamate monohydrate	3.7 g.
Thiamin hydrochloride	1 mg.
Nicotinic acid	1 mg.
Biotin	10 µg.

The pH was adjusted to 6.8 with 10 N - NaOH. The medium was sterilised by autoclaving at 10 lb./sq. in. for 10 min.

APPENDIX 6

Medium D : Growth Medium Used in the Investigation of the Nitrogen Sources for Rps.capsulatus

The following mixture was dissolved in 1 litre of water:

KH_2PO_4	500 mg.
$K_2H\ PO_4$	500 mg.
$Mg\ SO_4, 7H_2O$	200 mg.
$CaCl_2$	40 mg.
Iron citrate solution (appendix 7), 4×10^{-5} M	10 ml.
Sodium fumarate	6.4 g.
Thiamin hydrochloride	1 mg.
Nicotinic acid	5 mg.
Biotin	10 µg.

The pH was adjusted to 6.8 with 10 N - NaOH. The medium was sterilized by autoclaving at 10 lb./sq.in. for 10 min.

APPENDIX 7

Stock Solution of Iron Citrate (4×10^{-3}M)

(Lascelles, 1956a)

$(NH_4)_2$ Fe $(SO_4)_2$, 6 H_2O	157 mg.
Sodium citrate (dihydrate)	236 mg.

The mixture was dissolved in 100 ml. water and stored at 0 - 4°.

APPENDIX 8

Acid - Hydrolysed Casein

(Sneed & Kanneield, 1945)

For 1 litre of solution containing 10% (w/v) of original casein:

100 g. of casein ("alcohol extracted, vitamin free"; Genatosan, Ltd.) was boiled under reflux for 20 - 24 hr. with 800 ml. 5N - HCl. Excess HCl was removed by concentration in vacuo to yield a thick syrup which was then dissolved in 500 ml. H_2O. This concentration was repeated twice, and finally the syrup was dissolved in 800 ml. H_2O. The pH of the solution was adjusted to 2.0 - 2.5 with NaOH (about 20 g.) and 10 g. of absorbent charcoal ("Norite") added. Mixture was boiled for 30 min. and filtered through Whatma No. 50 paper. The pH of the filtrate was adjusted to 6.6 7.0 with NaOH (about 20 g) and the charcoal extraction repeated with a further 10 g. Norite. The filtrate was treated a third time with 10 g. Norite, for 2 hr. at room temperature, and refiltered. This filtrate was made up t 1 litre volume, a few drops of toluene added for preservat and stored at 0 - 4°.

APPENDIX 9

Method for Washing Ether to Remove Peroxides

Stock $FeSO_4$ solution was made up with 60 g. $FeSO_4$, $7H_2O$, 6 ml. conc. H_2SO_4, and 110 ml. H_2O, and kept in a stoppered bottle in the dark.

10 ml. stock $FeSO_4$ solution were diluted to 100 ml. with water and allowed to stand for about 5 min. with 1 - 2 litres ether, with repeated shaking. The ether layer was then washed three times with distilled water and kept in a stoppered brown glass bottle in the dark.

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A B S T R A C T O F T H E S I S

THE BIOSYNTHESIS OF PORPHYRINS BY SPECIES OF ATHIORHODACEAE

Introduction

(1) The biosynthetic reactions leading from the initial substrates for porphyrin biosynthesis, glycine and succinyl coenzyme - A, to the monopyrrole porphobilinogen have been studied in detail in work with animals, plants and photosynthetic bacteria. Less is known about the reactions from porphobilinogen to protoporphyrin. Recent studies with enzyme systems from animals and algae have shown that colourless precursors of porphyrins, resembling the porphyrinogens produced by chemical reduction of porphyrins, are synthesised from porphobilinogen, and that chemically prepared porphyrinogens may be utilised for synthesis of other porphyrins; little is known of the properties of either the chemically prepared porphyrinogens or the biologically synthesised precursors of porphyrins.

(2) The reactions leading from protoporphyrin to chlorophylls have been studied in detail only in mutant strains of Chlorella which excrete magnesium protoporphyrin and other intermediates containing magnesium.

(3) The conditions of incubation necessary for the synthesis of free porphyrins, haem and bacteriochlorophyll have been studied in detail with suspensions of Rhodopseudomonas spheroides, and provided a basis for the present study.

The present investigation.

(1) A photosynthetic bacterium was isolated from mud, using enrichment cultures incubated in the light, and identified as a strain of Rhodopseudomonas capsulatus, a member of the Athiorhodaceae, from its morphological and nutritional properties.

(2) To provide a basis for later work on the biosynthesis of porphyrins, the nutrition of Rps. capsulatus was investigated and compared with that of Rhodopseudomonas palustris; standard growth media were devised for each organism. When ammonium was the sole source of combined nitrogen, there was a delay of 4 days before cultures of Rps. capsulatus incubated in the light in the presence of air started to grow; no such delay was observed with Rps. palustris under similar conditions. This delay was prevented by adding 10^{-6} M methionine or 2×10^{-10} M cobalamin to growth media, or by incubating cultures anaerobically. When high concentrations of acid-hydrolysed casein were incorporated into growth media for Rps. capsulatus, growth yields of up to 8 mg. dry wt./ml. were obtained. The growth of Rps. palustris was sharply inhibited by concentrations of glycine greater than 4×10^{-3} M; no such inhibition was observed when glycine-leucine peptides were used in place of glycine.

