MECHANISMS AND STEREOCHEMISTRY OF BIOSYNTHESIS OF HAEM PRECURSORS

by

ZAHUR ZAMAN

Department of Physiology and Biochemistry

A Thesis submitted to the University of Southampton for the degree of DOCTOR OF PHILOSOPHY

September 1972
ACKNOWLEDGEMENTS

I should like to express my indebtedness to the Science Research Council for a research grant and Professor K.A. Munday for providing excellent facilities for research.

I also wish to offer profound thanks to Mrs. M. Akhtar for capably translating German scientific papers, Mrs. H. Chesters for competently typing, and Miss M.Y. Geddes for patiently reading through the manuscript.

The technical assistance of Miss O. Balderstone and Mrs. P. Lewis is gratefully acknowledged.

Words are a poor substitute to express my debt of gratitude to Professor M. Akhtar for his valued friendship, inspiration, encouragement and guidance in matters both scientific and temporal.
To the snow-capped mountains of Kashmir -

the guardians of my proud heritage.
Life is short, and the art of 'science' is long; the occasion fleeting, experience deceitful and judgement difficult.

Hippocrates
460?–357? B.C.
ABSTRACT

FACULTY OF SCIENCE

PHYSIOLOGY AND BIOCHEMISTRY

Doctor of Philosophy

MECHANISMS AND STEREOCHEMISTRY OF BIOSYNTHESIS

OF HAEM PRECURSORS

by Zahur Zaman

Incubations of crude and semipurified preparations of 5-aminolaevulinic acid synthetase from Rhodopseudomonas spheroides with $\beta,\delta$-tritiated 5-aminolaevulinic acid revealed the presence of a contaminant activity which catalyses the exchange of hydrogen atom(s) located at the $\delta$-carbon atom of 5-aminolaevulinic acid. This contaminant exchange activity was completely absent in 589-fold purified enzyme preparation which when used to biosynthesise 5-aminolaevulinic acid from stereospecifically tritiated glycines established that the formation of 5-aminolaevulinic acid involves the removal of the R hydrogen atom of glycine prior to the condensation of the latter with succinyl CoA. The use of $\beta,\delta$-tritiated 5-aminolaevulinic acid also indicated that our 13-fold purified L-alanine-4,5-dioxo-valerate transaminase does not catalyse stereospecific exchange of the $\delta$-hydrogen atom of 5-aminolaevulinic acid.

Stereospecifically tritiated samples of 2-oxoglutarate were prepared by an exchange reaction involving NADPH-dependent isocitrate dehydrogenase. Subsequent decarboxylation of the 2-oxoglutarate by hydrogen peroxide gave succinate. The use of these
compounds in the biosynthesis of haem by the haemolysed preparations of phenyl hydrazine-tritiated chick blood established that the vinyl groups of haem are formed through the loss of S hydrogen atoms located at the β-positions of the propionic acid side chains. The hydrogen atoms located at the α-positions of the side chains are not involved in the biosynthesis of haem. A mechanism for the reaction has been proposed.
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The following abbreviations might occur in this thesis:

ADP : adenosine diphosphate
AMP : adenosine monophosphate
ATP : adenosine triphosphate
ALA : 5-aminolaevulinic acid, or δ-aminolaevulinic acid
CoASH : coenzyme A
EDTA : ethylenediamine tetra-acetic acid
FAD : flavin-adenine dinucleotide
GSH : glutathione, reduced
GSSG : glutathione, oxidised
NAD : nicotinamide-adenine dinucleotide
NADH : reduced NAD
NADP : nicotinamide-adenine dinucleotide phosphate
NADPH : reduced NADP
Pi & PPi : orthophosphate and pyrophosphate respectively
tris : 2-amino-2 hydroxymethylpropane-1,3-diol.
CHAPTER 1

INTRODUCTORY REVIEW
Of the numerous pigments in the living matter on the earth, this chapter will deal mainly with the biosynthesis and control of haem, and also give a brief survey of the evidence for a common biosynthetic pathway for haem, chlorophyll and the vitamin B\textsubscript{12} group of compounds called cobalamins. The first indication that haem and chlorophyll are chemically related was obtained in 1880 by Hoppe-Seyler who showed that by drastic treatment of chlorophyll with alkali it could be converted to the red pigment. A tremendous amount of chemical work during the 1920's and 1930's established that haem and chlorophyll were both tetrapyroles. Historically, cobalamins entered the picture much later. However, soon after the isolation of vitamin B\textsubscript{12} in 1948, it was shown to be tetrapyrole. With the establishment of similarity in structure of haem, chlorophyll and cobalamins, it became apparent that a common pathway for the biosynthesis of these compounds might exist.

Our present knowledge of the porphyrin biosynthetic pathway, largely due to the works of Shemin et al and Neuberger et al is summarised in fig. 1.1.

**Biosynthesis of Common Precursors**

In a series of now classic papers, Shemin and his co-workers showed that \textsuperscript{15}N-glycine fed to a human was incorporated into the haem of newly biosynthesised erythrocytes (1, 2). While \textsuperscript{15}N from other amino acids appeared to be incorporated after
Fig. 1

A = -CH₂-COOH
P = -CH₂-C₂H₆-COOH

Cobalt branch?

CH₂-C₂H₆-COOH

PBG

CH₂-C₂H₆-COOH

Uroporphyrinogen - II

CH₂-C₂H₆-COOH

Protoporphyrinogen – II

CH₂-C₂H₆-COOH

Chlorophyll

HAEM

Mg²⁺

Fe²⁺
dilution by the total body nitrogen, $^{15}$N from glycine was shown to be incorporated more directly because there was minimal dilution of the labelled nitrogen. Experiments with [2-$^{14}$C]glycine showed that four carbon atoms at the $\alpha$-position of each pyrrole and four methylene bridge carbon atoms arise from the $\alpha$ carbon atom of glycine (3, 4, 5, 6). Further experiments with $^{14}$CH$_3$COOH and CH$_3$$^{14}$COOH revealed that before condensing with glycine, acetate was first converted via the tricarboxylic acid cycle to an asymmetric four carbon compound which was probably a derivative of succinic acid (7). Shemin et al (8) and Neuberger et al (9) later showed that succinyl CoA was the asymmetric intermediate of haem. From this knowledge, the condensation product of succinyl CoA and glycine was postulated by Shemin and Russell (10) to form $\alpha$-amino-$\beta$-ketoadipic acid. This compound being a $\beta$-keto acid decarboxylates spontaneously to form ALA (11). ALA, when supplied to a duck erythrocyte system and cell-free extracts of Rhodopseudomonas spheroides, was shown to be incorporated into haem more readily than succinate or glycine (10, 11, 12). Thus it was established that ALA is the first direct precursor of porphyrins.

**Biosynthesis of $\delta$-aminolaevulinic acid (ALA)**

The biosynthesis of ALA is catalysed by the enzyme ALA synthetase from glycine and succinyl CoASH (equation 1):

$$\begin{align*}
\text{NH}_2 — \text{CH} — \text{COOH} + \text{HOOC} — \text{CH}_2 — \text{CH}_2 — \text{CoSCoA} & \rightarrow \\
\text{NH}_2 — \text{CH} — \text{CO} — (\text{CH}_2)_2 — \text{COOH} + \text{Co}_2 + \text{CoASH} & \text{ALA} \quad \text{(Eq. 1)}
\end{align*}$$
It has long been known that nutritional deficiency in vitamin B₆ and/or pantothenate lead to anaemia which is caused by drastic decrease in porphyrin biosynthesis. These observations were accounted for when Neuberger, Laver and Udenfriend (13) first demonstrated the net synthesis of ALA from glycine and succinate or α-ketoglutarate by haemolysed preparations of anaemic chicken reticulocytes. The synthesis of ALA was stimulated by the addition of CoASH and pyridoxal phosphate. After freeze-drying the haemolysed reticulocyte preparation, CoASH and succinate could be replaced by the addition of chemically synthesised succinyl CoASH. These results were quickly confirmed by work with avian erythrocytes (14) and cell-free extracts of R. spheroides (15, 16). However, within a cell, the biosynthesis of ALA depends on the integrity of the tricarboxylic acid cycle and the electron transport chain as shown by studies with [1,4-¹⁴C] succinate and [2,3-¹⁴C] succinate (17) and also by inhibition studies with malonate, transaconitate, fluoroacetate (18) anaerobiosis and dinitrophenol (13). In addition to the tricarboxylic acid cycle, there are other enzymic reactions which can yield succinyl CoA. The enzyme succinyl CoA synthetase catalyses succinyl CoA formation from succinate, CoA and ATP (equation 2) (19). It is estimated that this reaction

\[
\text{HOOC} - \text{CH}_2 - \text{CH}_2 - \text{COOH} + \text{CoASH} + \text{ATP} \rightarrow \text{HOOC} - \text{CH}_2 - \text{CH}_2 - \text{COSCoA} + \text{ADP} + \text{Pi}
\]

(Eq. 2)
accounts for 50% of the succinyl CoA for ALA synthesis in chicken erythrocytes. (The heart muscle succinyl CoA synthetase requires GTP or ITP, but not ATP, (20, 21)). Acetoacetyl CoA transferase and methylmalonyl CoA transferase catalyse the transfer of CoA from acetoacetyl CoA and methylmalonyl CoA to succinate in reversible reactions (22).

Owing to greater activity of ALA synthetase in the bacterial extracts than in avian erythrocytes, cell-free extracts of bacteria, particularly R. spheroides, have been used in more detailed studies of the ALA synthetase. Any attempt in the past, to purify the enzyme always met with failure, largely due to the instability of the enzyme. This difficulty has now been overcome and the solution has involved the recognition of two important facts. Firstly the dual role of sulphhydril compounds which on the one hand are necessary for the stabilization of the enzymic protein and on the other hand inhibit the enzyme activity. Secondly, there are natural protein-like inhibitors of the enzyme in the bacterial extracts. Therefore care has to be taken to remove these inhibitors. The significance of the latter will be discussed under the section on control of haem biosynthesis.

The ALA synthetase has been highly purified from R. spheroides (23-26), rabbit reticulocytes (27) and partially purified from soybean callus (28). Studies on the purified enzyme have revealed that it is strongly inhibited by thiol group attacking reagents; e.g. p-chloromercuribenzoate (9, 13, 25); Hg Cl₂ (25); N-ethylmaleimide (27); and heavy metal ions,
e.g. Cu$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$ (25, 27). The inhibition due to both the thiol reagents and the heavy metal ions is easily reversed by β-mercaptoethanol which itself like CoASH, cysteine and GSH is inhibitory (9, 25, 27). The evidence for the apparent stimulatory effect of thiol compounds (24) and EDTA (9, 25), in my opinion, may be explained by the fact that these compounds might be involved in 'effective removal' of the endogenous inhibitory heavy metal ions. Furthermore, there is evidence that the ALA synthetase is inhibited by natural and unnatural haemins. The significance of this will be discussed under the section on control of haem biosynthesis.

While the enzyme protein from R. spheroides has been found to have (i) molecular weight of 60,000-80,000 (23, 24, 25) and (ii) $K_m$ for glycine, succinyl CoA and pyridoxal phosphate of 5-10 mM, 5-25 μM and 6 μM respectively, the rabbit reticulocyte ALA synthetase (27), which is a homogenous protein and differs from the bacterial enzyme in that it retains 60% of its activity in the absence of added pyridoxal phosphate and has molecular weight of 200,000, has $K_m$ for glycine and succinyl CoA of 10 mM, and 60 μM respectively.

The mechanism of action of the ALA synthetase will be given in another chapter.

**Biosynthesis of Porphobilinogen (PBG)**

The enzyme ALA dehydratase catalyses the Knorr-type condensation of two molecules of ALA to form porphobilinogen,
which was first isolated by Westall (29) from the urine of porphyric patients and its structure was established by Rimington and Cookson (30).

The ALA dehydratase is widespread in nature and has been partially purified from R. spheroides (19), ox liver (31, 32), erythrocytes (33, 34), mouse liver and spleen (35), rat Harderian glands (36), yeast (37), wheat leaves (38) and soybean callus (39). The studies of the properties of the enzyme from all these sources show that the enzyme activity is very susceptible to inactivation in the absence of thiol compounds and that it is strongly inhibited by thiol attacking reagents like p-chloromercuribenzoate and iodoacetamide. This inhibition can be reversed or prevented by thiols, e.g. β-mercaptoethanol, cysteine or dithiothreitol. Thus demonstrating that the \(-\text{SH}\) group(s) on the enzyme is essential for the activity.

In 1968, Nahdi and Shemin et al produced a series of excellent papers on ALA dehydratase from R. spheroides (40, 41, 42). Using highly purified (40) enzyme, they showed that the ALA dehydratase is an allosteric enzyme whose positive effectors are \(K^+\) and to a much lesser extent ALA (41), the
enzyme could be inactivated by sodium borohydride in the presence of the substrate, and that if the substrate was labelled with $^{14}$C, the radioactivity could be incorporated into the inactivated enzyme protein. From this it was deduced that a Schiff base is formed between the enzyme and the substrate (42).

In addition to this it was discovered that the ALA dehydratase could not only form Schiff base with laevulinic acid or its ester but also make mixed pyrroles by their condensation with ALA. Formation of such mixed pyrroles could only occur if the $\beta$-methylene carbon atom of laevulinic acid or its ester condensed with the carbonyl group of ALA (see structure I). If the condensation were to occur between the $\beta$-methylene carbon atom of ALA and carbonyl group of laevulinic acid or its ester, no pyrrole ring would result (see II). From this it follows therefore that in PEG, the acetic acid residue-carrying half of the molecule is derived from that ALA molecule which forms the Schiff base with enzyme (side A above).
On the basis of these and their previous findings that a carbonyl group γ to a free carboxylic group is necessary for binding with enzyme (38), Shemin et al proposed the following mechanism for PBG formation (Fig. 1,2).

Recent studies on the structure of the bacterial enzyme by Shemin (43) and the mouse liver enzyme by Doyle (44) showed that the ALA dehydratase from the mouse liver is similar in subunit structure to the R. spheroides’ enzyme. Both enzymes, in the native state, have molecular weight of 240,000-250,000 and can be dissociated with sodium dodesyl sulphate or 6M guanidine chloride into identical polypeptide chains of 39,000-40,000 molecular weight, suggesting that the enzymes are hexamers. The binding of 6 moles of ALA per 250,000 gm of the enzyme protein by Schiff base linkage, like that in the bacterial enzyme, also supports the hexameric structure for the mammalian enzyme. The latter, however, differs from the bacterial enzyme in that it shows no allosteric properties.

The bacterial enzyme on the other hand, is an allosteric protein which has $K^+$ (Li$^+$, Rb$^+$ or NH$_4^+$) ions as its positive effectors (40-43). In the absence of $K^+$ ions the bacterial ALA dehydratase exists in the form of monomeric subunits which associate in the presence of $K^+$ ions, to form hexamers (oligomers), di-, tri-, tetra-, or greater polyoligomers, depending on the concentration of the enzyme protein in the solution.
E-NH₂ + C=O → E-N=C + CH₂

E-N=C + CH₂ → E-N±C

E-N±C + CH₂ → COOH

COOH

E-NH₂ = Enzyme
Biosynthesis of Uroporphyrinogen-III

It has been known since 1953 that PBG is an intermediate in the biosynthesis of porphyrins (45, 46) and a good deal of information is available on the enzyme system - uroporphyrinogen-I synthetase and uroporphyrinogen-III cosynthetase - which catalyses the polymerization of PBG to uroporphyrinogen-III (structure III). The enzyme system has been isolated from wheat germs (47, 48), R. spheroides (49), rabbit and avian erythrocytes (33, 50), mouse spleen (51, 52, 53), cow liver (54) and soybean callus (55). It is generally agreed from all these studies that both uroporphyrinogen-I synthetase and uroporphyrinogen-III cosynthetase require presence of free thiol group(s) for optimal activity and it has also been discovered that the presence or the absence of air does not affect the porphyrin production from PBG (56, 47, 50, 54). However, Batlle et al (55) have demonstrated that soybean callus enzyme system produces little or no uroporphyrinogens under aerobic conditions, suggesting that air oxidises some intermediate(s) of the uroporphyrinogens.

Uroporphyrinogen-I synthetase and uroporphyrinogen-III cosynthetase can be distinguished easily because of the susceptibility of the uroporphyrinogen-III cosynthetase to heat inactivation. The uroporphyrinogen-I synthetase (or PBG deaminase) which is quite heat stable (46) catalyses the removal of the amino group from PBG and head-to-tail condensation of four PBG molecules to form uroporphyrinogen-I (structure IV). The
p = -CH₂·CH₂·COOH
A = -CH₂·COOH
enzyme from spinach leaves and avian erythrocytes is competitively inhibited by opsopyrrole dicarboxylic acid (structure V) and isoporphobilinogen (structure VI) (57). Hydroxylamine and high concentrations of NH$_4^+$ ions are inhibitors of a special class. They do not affect the rate of PBG consumption, but somehow force a temporary accumulation of linear pyrrolic intermediates which are converted to cyclic polypyrroles on prolonged incubations (58). Bogorad has isolated two such intermediates which are (i) a dipyrrylmethane (PA.PA$^+$) of structure VII (59) and (ii) a proposed tetrapyrrylmethane (PA.PA.PA.PA) (60). The latter can be converted apparently only non-enzymically to uroporphyrinogen-I. On the other hand, the dipyrrylmethane (VII) is a substrate for the uroporphyrinogen-I synthetase only in the presence of PBG.

The second enzyme, uroporphyrinogen-III cosynthetase, is heat labile, being rapidly inactivated at 60°. But addition of PBG or NH$_4^+$ ions afford protection against heat inactivation suggesting that -NH$_3^+$ group of PBG binds with the cosynthetase. The latter, in the absence of uroporphyrinogen-I synthetase, does not react with PBG. It has also been shown that the uroporphyrinogen-III cosynthetase alone or in the presence of uroporphyrinogen-I synthetase does not convert uroporphyrinogen-I (47, 61) or Bogorad's pyrrylmethanes (59, 60) to uroporphyrinogen-III. Since it is known that the rate of

Using A as a symbol of acetyl side chain and P for the propionyl side chain, the porphyrins of I series can be described as (AP.AP.AP.AP), whereas those of the III series would be (AP.AP.AP.PA).
enzymic consumption of PBG is increased when uroporphyrinogen-III cosynthetase is added, it was assumed that the substrate for this enzyme is a polypyrrole. This assumption was given credibility by Batlle et al (62, 63), who demonstrated that during the first three hours of incubation of soybean callus uroporphyrinogen-I synthetase - uroporphyrinogen-III cosynthetase system with PBG forms, yet unidentified, polypyrrolic intermediate(s). The latter, alone or in the presence of PBG, could be converted (i) by the uroporphyrinogen-III cosynthetase exclusively to uroporphyrinogen-III, and (ii) by uroporphyrinogen-I synthetase to uroporphyrinogen-III (80%) and uroporphyrinogen-I (20%). Similarly when PBG was incubated with uroporphyrinogen-I synthetase alone, the resulting intermediates could be converted, in the presence of PBG, by uroporphyrinogen-III cosynthetase predominantly to uroporphyrinogen-I and to some extent to uroporphyrinogen-III (30%).

Though the detection of these separate intermediates for the biosynthesis of uroporphyrinogen-I and uroporphyrinogen-III may prove to be a significant step towards discovering the mechanism of uroporphyrinogen-III formation, the conclusions drawn by Batlle et al (55) are completely misleading in view of the finding of Frydman et al (64). These workers have reported that a dipyrrylmethane (PA. AP) of the structure VIII (cf VII) is exclusively converted to uroporphyrinogen-III suggesting that the biosynthesis of uroporphyrinogen-I and uroporphyrinogen-III from porphobilinogen starts along different pathways from the beginning.

Of the existing postulated mechanisms (for the biosynthesis of uroporphyrinogen-III) two seem particularly plausible for not
only have they not yet been experimentally ruled out but also they incorporate the following known facts about the reaction:

(i) opsopyrroclic dicarboxylic acid is neither a cofactor nor a product of the reaction, (65); (ii) free formaldehyde is not involved in the reaction, (50, 66); and (iii) the stoichiometry of PBG to uroporphyrinogen-III reaction is 4:1 (33, 50, 67).

One of these mechanisms, first postulated by Robinson (69) involves migration of aminomethyl (-CH₂.NH₂) group from one position to the other position of the same pyrrole (intramolecular migration) or another pyrrole (intermolecular migration). Whether the migration of the aminomethyl group is intramolecular (a) or intermolecular (b), the resulting intermediate is chemically the same (i.e. PA.AP) as shown below.

This intermediate then condenses in stepwise manner with two more molecules of porphobilinogen to yield uroporphyrinogen-III (see fig.1.3). It is of interest to note here that the migration of aminomethyl group at each condensation step will still yield uroporphyrinogen-III.

The second mechanism for uroporphyrinogen-III biosynthesis was postulated by Mathewson and Corwin (70). One of the most attractive features of this mechanism is that a
Fig. 1.3
Fig. 1.4

Uroporphyrinogen-III

Corrin ring of B$_{12}$
minor modification in it can explain the formation of the co$_{\text{in}}$ ring of cobalamins (vitamin B$_{12}$) without postulating uroporphyrinogen-III as an intermediate (fig. 1.4). The key intermediate of the mechanism is the cyclic tetrapyrrolemethane in which the $\alpha$-position of one of the pyrroles is still free. This cyclic intermediate reopens, and the pyrrole with the free $\alpha$ position rotates such that the reclosure of the ring at the free $\alpha$ position yields uroporphyrinogen-III (or co$_{\text{in}}$ ring of vitamin B$_{12}$). This mechanism is more difficult to prove.

Biosynthesis of Coproporphyrinogen-III

The conversion of uroporphyrinogen-III to coproporphyrinogen-III (structure IX) is catalysed, optimally under anaerobiosis, by the enzyme uroporphyrinogen decarboxylase. The reaction involves decarboxylation of the four acetic acid side chains (49, 71, 72) (see below).
Since the uroporphyrin-III was the first visible condensation product of PBG, it was assumed that the former was an intermediate of haem biosynthesis. It was not until 1955 that Bogorad (73) demonstrated porphyrinogens to be the real intermediates in the biosynthesis of haem. This work has since been supported by the evidence of enzymic decarboxylation of uroporphyrinogen-I and uroporphyrinogen-III by crude preparations of Chlorella (67, 73), R. spheroides (59) and avian erythrocytes (74, 75). The biosynthetic products observed in these works were uroporphyrinogens with 7 carboxylic groups (7-COOH uroporphyrinogen-III), coproporphyrinogen-III and small amounts of 6-COOH- and 5-COOH-uroporphyrinogens. From this it was deduced that the decarboxylation of the acetic acid side chains of uroporphyrinogen-III proceeds in a stepwise manner (see below). More recently Grinstein et al, in addition to confirming this sequence of reaction by biosynthesising haem from extraneously added 7-COOH-, 6-COOH-, and 5-COOH-uroporphyrinogen-III (76, 77), have shown that the first decarboxylation step was much faster than the subsequent decarboxylations (78). This suggested that probably more than one enzyme
was involved in the decarboxylation of uroporphyrinogen-III.

However, since the purified enzyme from the avian erythrocytes (78) is a homogenous protein, it is possible that rather than the involvement of two or more enzymes, the decarboxylation occurs through participation of more than one active site in one enzyme.

The uroporphyrinogen decarboxylase from R. spheroides (49) and avian erythrocytes (79) unlike the enzyme from human erythrocytes (80) seem to have a co-factor requirement. A possible mechanism for decarboxylation of the acetic acid residues is shown below:

The route ab explains the co-factor (e.g. NADH) requirement.