(3) Rps. capsulatus, but not Rps. palustris, excreted magnesium protoporphyrin into culture fluids. This compound, previously reported only in work with algae, was identified by its absorption spectrum and that of the protoporphyrin derived from it, and by testing for magnesium.

(4) Both Rps. capsulatus and Rps. palustris formed coproporphyrin and bacteriochlorophyll in cultures incubated in the light; the formation of bacteriochlorophyll was reduced and that of coproporphyrin much increased when iron citrate was withheld from the medium.

(5) The synthesis of porphyrin (mainly coproporphyrin III), magnesium protoporphyrin and bacteriochlorophyll was investigated in suspensions of Rps. capsulatus incubated in the light. Incubation mixtures were devised to support the maximum synthesis of each of these compounds, and the

effect of omitting various components was recorded. The concentrations of added iron citrate supporting optimal synthesis of coproporphyrin, magnesium protoporphyrin and bacteriochlorophyll were, respectively, nil, 2×10^{-5} M and 2×10^{-4} M, suggesting that iron may be required for the conversion of magnesium protoporphyrin, or some related compound, to bacteriochlorophyll, as well as for earlier steps in the biosynthetic pathway. Added DL -methionine increased the synthesis of coproporphyin and, (at a lower concentration), of magnesium protoporphyrin, but not of bacteriochlorophyll. The anionic detergent, Tween 80, was essential in both cultures and suspensions if more than trace amounts of magnesium protoporphyrin were to be formed.

(6) Suspensions of Rps. capsulatus, incubated anaerobically in the light in the medium designed for optimal synthesis of coproporphyrin, gave rise to almost colourless supernatant fluids after centrifuging. However, when such supernatant fluids were allowed to stand in air, the colour changed to orange and then red, and the concentration of coproporphyrin increased 20-fold. When freshly prepared supernatant fluids were acidified, the increase in the content of coproporphyrin was accompanied by the appearance of a strong absorption band at 500 mμ which increased and then decreased in intensity. These changes were accelerated by light, oxidising agents, alkali, or organic solvents, and retarded by reducing agents.

(7) The time course of the changes in optical densities at 548 mμ (due to coproporphyrin) and at 500 mμ was followed when iodine or hydrogen peroxide was added to freshly prepared supernatant fluids from suspensions of Rps. capsulatus. The two oxidising agents produced similar sequences of changes in optical density, and in each case when the optical density at 500 mμ had begun to decrease, the ratio of decrease at 500 mμ to increase at 548 mμ was constant at 2.1; this same ratio was

also observed in acidified supernatant fluids under other conditions. It was concluded that a colourless precursor of coproporphyrin III (designated compound x) was produced in these suspensions of Rps. capsulatus; compound x was spontaneously oxidised to coproporphyrin III via. an intermediate absorbing at 500 mμ in acid solution (compound y). The probable molar extinction coefficient of compound y at 500 mμ was estimated from the above data.

(8) A method was devised for the extraction of compounds x and y from supernatant fluids into ether followed by re-extraction into aqueous alkali. The chemical and spectrometric properties of the extracted preparation were surveyed; three tests for bile pigments were applied with negative results. The spectrum in ether showed peaks at 481 mμ and 372 mμ probably due to compound y, and a further, sharp peak at 437 mμ.

(9) The preparation of compounds x and y was compared with coproporphyrinogen, the colourless tetrapyrrole produced when six hydrogen atoms are added to the coproporphyrin molecule, and prepared in the present work by reduction of coproporphyrin by sodium amalgam. The two preparations were identical in all observations made, including solubility, spectral and chemical properties. Hence compound x is probably coproporphyrinogen III; compound y may be a bilene, that is a tetrapyrrole containing one dipyrryl methene group.

(10) The following conversions were not observed when preparations of the substrates were added to suspensions incubated in the light and synthesis of the products compared with that in controls:

(i) coproporphyrinogen to magnesium protoporphyrin (Rps. capsulatus and Rps. spheroides)

(ii) coproporphyrinogen to bacteriochlorophyll (Rps. capsulatus and Rps. spheroides)

(iii) magnesium protoporphyrin to bacteriochlorophyll (Rps. capsulatus)

(11) ^{14}C - labelled coproporphyrinogen and bacteriopheophytin were prepared by incubating suspensions of Rps. capsulatus with 2 - ^{14}C glycine. The molar specific activity of the pure bacteriopheophytin, prepared by chemical removal of the magnesium atom of bacteriochlorophyll followed by column chromatography, was 7 - 8 times that of the original 2 - ^{14}C glycine. The corresponding ratio for the coproporphyrinogen preparation was 10 - 11, but the purity of this preparation was uncertain.

(12) The radioactivity of bacteriopheophytin synthesised when suspensions of Rps. capsulatus were incubated in the presence of the ^{14}C - labelled coproporphyrinogen was negligible. Pyrrole compounds may be unable to penetrate from the external medium to the sites of porphyrin and bacteriochlorophyll synthesis in this organism.