**Biosynthesis of Protoporphyrin-IX**

The conversion of coproporphyrinogen-III to protoporphyrin-IX occurs in two steps; firstly the stepwise decarboxylation (81) of two propionate residues at positions 2 and 4 to give protoporphyrinogen-IX (structure X), and secondly, the oxidative removal of six hydrogen atoms from protoporphyrinogen-IX to yield protoporphyrin-IX (structure XI) as shown below:
Although the possibility of spontaneous oxidation of protoporphyrinogen-IX to protoporphyrin-IX can not as yet be ruled out, the experiments of Sano and Granick (81) indicate that the reaction is enzyme catalysed. On the other hand it is fairly certain that the oxidative decarboxylation of both propionate residues is the property of a single enzyme called coproporphyrinogen oxidase (82, 83). This enzyme, which is associated with mitochondria and can be solubilised therefrom by thioglycollate (81), has been demonstrated in Euglena gracilis, avian erythrocytes (74), a wide variety of mammalian tissues (81, 84), chromatium strain D (85) and 60-fold purified from rat liver mitochondria (82). The enzyme from all these sources was highly
specific for coproporphyrinogen-III, explaining the inexistence of the series I porphyrins in living matter, and had a reaction requirement for iron which accounts for accumulation of coproporphyrin in R. spheroides under iron-deficient conditions.

Apart from a report of anaerobic activity in extracts of a species of Pseudomonas (86), the coproporphyrinogen oxidase activity detected in all other cells, even in photosynthetic anaerobe Chromatium strain D (85), had an absolute requirement for molecular oxygen. However, Tait (83) recently reported the existence of aerobic and anaerobic coproporphyrinogen oxidase activity in R. spheroides and only anaerobic activity in Chromatium strain D (cf (85) above). The aerobic activity from R. spheroides, like that from other cells, had no co-factor requirement, but differed from others in that it was not inhibited by chelating agents such as 1,10-phenanthroline and α,α'-dipyridyl.

On the other hand the anaerobic activity from R. spheroides required ATP, Mg$^{2+}$ and S-adenosylmethionine in addition to Fe$^{2+}$, NADH, NADP and suggested that possibly adenine or flavine (or both) dinucleotides are acting as hydrogen acceptors.

The mechanism of oxidative decarboxylation of propionate residues is discussed in another chapter.

**Biosynthesis of Proto-haem (Haem)**

The final step in the biosynthesis of proto-haem (structure XII) is the insertion of Fe$^{2+}$ into the molecule of protoporphyrin-IX as shown below. Such an incorporation of Fe$^{2+}$ can occur not only enzymically but also non-enzymically under anaerobic and
semi-physiological conditions (i.e. room temperature and neutral pH) provided the aggregation of protoporphyrin-IX is prevented by addition of solubilising agents, e.g. detergents. Sano and Tokunaga (87) have shown that sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) considerably enhances the non-enzymic incorporation of iron, not by acting as a reducing agent (i.e. by preventing Ferrous to ferric change) but by providing an "active iron" compound (probably FeS). It is doubtful whether the non-enzymic proto-haem formation has any physiological significance.

The enzymic reaction is catalysed by the enzyme ferrochelatase which has been demonstrated in rat liver (88-90), pig liver (91, 92), duck erythrocytes (93) and R. spheroides (94) which appears to contain two enzymes, a soluble and an insoluble one. The enzyme from all these sources seems to have certain constant features. Firstly, it is inhibited by aerobiosis but this can be prevented by addition of reducing agents such as GSH, ascorbate or flushing with $\text{N}_2$, suggesting that probably air oxidises...
ferrous ion to ferric form. Secondly, thiol attacking compounds, e.g. p-chloromercuribenzoate inhibit the enzyme indicating that the integrity of thiol groups is essential for activity. Thirdly, the enzyme shows no specificity towards protoporphyrin-IX. In fact the order of enzymic affinity for the porphyrins is deutero-meso-, proto-, 2,4-dibromodeutero-, and haemato-porphyrin (89). Fourthly, iron is not incorporated into porphyrinogens (89, 91). Finally, although nutritional requirements for certain microorganisms suggest that sideramines may be involved as iron carrying agents (95, 96), there is no direct evidence of a co-enzyme requirement for the chelatase reaction. This discrepancy may be explained after studies have been done with highly purified enzyme preparations.

**Demonstration of a Common Pathway**

In order to demonstrate that haem and chlorophyll have a common biosynthetic pathway, Granick (97-101) prepared Chlorella vulgaris mutants by x-irradiation. The different mutants that were unable to biosynthesise chlorophyll accumulated (i) protoporphyrin-IX (97, 100); (ii) Mg-protoporphyrin-IX (98); (iii) Mg-2-vinyl-pheoporphyrin Cl5 (99); and (iv) protoporphyrin-IX monomethyl ester and Mg-protoporphyrin-IX monomethyl ester (100). The accumulation of protoporphyrin-IX and its derivatives in these mutants showed that protoporphyrin-IX is a common precursor for both chlorophyll and haem. However, accumulation of a
compound by a mutant organism is no proof that the compound is a biosynthetic precursor of an end product whose biosynthesis is blocked. An example of this was provided again by the work of Granick (102) who showed that haematoporphyrin (structure \textsuperscript{1-XIII}) which accumulates in a Chlorella mutant is a metabolic artefact (81) whose significance is not understood yet.

\textsuperscript{1-XIII}

The evidence that cobalamins also share a common biosynthetic pathway with haem and chlorophyll has again come from experimental micro-organisms. Using [\textsuperscript{4-}\textsuperscript{14}C]ALA (103) and \textsuperscript{14}C-PBG (104) as substrates it has been shown that vitamin B\textsubscript{12}-biosynthesising Actinomycetes rapidly convert these compounds to cobinamides and that in the latter the pattern of \textsuperscript{14}C distribution from [\textsuperscript{4-}\textsuperscript{14}C]ALA is identical to that in porphyrins (103). More recently Porra (105) has shown that when cell-free extracts of Clostridium tetanomorphum are incubated anaerobically in the presence of ALA, only uroporphyrinogen-III is detected under the incubation conditions. The detection of uroporphyrinogen-III and
not of coproporphyrinogen-III or protoporphyrinogen suggests that the pathway for porphyrin and vitamin $\text{B}_{12}$ diverge after uroporphyrinogen-III but prior to the coproporphyrinogen-III stage.

Two characteristic features of cobalamin compared with porphyrins are (i) that $\delta$-methylene bridge of the macro ring is missing and (ii) that there are supernumerary methyl groups. According to Shemin et al (106) the six methyl groups marked (o) originate from methionine (S-adenosylmethionine is the actual intermediate involved in the reaction). The methyl group marked (e) does not arise from the $\delta$carbon atom of ALA (106a). At present there is no direct evidence available as to when the methyl groups are incorporated during the process of biosynthesis.

Budkin et al (107) actually consider that the macro ring of vitamin $\text{B}_{12}$ is not formed from ALA but from methylated ALA. Their conclusion is based on the following observation on Propioni-
bacterium shermanic. (i) Toxopyridine (XV) does not inhibit vitamin $B_{12}$ formation but does inhibit porphyrin biosynthesis presumably by inhibiting pyridoxal phosphate-dependent ALA synthetase; (ii) addition of ALA stimulated porphyrin production but not vitamin $B_{12}$ synthesis; (iii) iron salts, e.g. FeSO$_4$$\cdot$7H$_2$O, inhibited porphyrin biosynthesis but had no effect on vitamin $B_{12}$ biosynthesis; and (iv) amino pterin, an antagonist of folic acid, suppressed vitamin $B_{12}$ biosynthesis 20-25 times without affecting porphyrin biosynthesis.

Control of tetrapyrrole biosynthesis

The overproduction of intermediates of the tetrapyrrole biosynthetic pathway in conditions such as porphyria is unambiguous evidence of the fact that higher organisms have regulatory mechanism(s) for controlling the supply of these intermediates. In spite of this the control mechanisms have mostly been studied in bacteria because of their faster growth rate and specified growth requirements which can be easily varied. The control of tetrapyrrole biosynthesis has been studied most of all in the photosynthetic bacterium $R$. spheroides. Therefore the major part of this section will be devoted to results from this organism.

It is well established that metabolic pathways are controlled
in general by two mechanisms which are negative feedback and repression. Both types of control mechanisms are affected by an end product which inhibits either the activity (negative feedback inhibition) or the formation at gene level (repression) of an enzyme catalysing an early step leading specifically to the end product. The negative feedback inhibition comes into play very quickly and exerts a fine control on the flow of intermediates, whereas the repression of an enzyme formation is a slow process designed to prevent wasteful synthesis of unnecessary enzymes.

Control by negative feedback inhibition and repression: Evidence for negative feedback inhibition in R. spheroides was first provided by Lascelles (108) when she showed that iron-deficient suspensions of the organism under semi anaerobic conditions accumulated large quantities of coproporphyrin-III at the expense of bacteriochlorophyll and that addition of catalytic amounts of iron could prevent the accumulation of coproporphyrin-III with concomittent restoration of bacteriochlorophyll to normal. The implication of these results, that iron was necessary for synthesis of a compound which controlled an earlier step in the tetrapyrrole biosynthesis by feedback inhibition, was proved correct when it was demonstrated that ALA synthetase is inhibited by haem and haemoproteins, e.g. haemoglobin and myoglobin and other haemoproteins: (actual inhibition of ALA synthetase is produced by chelation of the enzyme with haem iron (109). These results have been confirmed by many workers with the solitary exception of
Neuwirt et al (110) who suggest that at least in rabbit reticulo-
cytes haem exerts negative feedback control by preventing entry
of iron into reticulocytes. These workers observed no inhibitory
effect of exogenous haem on the biosynthesis of haem from glycine
or ALA but it is still possible that haem controls the entry of
iron into rabbit reticulocytes in addition to inhibiting ALA synthetase.

Therefore one mechanism of control over excessive production
of the intermediates of tetrapyrrole synthesis is through feedback
inhibition of ALA synthetase by haem whose formation is, in turn,
controlled by the availability of iron. The fact that iron is also
needed for the enzymic conversion of coproporphyrinogen-III to
protoporphyrin-IX formation explains the accumulation of copro-
porphyrin-III rather than protoporphyrin-IX under iron-deficient
conditions.

A regulatory mechanism involving repression of ALA
synthetase formation was again first shown by Lascelles (97). She
found that addition of as little as 10 µm of haem to cultures
of R. spheroides growing in the presence of glycine and α-keto-
glutarate repressed ALA synthetase formation. These results
have been confirmed and augmented by detailed studies of
Granick (111) on the mechanism of induction of ALA synthetase
by chemicals such as allylisopropyl acetamide and steroids in
primary cultures of embryonic chick liver. The results have
revealed that (a) inducing agent caused an increase in ALA syn-
thetase and that this increase was caused by de novo biosynthesis
of the enzyme; (b) the action of the inducers was reversible, and
(c) the rate of ALA synthetase formation was controlled by haem.
In fact in the presence of haem, the amount of porphyrin produced after 18 hrs was less than half that observed in the controls incubated with the inducer alone. Based on these and other observations, Granick has proposed the following scheme (fig. 1.5) for control of porphyrin biosynthesis. In this scheme haem, acting as co-repressor, binds with the apo-repressor and prevents ALA synthetase formation. In the presence of an inducer, there is competition between haem and the inducer molecule which when bound with the apo-repressor permits ALA synthetase synthesis. It is also proposed that depending on their concentration, the inducer and haem can displace each other from the apo-repressor.

Fig. 1.5: RG is regulatory gene; O, operator and SG, structural gene. -> represents repression and -+ induction.

From the fact that ALA dehydratase is inhibited by haem (40-42), repressed by exogenous haem (108) and is an allosteric
enzyme (40-42), it has been suggested that this enzyme is also involved in the regulation of the porphyrin biosynthesis. However, there is no evidence to support this hypothesis from any source except Neurospora crassa in which ALA dehydratase instead of ALA synthetase has been shown to be the regulatory enzyme of the haem-biosynthetic pathway (112, 113).

Control by environmental factors: It is well known that pigment synthesis in R. spheroides is inversely proportional to the concentration of \( O_2 \) and the intensity of light suggesting that the ultimate controlling mechanism is the same in each case. Another well established fact is that when R. spheroides, adapted to semi-anaerobic photosynthetic growth, is oxygenated there is an immediate decrease in ALA synthetase activity and bacteriochlorophyll biosynthesis. This situation is readily reversed by the restoration of semi-anaerobic conditions (108) (see below).

This phenomenon was erroneously explained by assuming that
ALA synthetase was rapidly destroyed after its repression by high concentrations of oxygen. A very plausible explanation was recently provided by the work of Neuberger et al (114, 115) who showed that (i) at least two heat-stable low molecular weight compounds, an activator and an inhibitor, participate in the control of ALA synthetase of *R. spheroides*; (ii) although activation was dependent on air (or light) it was prevented and (or) stopped by oxygenation, the cessation of ALA synthetase activation being complete 10 min after the start of oxygenation; and (iii) the ALA synthetase inactivated by oxygenation could be activated by extracts from non-oxygenated cells. These results indicate that enzyme activity is controlled by the ratio of the concentrations of the activator and inhibitor and therefore rapid inactivation of ALA synthetase by oxygenation is not due to repression or protein degradation but to modification of the existing enzyme to give low activity. This view has been re-affirmed by Tuboi et al (23, 116, 117) recently in a series of papers on ALA synthetase (118) of *R. spheroides*. These workers have demonstrated that (i) *R. spheroides* contains two types of independently controlled ALA synthetase, Fraction I and Fraction II; (ii) in crude extracts the Fraction I itself exists in two forms, active and inactive and that the latter, when crude, could be converted to the active form by the addition of rat liver mitochondria (117) or disulphide compounds such as GSSG, and cysteine (118) but when 100-fold purified the inactive form required for activation not only disulphide compounds but also
a protein fraction of the cell-free extracts (118).

Cumulatively, the results presented above suffice to emphasize the complexity of control of porphyrin - in particular ALA-biosynthesis which involves (i) repression, feedback inhibition of ALA synthetase, (ii) control of the ratio of the concentration of naturally occurring activator and inhibitor, and (iii) the conversion of Fraction I ALA synthetase from the inactive to the active form (and probably reverse). All these factors are schematically presented below.
CHAPTER 2

MECHANISM OF ACTION OF

ALA SYNTHETASE
INTRODUCTION

It has already been mentioned in Chapter 1 (page 4) that ALA synthetase catalyses the condensation of glycine with succinyl CoA to give ALA (structure 2.1) according to the following equation:

\[
\begin{align*}
\text{(Succinyl CoA)} & \quad \text{COOH} \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CoAS} \quad \text{C} = O & \quad \text{C} = O + \text{CoASH} + \text{CO}_2 \\
+ & \\
\text{COOH-CH}_2 & \quad \text{NH}_2
\end{align*}
\]

Glycine (2.1)

This reaction requires pyridoxal phosphate (structure 2.11) which has been suggested to form glycine-pyridoxalphosphate-enzyme complex. By analogy with other pyridoxal phosphate dependent enzyme reactions (119), at least two broad mechanisms may be considered for the synthesis of ALA.

In mechanism 1 (fig. 2.1) glycine-pyridoxalphosphate-enzyme complex (2.11) first undergoes decarboxylation to furnish the stabilised carbanion (2.11) which then condenses with succinyl CoA to give the ALA-pyridoxalphosphate-enzyme intermediate (2.11). The latter intermediate on hydrolysis yields ALA (2.1). The important aspect of this mechanism is that both \(\alpha\)-hydrogen atoms (i.e. C-2) of glycine are carried through undisturbed into ALA (see hydrogen atom enclosed by triangles and circles in fig. 2.1).

In mechanism 2 (fig. 2.2) an initial deprotonation,
Throughout

\[ \text{R} = \text{CH}_2\text{OPO}_3\text{H}_2 \]

\[ \text{R} = \text{OH} \]

\[ \text{R} = \text{CH}_3 \]

Fig. 2.1. Mechanism 1.
rather than decarboxylation, of glycine-pyridoxal phosphate-
enzyme complex (2. III) gives a stabilised carbanion (2. VI)
which after condensation with succinyl CoA yields \( \alpha \)-amino-\( \beta \)-
keto adipic acid, bound through the Schiff base linkage of pyri-
doxal phosphate, to the enzyme (2. VII). This intermediate can
then be converted to ALA by one of two mechanisms. In
mechanism (2a) decarboxylation of \( \alpha \)-amino-\( \beta \)-keto adipic acid
occurs whilst it is still bound to the enzyme, to give ALA-
pyridoxal phosphate-enzyme complex (2. IX) which on hydrolysis
furnishes ALA. In mechanism 2b, on the other hand, (2. VII)
is hydrolysed to liberate \( \alpha \)-amino-\( \beta \)-keto adipic acid (2. VIII),
which being the unstable \( \beta \)-keto dicarboxylic acid, spontaneously
decarboxylates to yield ALA (11). The significant feature of the
mechanism 2 (in contrast to mechanism 1), whether it operates
via 2a or 2b, is that only one of the original two \( \alpha \)-hydrogen
atoms of glycine is retained in ALA (see circled hydrogen atoms
in fig. 2.2).

Since the \( \delta \)-carbon (C-5) of ALA arises exclusively from
the \( \alpha \)-carbon (C-2) of glycine, to elucidate the mechanism of
action of ALA synthetase, it is therefore necessary to incubate
\( [2RS-^3H_2] \) glycine with the enzyme system and then determine the
extent of tritium radioactivity retained in the \( \delta \)-carbon atom of
the biosynthesised ALA. However, owing to the presence of a
functional carbonyl group at the \( \gamma \)-position (C-4) of ALA, the
hydrogen atoms located at the \( \beta \)- (C-3) and \( \delta \)- (C-5) carbon atoms
of ALA would be expected to equilibrate with the protons of the
Fig. 2.2 Mechanism 2.
medium according to the following enolization reaction:

\[
\begin{align*}
\text{COOH} & \quad \text{COOH} \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CH}_2 & \quad \text{CH}_2 \\
C = O & \quad C = O \\
H - C - H & \quad +H^+ \\
\text{NH}_2 & \quad \text{NH}_2 \\
& \quad -H^+ \\
& \quad \text{H}^+ \\
& \quad \text{H}^+ \\
& \quad \text{H}^+ \\
& \quad \text{H}^+ \\
\end{align*}
\]

Therefore any tritium incorporated into ALA, from [2RS-\(^{3}\)H\(_2\)]glycine, may be lost either (i) soon after the biosynthesis of ALA or (ii) during the isolation of the ALA from the incubation mixture. The first difficulty (i.e. (i)) was overcome by performing the incubation for a short period of time (usually 10 min), while the second problem was circumvented by stabilizing the \(\delta\)-hydrogen atoms of the biosynthesised ALA through reduction of the carbonyl group with sodium borohydride to alcohol (120) (structure 2.X which will be called dihydro ALA).

\[
\begin{align*}
\text{COOH} & \quad \text{COOH} \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CO} & \quad \text{H} - \text{C} - \text{OH} \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{NH}_2 & \quad \text{NH}_2 \\
\end{align*}
\]

In order to measure the radioactivity incorporated into ALA from glycine, the \(\delta\)-carbon atom of ALA was oxidised with sodium periodate to formaldehyde which was then isolated as the
formaldehyde dimedone derivative as illustrated in the sequence of reactions below:

\[
\begin{align*}
\text{HOOC} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CHO} & \xrightarrow{\text{IO}_3^-} \begin{array}{c}
\text{H} \\
\text{C} = \text{O} \\
\text{H}
\end{array} \\
\text{COOH} & \xrightarrow{\beta \text{CH}_2} \begin{array}{c}
\text{H} \\
\text{C} = \text{O} \\
\text{H}
\end{array}
\end{align*}
\]

\[
\begin{align*}
\text{COOH} & \xrightarrow{\alpha \text{CH}_2} \\
\text{HOOC} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CHO} & \xrightarrow{\text{IO}_4^-} \begin{array}{c}
\text{H} \\
\text{C} = \text{O} \\
\text{H}
\end{array}
\end{align*}
\]

It was deduced from P.M. Jordan's work (120) that all the tritium radioactivity located at the δ-carbon atom of ALA is retained in the formaldehyde dimedone derivative. (This feature is illustrated by encircling the original hydrogen atoms in the above reactions). In addition to this, the study of P.M. Jordan and M. Akhtar (121) using cell-free extracts of R. spheroides (crude enzyme), suggested that during the conversion of glycine and succinyl CoA to ALA, one of the α-hydrogen atoms of glycine is stereospecifically removed, thus indicating that a mechanism of the type 2 (fig. 2.2, p 40) is involved in this process. However, this work is open to a very serious criticism which is that it does not consider the possibility that the loss of the hydrogen atom under consideration occurred not during the biosynthesis of ALA but in a subsequent event in which another contaminant enzyme, present in the crude
ALA synthetase preparation, participated in stereospecific exchange of one of the δ-hydrogen atoms of the biosynthesised ALA with the medium as shown below.

\[
\begin{align*}
\text{H} & \quad \text{C} \quad \text{COOH} \\
\text{NH}_2 & \quad \rightarrow \quad \text{NH}_2 & \quad \text{C} \quad (\text{CH}_2)_2 & \quad \text{COOH} \\
\text{O} & \quad + \quad \text{C} \quad (\text{CH}_2)_2 & \quad \text{COOH} \\
\text{CoAS} & \quad \text{Enzyme 'X'} \quad \rightarrow \quad \text{NH}_2 & \quad \text{C} \quad (\text{CH}_2)_2 & \quad \text{C} \quad \text{OH} \\
& \quad + \quad \text{H}^+ \\
\end{align*}
\]

Such a possibility was exhaustively investigated by studying the biosynthesis of ALA from \([2RS-^3H_2]\)glycine in parallel with the study of the exchange of δ-tritium atoms of \([\beta,\delta-^3H_4]\)ALA by crude-, partially purified-, and highly purified-ALA synthetase.

Results

The Exchange of tritium located at the δ-carbon atom of \([\beta,\delta-^3H_4]\)ALA by the cell-free extracts of \(R. \ spheroides\).

In order to establish first of all whether or not there was an exchange of δ-hydrogen atom(s) of ALA with the protons of the medium under the incubation conditions employed by Jordan et al (121), 80 µg of neutral \([\beta,\delta-^3H_4]\)ALA (13 x 10^6 cpm/mg, 58% of \(^3\text{H}\) was located at the δ-carbon atom) was incubated with the
cell-free extracts (crude enzyme) in the presence of the complete ALA synthetase system at 37°C. The control experiments contained either boiled extracts or none at all. Aliquots were removed from the incubation mixtures at different time intervals and ALA was degraded to obtain formaldehyde dimedone derivatives. The results, displayed in table 2.1 and fig. 2.3, show that there is significant time-dependent tritium loss from the δ-carbon atom of [β,δ-3H₄]ALA suggesting that the basic premise of the present study, that a contaminant enzyme may be involved in the removal of one of the original α-hydrogen atoms of glycine, was feasible. These results also underlined the need for further purification of ALA synthetase.

Biosynthetic and exchange studies using 33-fold purified ALA synthetase.

Having established that the δ-hydrogen atom(s) of ALA is enzymically labilized, it was necessary to purify the ALA synthetase and determine whether this exchange activity had any effect on the net loss of the α-hydrogen atom during the conversion of [2RS-3H₂]glycine into ALA. Therefore a series of experiments on the biosynthesis of ALA from [2RS-3H₂: 2-¹⁴C]glycine (¹⁴C = 4 x 10⁶ cpm) (table 2.2) were carried out in parallel with the study of the exchange of the tritium located at the δ-carbon atom of [β,δ-3H₄]ALA (table 2.3 and fig. 2.4).

The results of the biosynthetic experiments, particularly that of experiment 4 (table 2.2) whose chemical composition was identical to that of the exchange experiments in table 2.3, showed
The exchange of tritium located at the \( \delta \)-carbon atom of \( [\beta,\delta-^3\text{H}_4] \text{ALA} \) by the cell-free extracts of \( \text{R. spheroides} \).

Each flask contained 80 \( \mu \)g of neutral \( [\beta,\delta-^3\text{H}_4] \text{ALA} \) (13 x 10^6 cpm/mg, 58\% \( ^3\text{H} \) was located at the \( \delta \)-carbon atom); glycine, 150 \( \mu \)moles; sodium succinate, 150 \( \mu \)moles; \( \text{MgSO}_4 \), 10 \( \mu \)moles; \( \text{MnSO}_4 \), 0.025 \( \mu \)moles; pyridoxal phosphate, 0.2 \( \mu \)moles; \( \beta \)-mercaptoethanol 1 \( \mu \)mole; co-enzyme A, 1.3 \( \mu \)mole; ATP, 7.5 \( \mu \)moles; tris-HCl buffer (pH 7.5) 75 \( \mu \)moles; and enzyme, 0.41 units (i.e. cell-free extracts) in a total volume of 1.5 ml. Control experiments contained the cell-free extracts which had been boiled for 15 min. 0.3 ml aliquots were removed at different time intervals and ALA was degraded to isolate the formaldehyde dimedone derivative (FDD) as described in Methods.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/10 mg FDD</td>
<td>% ( ^3\text{H} ) loss</td>
</tr>
<tr>
<td>0</td>
<td>1,400</td>
<td>0.0</td>
</tr>
<tr>
<td>20</td>
<td>1,390</td>
<td>0.71</td>
</tr>
<tr>
<td>40</td>
<td>1,400</td>
<td>0.0</td>
</tr>
<tr>
<td>60</td>
<td>1,430</td>
<td>-2.1</td>
</tr>
<tr>
<td>90</td>
<td>1,400</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Fig. 2.3

Exchange of δ-tritium atoms of [δδ, δ-^3H]ALA by crude ALA synthetase.
Table 2.2

The biosynthesis of ALA from [2RS-\(^3\)H\(_2\); 2-\(^{14}\)C]glycine

by 33-fold purified ALA synthetase.

The incubation mixture contained [2RS-\(^3\)H\(_2\); 2-\(^{14}\)C]glycine (\(^3\)H: \(^{14}\)C ratio of 4.02, \(^{14}\)C = 4 \times 10^6 cpm), 56 \(\mu\)moles; MgSO\(_4\), 10 \(\mu\)moles; MnSO\(_4\), 0.025 \(\mu\)moles; pyridoxal phosphate, 0.2 \(\mu\)moles; \(\beta\)-mercaptoethanol, 1 \(\mu\)mole; synthesised succinyl CoA, 2 \(\mu\)moles; tris-HCl buffer (pH 7.5), 75 \(\mu\)moles and 33-fold purified ALA synthetase (7.9 units) in a total volume of 1.5 ml. The incubations were carried out at 37° for 10 min and terminated by the addition of 3 mg of non-radioactive ALA and 6 mg of sodium borohydride at 0°. The samples were then processed, as described in Methods, to make the formaldehyde dimedone derivative (FDD).

<table>
<thead>
<tr>
<th>No.</th>
<th>Incubation conditions</th>
<th>(^3)H: (^{14})C ratio of glycine</th>
<th>(^3)H: (^{14})C ratio of FDD</th>
<th>% retention of (^3)H</th>
<th>Found</th>
<th>Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>complete system</td>
<td>4.02</td>
<td>1.53</td>
<td></td>
<td>38</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>No succinyl CoA</td>
<td>4.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Boiled enzyme</td>
<td>4.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>complete system + 50 (\mu)g ALA</td>
<td>4.02</td>
<td>1.53</td>
<td></td>
<td>38</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 2.3
The exchange of $\delta$-tritium atoms of $[\beta,\delta-^3H_4]ALA$
by 33-fold purified ALA synthetase.

The incubation mixtures contained $[\beta,\delta-^3H_4]ALA$
($13 \times 10^6$ cpm/μg; 58% of the $^3H$ was located at $\delta$-carbon atom),
50 μg; glycine, 56 μmoles; MgSO$_4$, 10 μmoles; MnSO$_3$,
0.025 μmole; pyridoxal phosphate, 0.2 μmole; β-mercapto-
ethanol, 1 μmole; synthesised succinyl CoA, 2 μmoles; tris-
HCl buffer (pH 7.5), 75 μmoles and 33-fold purified ALA
synthetase (7.9 units) in a total volume of 1.5 ml. The control
incubations contained the enzyme that had been boiled for 15 min.
The incubations were carried out at 37°C. Aliquots were re-
moved at different time intervals and ALA was degraded to
obtain formaldehyde dimedone derivative (FDD).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/10 mg FDD</td>
<td>$^3H$ loss</td>
</tr>
<tr>
<td>0</td>
<td>1,000</td>
<td>0.0</td>
</tr>
<tr>
<td>10</td>
<td>998</td>
<td>0.2</td>
</tr>
<tr>
<td>15</td>
<td>990</td>
<td>1.0</td>
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<td>1,010</td>
<td>-1.0</td>
</tr>
<tr>
<td>30</td>
<td>997</td>
<td>0.3</td>
</tr>
</tbody>
</table>
ATP synthetase.

Exchange of $^3$H atoms of $p^3H$-ATP by 33-fold purified $\text{ATPase}$.
that only 38% (compared with the predicted value of 50%) of the original glycine tritium radioactivity was retained in the biosynthesised ALA, whilst under identical conditions 12% of the δ-tritium radioactivity from $[\beta,\delta-\text{H}_2]\text{ALA}$ had exchanged with the medium. Since there was no chemical equilibration of the δ-hydrogen atoms of ALA under the biosynthetic incubation conditions, these results were interpreted as being indicative of the fact that after its biosynthesis from $[2RS-\text{H}_2]\text{glycine}$, ALA enzymically loses (in a period of 15 min) 12% of its tritium radioactivity thus reducing 50% retention of the original glycine tritium, as predicted by the mechanism 2 (p.40), to 38%.

In this experiment the total incorporation of radioactivity from $[2-^{14}\text{C}]\text{glycine}$ into ALA isolated as the formaldehyde dimedone derivative of the δ-carbon atom of ALA was 38,400 cpm, representing a radiochemical conversion of 1%. In a similar non-radioactive incubation carried out simultaneously on colorimetric assay indicated 2.3% conversion of glycine into ALA.

**Biosynthetic and exchange studies using 589-fold purified ALA synthetase.**

The results shown above demonstrated that 33-fold purified ALA synthetase still contained the contaminant exchange activity. Therefore the enzyme was further purified to obtain 589-fold purified preparation which was used to carry out a series of parallel biosynthetic and exchange studies (26).

$[2RS-\text{H}_2; 2-^{14}\text{C}]\text{glycine}$ of $^{3}\text{H}/^{14}\text{C}$ ratio of 4.3 ($^{14}\text{C}=4 \times 10^6$ cpm) was incubated with 589-fold purified ALA synthetase and the
δ-carbon atom of the biosynthesised ALA was isolated as the formaldehyde dimedone derivative. The results (displayed in table 2.4) showed that an average of 50% of the original glycine tritium radioactivity was retained in the biosynthesised ALA. Under identical conditions (particularly attention is drawn to experiment 2 in table 2.4) when [β,δ-H\textsubscript{3}]ALA was incubated with this highly purified enzyme, no exchange of the tritium located at the δ-carbon atom of [β,δ-H\textsubscript{3}]ALA was observed (table 2.5) suggesting that the contaminant enzyme had been removed during the purification of ALA synthetase.

Cumulatively, the results considered above establish conclusively that during the biosynthesis of ALA one of the α-hydrogen atoms of glycine is eliminated as predicted by the mechanism 2 (fig. 2.2, p. 40) and that a contaminant enzyme, in the crude preparations of ALA synthetase, can exchange δ-hydrogen atom(s) of ALA with the medium.

The total incorporation of radioactivity into ALA from [2-\textsuperscript{14}C]-glycine isolated as dimedone derivative of the δ-carbon atom of ALA was 92,000 cpm. This represented a radiochemical conversion of 2.3%. Under parallel conditions non-radioactive incubation on colorimetric assay indicated 3.6% conversion of glycine into ALA.

The stereochemistry of proton elimination from glycine during the enzymic synthesis of ALA.

In order to determine which of the two α-hydrogen atoms
Table 2.4

The biosynthesis of ALA from $[2RS-^3H_2;2-^{14}C]$glycine

by 589-fold purified ALA synthetase.

The incubation conditions were identical to those of table 2.2 (p.47) except that $[2RS-^3H_2;2-^{14}C]$glycine had $^{3}H/^{14}C$ ratio of 4.30 ($^{14}C = 4 \times 10^6$ cpm) and 8.2 units of enzyme were used.

<table>
<thead>
<tr>
<th>No.</th>
<th>Incubation conditions</th>
<th>$^{3}H:^{14}C$ ratio of glycine</th>
<th>$^{3}H:^{14}C$ ratio of FDD</th>
<th>% retention of $^{3}H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>complete system</td>
<td>4.30</td>
<td>2.07</td>
<td>52.00</td>
</tr>
<tr>
<td>2</td>
<td>complete system + 50 μg ALA</td>
<td>4.30</td>
<td>2.28</td>
<td>47.00</td>
</tr>
</tbody>
</table>

† In order to ensure that the biosynthesis of labelled ALA was carried out under conditions identical to those used for assessing the extent of the exchange reaction, non-radioactive ALA was included.
Table 2.5

The exchange of $\delta$-tritium atoms of $[^{\beta,\delta-3}\text{H}_4]ALA$ by 589-fold purified ALA synthetase.

The incubation conditions were the same as those described in table 2.3 (p.48) except that 50 $\mu$g of doubly labelled ALA (i.e. $[^{\beta,\delta-3}\text{H}_4;5-^{14}\text{C}]ALA$) with $^{3}\text{H}:^{14}\text{C}$ ratio of 23.8 ($^{14}\text{C} = 2.5 \times 10^6$ cpm) and 8.2 units of 589-fold purified ALA synthetase were used.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{3}\text{H}:^{14}\text{C}$ ratio of ALA</td>
<td>$^{3}\text{H}$ loss</td>
</tr>
<tr>
<td>0</td>
<td>23.80</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>23.82</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>23.80</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>23.80</td>
<td>-</td>
</tr>
</tbody>
</table>
of glycine was eliminated during the biosynthesis of ALA, [2R-\(^3\)H]-glycine and [2S-\(^3\)H]glycine (these were prepared by P.M. Jordan) were incubated with 589-fold purified ALA synthetase. The δ-carbon atom of the biosynthetic ALA was recovered as the formaldehyde dimeredone derivative. The results, displayed in table 2.6, showed that ALA synthesised from [2S-\(^3\)H]glycine contained 100% of the original glycine tritium activity whereas that synthesised from [2R-\(^3\)H]glycine retained only 3.0% of the tritium radioactivity.

These results establish that the biosynthesis of ALA is accomplished by means of the mechanism 2 which was favoured (fig. 2.2, p.40) by previous workers. In this mechanism, glycine bound to pyridoxal phosphate through a Schiff base linkage (structure 2.III) loses the R-hydrogen atom, as a proton, to furnish the stabilised carbanion (2.VI). The condensation of succinyl CoA with this carbanion yields the \(\alpha\)-amino-\(\beta\)-keto adipic acid-pyridoxal phosphate schiff base enzyme complex (2.VII).

**Discussion.**

Previous studies with the crude ALA synthetase by Jordan et al (121) had suggested that in the biosynthesis of ALA, the \(\alpha\)-hydrogen atom of glycine with R configuration was lost. Since in this study the workers had not investigated the magnitude of equilibration of the δ-hydrogen atom of ALA under the incubation condition, another explanation for their results was left open. This explanation involved the possibility of stereospecific loss of one of the δ-hydrogen atoms of ALA by a contaminant enzyme after, rather than during, its biosynthesis as illustrated
The stereochemistry of proton elimination from glycine during the enzymic synthesis of ALA.

The incubations were carried out as described in table 2.2 (p. 12) except that [2R-3H]glycine and [2S-3H]glycine indicated below, contained 1 x 10^6 cpm and 1.5 x 10^6 cpm of 14C respectively and 8.2 units of 589-fold purified ALA synthetase were used.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>3H:14C ratio of initial glycine</th>
<th>3H:14C ratio of FDD</th>
<th>% 3H retained in ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2R-3H:2-14C] glycine</td>
<td>1.55</td>
<td>0.04</td>
<td>3.00</td>
</tr>
<tr>
<td>[2S-3H:2-14C] glycine</td>
<td>5.80</td>
<td>5.80</td>
<td>100.00</td>
</tr>
</tbody>
</table>
The investigation of this possibility was permitted by the availability of $[\beta,\delta - ^3\text{H}_4]\text{ALA}$. The incubation of the latter with the crude ALA synthetase under the conditions identical to those of Jordan et al (121) revealed that during 60 min of incubation, there was a linear exchange of the $\delta$-hydrogen atom of ALA with the protons of the medium (fig. 2.3, table 2.1). Although these results did not necessarily invalidate the conclusion of Jordan et al (121), they emphasised the need for a new approach to the problem using more purified enzyme.

Therefore a new series of biosynthetic experiments, in parallel with the exchange studies, was performed using ALA synthetase at different stages of purification, to determine on the one hand the loss of hydrogen atom in conversion of $[2\text{RS-}^3\text{H}_2]\text{glycine}$ into ALA and on the other hand, to estimate the exchange of $\delta$-hydrogen atom of ALA with the medium protons. 33-fold purification of ALA synthetase failed to remove all the contaminant exchange activity as shown by the approximately linear loss of $\delta$-tritium from $[\beta,\delta - ^3\text{H}_4]\text{ALA}$ during the first 20 min of incubation (fig. 2.4). In view of the fact that there is no chemical exchange of the $\delta$-hydrogen atoms of ALA (tables 2.1 and 2.3) under the incubation conditions, it was assumed that the retention of 38%, instead of 50%, of the original glycine tritium activity was due to the exchange of the $\delta$-tritium atom(s) of ALA after its biosynthesis from $[2\text{RS-}^3\text{H}_2]\text{glycine}$. This assumption was born out by the results obtained from experiments performed
with 589-fold purified enzyme when it was observed that one α-hydrogen atom of glycine is removed during the biosynthesis of ALA (table 2.4) under the conditions when there was no exchange of δ-hydrogen atoms of ALA with the medium (table 2.5).

The results of the study with 589-fold purified ALA synthetase are very remarkable not only in endorsing the conclusions of Jordan et al (121) but also for the close agreement between the theoretically predicted loss of hydrogen and the value experimentally determined in the biosynthetic experiments (table 2.4).

Another reward of using the highly purified enzyme was an impressive improvement over the stereochemical results of Akhtar et al (26). When the biosynthetic experiments were carried out using this preparation and stereospecifically tritiated glycine, it was observed that [2R-\(^3\)H]glycine lost 97% of tritium and [2S-\(^3\)H]glycine retained 98% of its tritium during the biosynthesis of ALA (table 2.6).

Thus the present work showing the loss of the α-hydrogen atom with the R configuration establishes conclusively the mechanism 2 (p40) for the biosynthesis of ALA. This mechanism involves deprotonation of glycine-pyridoxal phosphate-enzyme complex (2.III) to give a stabilised carbanion (2.VI) which after condensation with succinyl CoA yields α-amino-β-ketoadipic acid, bound through the schiff base linkage of pyridoxal phosphate, to the enzyme (2.VII). The latter intermediate may then produce ALA either via (2.VII)\(\rightarrow\) (2.VIII)\(\rightarrow\) ALA, or through the sequence (2.VII)\(\rightarrow\) (2.IX)\(\rightarrow\) ALA. This feature will be discussed in the next chapter.
It seems that the alternative mechanism of the type 1 (p38) is also operative in the natural systems, as shown by Weiss (122) in the formation of 3-ketosphinganine (an intermediate in the biosynthesis of sphingolipid bases) from pamitoyl CoA and serine. He found that when [2, 3-\textsuperscript{3}H\textsubscript{3}]serine was injected into rats during active myelination, all the tritium located at the C-2 was retained in 3-ketosphinganine. Therefore it was proposed that in this particular case the serine-pyridoxal phosphate-enzyme complex undergoes decarboxylation, rather than deprotonation, before condensing with pamitoyl CoA to form a new C-C bond, as illustrated in the sequence below:

\[
\begin{align*}
\text{HO-CH}_2-\text{COOH} + \text{NH}_2 \rightarrow \text{HO-CH}_2-\text{COOH} - \text{CO}_2 \\
\end{align*}
\]

3-ketosphinganine.
Another interesting stereochemical diversity to note is that the biosynthesis of ALA from glycine involves the removal of the R-hydrogen atom whereas the biosynthesis of serine and threonine occurs through the loss of the S-hydrogen atom of glycine.
Experimental.

Materials.

$\left[2-^3H_2\right]$glycine, $\left[2-^{14}C\right]$glycine and tritiated water were obtained from the Radiochemical Centre, Amersham, U.K. $\left[5-^{14}C\right]$ALA HCl was purchased from NEN Chemicals, Germany. Non-radioactive ALA, pyridoxal phosphate and ATP were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Co-enzyme A and Trizmabase (tris) were purchased from Sigma (London) Chemical Co.Ltd., London S.W.6., U.K. Sephadex G-100 and DEAE-Sephadex A-25 were purchased from Pharmacia Co., London W.5., U.K. The rest of the chemicals and solvents were obtained from the British Drug Houses Ltd., Poole, Dorset, U.K. Rhodopseudomonas spheroides (N.C.I.B. 8253) was obtained from Torrey Research Station, Aberdeen, Scotland.

$\left[2-^3H\right]$- and $\left[25-^3H\right]$glycines and 589-fold purified ALA synthetase were prepared by P.M. Jordan.

Methods.

Growth and harvesting of Rhodopseudomonas spheroides.

The organism (N.C.I.B.8253) was maintained in stab cultures in medium containing yeast extract (0.2% w/v) and agar (1.5% w/v). These cultures were subcultured monthly and incubated for 48 h in the light at 32-33°; the stab cultures were stored at 0-4°.

Cells for experimental work were grown in Medium MS of Lascelles (123). The medium contained per litre final volume:
monohydrate sodium L-glutamate, 1.8 g; DL-malic acid, 2.7 g; potassium dihydrogen orthophosphate, 500 mg; dipotassium hydrogen orthophosphate, 500 mg; di-ammonium hydrogen orthophosphate, 800 mg; MgSO\(_4\)\(_7\)H\(_2\)O, 400 mg; CaCl\(_2\) 6H\(_2\)O, 79 mg; MnSO\(_4\)\(_4\)H\(_2\)O, 1.5 mg; thiamine hydrochloride, 1 mg; nicotinic acid, 1 mg; D-biotin, 0.05 mg. The pH was adjusted to 6.8 with about 40 ml of 1 M NaOH. The medium was sterilized by autoclaving for 15 min at about 15 lb/sq.in.

100 ml of the MS medium, supplemented with a yeast extract (0.2% w/v) contained in a 250 ml flask plugged with cotton wool, was inoculated from the stab cultures and the organism was incubated in light (from two 60W bulbs) at 30-33° for 48 hrs. The red-brown suspension was poured into a 5 l. flask containing 4.5 l. of the medium MS and incubation was continued for 72 hrs at 30-33° under illumination from two 60W bulbs. The cultured cells were harvested by centrifugation, and washed with 0.02 M phosphate buffer (pH 7.0). The packed cells (about 8 g wet weight/l.) were stored at -16 to -18° until they were needed for the enzyme preparation.

Purification of ALA synthetase: All operations were carried out at 0-4° unless otherwise stated.

Crude enzyme: 2-4 g wet weight of Rhodopseudomonas spheroides cells were suspended in 10 ml of 0.04 M potassium phosphate buffer (pH 7.0) and disrupted at 0° for 10-15 min, using MSE ultrasonicator. The disrupted cells were centrifuged at 105,000g for 90 min. The clear supernatant from the top was used in
experimental work within 4 hrs after centrifugation. The specific activity of the enzyme was always between 0.04-0.06 units/mg. 

33-fold purified enzyme (23): About 200 g wet weight of Rhodopseudomonas spheroides cells were suspended in 0.05 M Tris-HCl buffer (pH 7.4) to give a final volume of 75 ml. The suspended cells were disrupted at 0° for 25-30 min by means of an MSE ultrasonicator. The broken cells were centrifuged at 100,000 g for 90 min and the clear supernatant was collected and diluted to 100 ml (cell-free extracts).

To the cell-free extract (from above) was added β-mercaptoethanol to 0.01 M which was followed by gradual addition (over 20-25 min period) of finely ground solid (NH₄)₂SO₄ up to 40% saturation (22.6 g). The solution was stirred for 30 min, and then centrifuged. The precipitate obtained was dissolved in 10 ml of 0.05 M Tris-HCl buffer (pH 7.4) containing 0.01 M β-mercaptoethanol and against 2.5 l. of 0.05 M potassium phosphate buffer (pH 7.4) containing 0.01 M mercaptoethanol (ammonium sulphate fraction).

The dialysed ammonium sulphate fraction was applied onto a column of sephadex G-150 (2.5 x 36 cm) which had been equilibrated with 0.05 M potassium phosphate buffer (pH 7.4) containing 0.005 M β-mercaptoethanol. Elution was carried out with the same buffer and 5 ml fractions were collected. All the enzyme activity was confined between fraction nos. 48-57. The During sonication the temperature was maintained at 0° by keeping the sonication vessel immersed in ice-potassium chloride mixture.
contents of the tubes containing these fractions were combined and concentrated to 5 ml by dialysing against 30% solution of polyethylene glycol (m. wt 6,000) (G-150 fraction) in the same buffer.

### Purification of ALA Synthetase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Sp. Activity (units/mg)</th>
<th>Total Units</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>100</td>
<td>1,480</td>
<td>0.056</td>
<td>82.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Fraction before dialysis</td>
<td>20</td>
<td>550</td>
<td>0.34</td>
<td>182.0</td>
<td>6.0</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Fraction after dialysis</td>
<td>20</td>
<td>540</td>
<td>0.57</td>
<td>308.0</td>
<td>10.4</td>
</tr>
<tr>
<td>G-150 Fraction</td>
<td>5</td>
<td>42</td>
<td>1.86</td>
<td>78.0</td>
<td>33.0</td>
</tr>
</tbody>
</table>

Unit of the enzyme activity: This is defined as 1 μmole of ALA formed per hour at 37°.

The enzyme assay: The assay mixture contained glycine, (50 μmoles); pyridoxal phosphate, (0.1 μmoles); potassium phosphate buffer (pH 6.9), (50 μmoles); EDTA, (3 μmoles); β-mercaptoethanol, (3 μmoles); bovine serum albumin (5%); enzyme (0.1 ml) and succinyl CoA (2 μmoles) in a total volume of 0.375 ml. After incubation for 10 min, the reaction was stopped by addition of
0.2 ml of 20% trichloroacetic acid. The sample was centrifuged and 0.5 ml of the clear supernatant assayed as follows: 0.5 ml supernatant from above was mixed in a test tube with 0.2 ml acetylacetone and 9.3 ml of 1 M acetate buffer (pH 4.6). The tube was then heated in a boiling water bath for 10 min. After cooling, 2 ml of this solution were added to 2 ml of modified Ehrlich reagent and the optical density was measured at 553 after 15 min.

Preparation of succinyl CoA: Succinyl CoA was prepared by stirring succinic anhydride (1 mg), sodium bicarbonate (4.5 mg) and co-enzyme A (8 mg) in 1 ml of water for 30 min at 0°.

The enzymic synthesis of ALA: The incubation mixtures contained tris-HCl buffer (pH 7.5), 75 μmoles; glycine, 56 μmoles; MgSO₄·7H₂O, 10 μmoles; MnSO₄·4H₂O, 0.025 μmoles; pyridoxal phosphate, 0.2 μmoles; β-mercaptoethanol, 2 μmoles; synthesised succinyl CoA, 2 μmoles and ALA synthetase (0.41 units of crude, 7.9 units of 33-fold purified or 8.2 units of 589-fold purified) in a total volume of 2.0 mls. The incubations were started by the addition of succinyl CoA and carried out at 37° for 15 min. The control incubations contained either boiled enzyme or no succinyl CoA. Aliquots of the incubation mixture were taken to assay the biosynthesised ALA, while the rest was treated to obtain the formaldehyde dimedone derivative of the δ-carbon atom of the ALA as described below.

Isolation of δ-carbon atom of biosynthetic ALA as formaldehyde dimedone derivative:

Incubation mixtures or aliquots therefrom were transferred to a 50 ml flask containing 3 mg of non-radioactive ALA and
6 mg of sodium borohydride and the reaction was allowed to proceed in ice for 30 min. The contents of the flask were then transferred to a centrifuge tube and the flask was washed with 2-3 ml methanol. The washing was added to the centrifuge tube. After centrifugation, the supernatant was evaporated to a small volume below 40°. The sample was then spotted on a silica gel thick-layer plate which was developed in methanol-acetone-ammonia (0.88) (100:50:15 v/v) to separate glycine from δ-amino dihydrolevulinic acid. After drying thoroughly in a current of air, a small strip of the plate was sprayed with 0.5% ninhydrin in acetone (w/v). The bands corresponding to glycine ($R_f$ 0.27) and δ-amino dihydrolevulinic acid ($R_f$ 0.59) were scraped and thoroughly eluted with 10% NH$_4$OH and methanol respectively. The methanol solution of δ-amino dihydrolevulinic acid was evaporated to a small volume below 40° and rechromatographed and eluted with methanol. The methanol solution was evaporated to dryness and treated with saturated sodium bicarbonate (10 ml), sodium metaperiodate (1 ml from 100 mg/ml solution) and formaldehyde (10 mg, 0.025 ml from 40% solution of formaldehyde) and the flask was left in complete darkness for 8 hrs. After this time the pH of the contents of the flask was adjusted to about 6 by dropwise addition of 50% acetic acid (v/v), (if after 8 hrs the flask had a precipitate, the contents were filtered before adjusting the pH), and then 10 ml of dimedone reagent were added. The precipitation of the formaldehyde dimedone which appeared immediately was allowed to proceed for 1 hr in ice.
The precipitate was filtered, washed with 100 ml of chilled water and crystallised slowly from hot methanol. The crystals were washed with 100 ml of chilled water and dried at 100° (m.pt. 182).

**Dimedone reagent:** The reagent was made by dissolving, on a steam bath, 0.71 g NaH$_2$PO$_4$$ \cdot$2H$_2$O, 0.93 g Na$_2$HPO$_4$ and 1 g of dimedone (which had been recrystallised twice from acetone) in 100 ml of water.

**Preparation of $[\beta,\delta-^3$H$_4]$ALA:** 50 mg of ALA, 0.15 ml T$_2$O (1 Ci/ml) and 0.15 ml concentrated HCl were autoclaved in a sealed and evacuated tube for 1 hr at 15 lb/sq.in. After this the contents of the tube were freeze dried. The residue was dissolved in 2 ml H$_2$O and refreeze dried. This was repeated twice. 58% of the total tritium incorporated into ALA was found to be located at the $\delta$-carbon atom by the following method.

0.5 mg of tritiated ALA and 1 $\mu$Ci of $[5-^{14}$C]$ALA$ were dissolved in 0.3 ml of H$_2$O. 0.25 ml of this solution and 1-1.5 mg of ALA were reduced with sodium borohydride (3 mg) at 0° for 30 min. 0.1 ml of this solution was chromatographed, only once, to separate $\delta$-amino dihydrolevulinic acid (as described above). Another 0.1 ml of the solution was degraded to obtain formaldehyde dimedone derivative of the $\delta$-carbon atom (as described above). The amount of tritium located at the $\delta$-carbon atom of ALA was estimated by comparing the $^3$H:$^{14}$C ratio of $\delta$-amino dihydrolevulinic acid with that of the formaldehyde dimedone derivative.
Since it is assumed that acid catalysed labilization of hydrogen atoms is more likely to occur at methylene groups adjacent to a carbonyl group, the tritium radioactivity in excess of 58% would be expected to be located at the \( \beta \)-carbon atom of ALA. However, since the present biosynthetic and exchange studies required specific isolation of \( \delta \)-carbon atom of ALA, the knowledge of tritium located at the other carbon atoms was not necessary.

**Determination of exchange of \( ^3H \) located at the \( \delta \)-carbon atom of \( [\beta, \delta-^3H_4]\text{ALA} \).**

To study the exchange of the \( ^3H \) atom located at the \( \delta \)-carbon atom of \( [\beta, \delta-^3H_4]\text{ALA} \), ALA synthetase (0.41 units of crude, 7.9 units of 33-fold purified or 8.2 units of 589-fold purified) were incubated at 37\(^\circ\) with all the constituents necessary for the biosynthesis of ALA in the presence of 80 \( \mu \)g \( [\beta, \delta-^3H_4] \) ALA for table 2.1, fig. 2.3, 50 \( \mu \)g of \( [\beta, \delta-^3H_4]\text{ALA} \) for table 2.3 and fig. 2.4 and 50 \( \mu \)g of \( [\beta, \delta-^3H_4;5-^{14}C]\text{ALA} (^{14}C=2.6 \times 10^6 \text{ cpm}) \) for table 2.5.

Aliquots of the incubation mixtures were taken at various time intervals and added into a flask containing 3 mg of non-radioactive ALA and 6 mg of sodium borohydride at 0\(^\circ\). After 30 min the contents of the flask were acidified to pH 6.0 with 50% acetic acid and treated with a saturated solution of sodium bicarbonate (10 ml), sodium metaperiodate (1 ml from 100 mg/ml solution) and formaldehyde (10 mg) for 8 hrs in complete
darkness. The contents of each flask were processed (as described above) to make the formaldehyde dimedone derivatives. A known weight of each sample of the dimedone derivative was measured for radioactivity.

The percentage exchange for table 2.1 was calculated from the differences in specific activities (cpm/1 mg) of the dimedone derivatives at zero time and at other respective times. For table 2.5 the percentage exchange was calculated from the differences in $^3\text{H}:^{14}\text{C}$ ratios of the dimedone derivatives at zero time and at any other time shown in the table.
CHAPTER 3

STUDIES ON

L-ALANINE-4, 5-DIOXOVALERATE TRANSAMINASE
Independent work from many laboratories (17 and references therein) has established that the \( \alpha \)-carbon atom of glycine is used not only for the biosynthesis of porphyrins but also for the ureido groups of purines, the \( \beta \)-carbon atom of serine \((\text{HO-}^\beta\text{CH}_2\text{-}^\alpha\text{CHNH}_2\text{-COOH})\) and methyl groups of compounds such as S-adenosyl methionine. The incorporation of the \( \alpha \)-carbon atom of glycine into these apparently unrelated compounds suggests the possibility that glycine is metabolised via a pathway in which intermediates are produced which can then be utilized for the biosynthesis of these different compounds.

The evidence for such a pathway was provided by Shemin et al (17, 124). When they injected \([5-^{14}\text{C}]\text{ALA}\) into ducks, C-5 was incorporated into the ureido groups of purines in the erythrocytes and was also excreted as formic acid (HCOOH). The injection into rats of \([1:4-^{14}\text{C}]\text{ALA}\) together with malonate led to the excretion, into the urine, of succinate labelled only in carboxylic groups. The injection of \([5-^{14}\text{C}]4,5\text{-dioxovalerate (4-ketoglutaraldehyde)}\) in rats led to the excretion of HCOOH and in pigeons to labelled uric acid.

Based on their results, Shemin et al (17, 124) proposed a series of reactions called the succinate-glycine cycle (fig. 3.1). In this pathway, it is postulated that succinyl CoA to give ALA(\(\text{I}\)) (I) condenses with glycine (II) which can be either utilized for porphyrin biosynthesis or be deaminated to furnish 4,5-dioxovalerate (4-ketoglutaraldehyde V). This compound, on losing terminal-CHO, (originating from C-2 atom of glycine) regen-
tricarboxylic acid cycle

Ureido group of purines, formate etc.

\[ \text{HOOC.CH}_2\cdot\text{CH}_2\cdot\text{COSCoA} \]

\[ \alpha \quad \beta \quad \gamma \]

\[ \text{HOOC.CH}_2\cdot\text{CH}_2\cdot\text{COOH} \]

\[ \delta \text{carbon atom to} \]

\[ \text{Succinate-Glycine Cycle} \]

\[ \text{NH}_2\cdot\text{CH} \cdot \text{COOH} \]

\[ \text{II} \]

\[ \text{HOOC.CH}_2\cdot\text{CH}_2\cdot\text{CO} \cdot \text{CHO} \]

\[ \text{IV} \]

\[ \text{[O]} \]

\[ \text{2-Oxoglutarate} \]

\[ \text{Porphyrrins} \]

\[ \text{HOOC.CH}_2\cdot\text{CH}_2\cdot\text{CO} \cdot \text{CH}_2\cdot\text{NH}_2 \]

\[ \text{III} \]

Fig. 3.1 Succinate-Glycine Cycle.
erates succinate (V) and also yields a one-carbon fragment capable of undergoing further reactions, e.g. utilization in the biosynthesis of ureido groups of purines, the β-carbon atom of serine and methyl groups. Some experimental support for this cycle has come from the work of Neuberger's group. These workers have isolated (from Rhodopseudomonas spheroides) and extensively studied the enzyme L-alanine-4,5-dioxovalerate transaminase (125, 125a) which catalyses the transamination of ALA to yield 4,5-dioxovalerate (an intermediate in the succinate-glycine cycle) as shown below.

\[
\begin{align*}
\text{pyruvate} & \quad + \\
\text{H—C—NH}_2 & \quad \text{L-alanine} + \text{NH}_2 \\
\text{CH}_3—\text{CO}—\text{COOH} & \quad + \quad \text{H—C—COOH} \\
\text{H} & \quad \text{C} = \text{O} \\
\text{C} & \quad \text{C} = \text{O} \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{COOH} & \quad \text{COOH} \\
\end{align*}
\]

ALA \quad 4,5\text{-dioxovalerate}

This reaction can be demonstrated in both directions, i.e. the formation and disappearance of ALA.

Like all other transaminases, this enzyme requires pyridoxal phosphate (which is tightly bound to the enzyme protein) and therefore would be expected to catalyse the equilibration of one of the β-hydrogen atoms of ALA with the protons of the medium (see below) and hence afford a method for preparing
stereospecifically labelled \([5^3\text{H}]\text{ALA}\). The latter may then be used to study the stereochemistry of the \(\text{ALA dehydratase} \) reaction.
A  **Properties of L-alanine-3, 4, 5-dioxovalerate transaminase.**

The comparison of the specific activities of our preparation of the enzyme (see experimental p87) with those obtained by Neuberger et al (125, 125ₐ) revealed that our values were about 10-fold higher than those of the latter. The explanation of this discrepancy may lie in the fact that our 4, 5-dioxovalerate was much purer than that of these workers.

i) **Substrate specificity:** L-alanine, D-alanine and glycine were tested as amino group donors. The results displayed in the table 3.1 and fig. 3.2 showed that D-alanine did not serve as a substrate at all. Glycine was, however, 34% as active as L-alanine which, being the natural substrate, proved to be the best.

ii) **Inhibition:** Previous studies (125, 125ₐ) had revealed that thiol attacking reagents such as iodoacetate and p-chloromercuribenzoate and 2-oxo acids were inhibitors of the enzyme activity. Therefore iodoacetate and glyoxylic acid (CHO-COOH) were tested for their effect on the enzyme. The results, displayed in table 3.2, showed that iodoacetate inhibited the enzyme action by 75% at 2 mM whereas at the same concentration glyoxylic acid produced 100% inhibition. These results were in close agreement with those of Neuberger et al (125, 125ₐ).

B  **Studies on the exchange of the 8-hydrogen atoms of ALA with the tritium of the medium.**

i) **Chemical exchange:** In this series of experiments it was intended to evaluate the chemical equilibration between the hydrogen
The substrate specificity of L-alanine-4,5-dioxovalerate transaminase.

The incubation mixture contained L-alanine, (48 μmoles); phosphate buffer, pH 7.0, (72 μmoles); 13-fold purified enzyme, (0.24 units); and 4,5-dioxovalerate, (1.6 μmoles) in a total volume of 1.2 ml. Reactions were started by the addition of 4,5-dioxovalerate. Incubations were carried out at 37°. 0.3 ml aliquots were removed at various time intervals and quenched by the addition of 0.2 ml of 20% (w/v) trichloroacetic acid and 0.08 ml of freshly made iodoacetic acid (0.01 M). 0.5 ml of the clear supernatant was assayed for ALA biosynthesised. Optical density (O.D.) at 553 μm is the measure of the amount of ALA.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>O.D. at 553 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-ala</td>
</tr>
<tr>
<td>15</td>
<td>0.34</td>
</tr>
<tr>
<td>30</td>
<td>0.48</td>
</tr>
<tr>
<td>60</td>
<td>0.63</td>
</tr>
<tr>
<td>90</td>
<td>0.73</td>
</tr>
</tbody>
</table>
Fig. 3.2  Biosynthesis of ALA from
4,5-dioxovalerate & alanine (or gly).
Table 3.2

The inhibition studies on L-alanine-4,5-dioxovalerate transaminase.

The 13-fold purified enzyme (0.24 units) was preincubated, for 15 min at 37°, with inhibitors (iodoacetate and glyoxylic acid) and then the incubation mixture was made up to 0.75 ml containing L-alanine, (30 μmoles); phosphate buffer, pH 7.0, (45 μmoles); and crystalline 4,5-dioxovalerate, (1 μmole). Incubations were carried out for 15 min at 37° and stopped by the addition of 0.25 ml of trichloroacetic acid (20% w/v) and 0.2 ml of iodoacetic acid (0.01 M). 1 ml of the clear supernatant was assayed for the biosynthesised ALA.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>O.D. at 553 μm</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. complete system</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>2. complete system + 0.002 mmoles iodoacetate</td>
<td>0.125</td>
<td>75</td>
</tr>
<tr>
<td>3. complete system + 0.002 mmoles glyoxylic acid</td>
<td>0.00</td>
<td>100</td>
</tr>
</tbody>
</table>
atoms at the $\delta$-carbon atom of ALA and the medium. This involved the incubation of ALA at various pH's in the presence of tritiated water (table 3.3).

Since enolization is an acid/base catalysed reaction (see below), it was not unreasonable to find that equilibration in the acidic medium was about four times greater than under neutral conditions (table 3.3).

\[
\begin{align*}
\text{COOH} & \quad \text{COOH} \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{C} \quad \text{C} & \quad \text{C} \\
\text{O} & \quad \text{O}’ \\
\text{NH}_2 & \quad \text{NH}_2
\end{align*}
\]

\[\text{acid}\]

ii) The enzymic exchange of the $\delta$-hydrogen atom of ALA with the medium: ALA was incubated with tritiated water in the presence of L-alanine-4,5-dioxovalerate transaminase under various conditions at 37° for 1 hr. The results (displayed in table 3.4) were equivocal. On the one hand they suggested an enzyme catalysed exchange of hydrogen atoms between the $\delta$-hydrogen atoms of ALA and the medium, because this exchange was reduced to only 29% in the presence of glyoxylic acid (4 $\mu$moles) which is a known inhibitor of L-alanine-4,5-dioxovalerate transaminase and diminished to a mere 10% in the absence of the enzyme. On the other hand, in the presence of the boiled enzyme (boiled for 15 min at 100°) this exchange
Table 3.3

The effect of pH on exchange of δ-hydrogen atom of ALA with the medium tritium.

The incubation mixtures contained phosphate buffers, 75 μmoles; ALA, (6 μmoles); T₂O, (20 mCi to give specific activity of 22.2 x 10⁶ cpm per μg atom of hydrogen); in a total volume of 1.15 ml. The incubations were carried out at 37° for 1 hr and terminated by the addition of 2 mg of non-radioactive ALA and freeze-drying of the contents. Finally, ALA was degraded to obtain formaldimedone (FD) as described in Methods.

<table>
<thead>
<tr>
<th>pH</th>
<th>Total $^3$H cpm incorporation into ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>246,000</td>
</tr>
<tr>
<td>6</td>
<td>220,000</td>
</tr>
<tr>
<td>7</td>
<td>65,000</td>
</tr>
<tr>
<td>7.2</td>
<td>43,600</td>
</tr>
<tr>
<td>7.8</td>
<td>50,800</td>
</tr>
</tbody>
</table>
Table 3.4
The exchange of 6-hydrogen atom of ALA with tritium of the medium by L-alanine-4,5-dioxovalerate transaminase.

Incubation mixtures contained phosphate buffer, pH 7.4, (75 μmoles); ALA, (10 μmoles); T$_2$O (20 mCi to give a specific activity of 22.2 x 10$^6$ cpm per μg atom of hydrogen); enzyme, (0.72 units); in a total volume of 1.15 ml. The incubations were carried out at 37° for 1 hr. To measure tritium incorporated into ALA, the latter was degraded to obtain formaldimedone (FD) which was then counted for radioactivity.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Total $^3$H cpm in FD</th>
<th>% $^3$H incorporation in ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>complete system</td>
<td>1,016,900</td>
<td>100</td>
</tr>
<tr>
<td>boiled enzyme</td>
<td>770,000</td>
<td>76.0</td>
</tr>
<tr>
<td>minus enzyme</td>
<td>102,400</td>
<td>10.1</td>
</tr>
<tr>
<td>complete system + glyoxylate (4 μmoles)</td>
<td>298,000</td>
<td>29.3</td>
</tr>
</tbody>
</table>
was as much as 76% in comparison with the enzymic exchange, suggesting that perhaps the exchange was not an enzymic but a non-specific protein-catalysed phenomenon.

This ambiguity was resolved as follows. $[\beta,\delta^{3}\text{H}_{4}:4^{14}\text{C}]-\text{ALA}$ was incubated with L-alanine-4,5-dioxa-valerate transaminase and the loss (rather than incorporation) of tritium from the $\delta$-carbon atom of ALA was estimated from the $\text{H}/\text{C}$ ratios of formaldehydeone at various time intervals. The results (table 3.5 and fig. 3.3) revealed that though there was negligible chemical equilibration, in enzymic incubations after about an hour almost all the tritium radioactivity from $\delta$-carbon atom was removed. These results were unchanged even when the incubation mixtures contained catalytic amounts of pyruvate (1 nmole). This suggested that no 'co-factor' other than pyridoxal phosphate was necessary for the half reaction under investigation.

Discussion.

In their study of the properties of L-alanine-4,5-dioxa-valerate transaminase Neuberger et al (125, 125a showed that most natural amino acids could not replace L-alanine as substrate except glycine which manifested about 10% of the L-alanine activity. In our study (table 3.1 and fig. 3.2) glycine at concentrations similar to those utilized by these workers proved a better substrate. It had 34% of the L-alanine activity. This discrepancy may be explained as pointed out elsewhere, by the fact that our 4,5-dioxo-valerate was much purer than that of Neuberger et al. Inhibition of the enzyme (table 3.2) by glyoxylate (100% at 2 mM) and by iodoacetate (75% at 2 mM) also essentially confirmed the findings of the
The enzymic exchange of \( \delta \)-tritium atoms of \([\beta,\delta-^3H_4:5\beta-^{14}C]\)ALA.

The incubation mixture contained \([\beta,\delta-^3H_4:5\beta-^{14}C]\)ALA, (0.8 \( \mu \)moles, 75\% tritium was located at \( \delta \)-carbon atom and \( ^{14}C = 6 \times 10^6 \) cpm); phosphate buffer, pH 7.0, (120 \( \mu \)moles); enzyme, (1.2 units) in a total volume of 2 ml. 0.5 ml aliquots were removed at various time intervals and formal-dimedone (FD) prepared from the ALA.

In some incubation mixtures pyruvate (1 nmole) was also added.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Active Enzyme</th>
<th>Boiled Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( ^3H/^{14}C ) ratio of FD</td>
<td>( ^3H ) loss</td>
</tr>
<tr>
<td>0</td>
<td>44.99</td>
<td>0.00</td>
</tr>
<tr>
<td>15</td>
<td>24.59</td>
<td>45.2</td>
</tr>
<tr>
<td>30</td>
<td>13.75</td>
<td>70.0</td>
</tr>
<tr>
<td>60</td>
<td>4.00</td>
<td>91.0</td>
</tr>
</tbody>
</table>
Fig. 3.3  Enzymic exchange of $\delta$-tritium atoms of $[\beta, \delta-^3H_4:5-^{14}C]ALA$. 
previous workers (125, 125a) who found that inhibition by p-
chloromercuribenzoate and iodoacetate could be reversed by
reduced glutathione or β-mercapto ethanol, suggesting that in-
tact thiol group(s) in the enzymes are essential for the activity.

By analogy with other transaminase enzymes L-alanine-
4,5-dioxovalerate transaminase was expected to catalyse the
exchange between the medium protons and the 5-hydrogen
atom(s) of ALA (as illustrated on page 73). The investigation
of this possibility revealed (table 3.4) that although the enzyme
could catalyse incorporation of more than 1 x 10^6 cpm ^3H/10
μmoles ALA per hour, 76% of this radioactivity could be incor-
porated in the presence of boiled enzyme. This suggested that
some functional groups, such as -SH, in the denatured protein
were capable of catalysing nonspecific incorporation of the med-
ium tritium into ALA. (It will be remembered that enolization
is an acid/base catalysed reaction (see p78) and -SH is the con-
jugate acid of the base -S^-).

Since, in terms of reliability, the work with tritiated water
is always suspect except in a few well defined systems, new
experiments were designed, using [β,δ-^3H_4:5^14C]ALA to test
the possibility of nonspecific protein catalysed equilibration of
the 5-hydrogen atom(s) of ALA with the proton of the medium.
In these experiments loss, rather than incorporation, of tritium
from the 5-carbon atom of ALA was measured. The results
(table 3.5 and fig. 3.3) showed that the boiled enzyme did not
catalyse the exchange between the medium protons and the 
\( \delta \)-hydrogen atoms of the ALA. On the other hand in the enzymic 
incubations almost all the tritium radioactivity located at the 
\( \delta \)-carbon atom of ALA was removed (table 3.5 and fig. 3.3).

Unless L-alanine-4,5-dioxovalerate transaminase is a 
unique transaminase, the exchange of both of the \( \delta \)-hydrogen 
atoms of ALA cannot be the function of this enzyme. It is 
possible that one \( \delta \)-hydrogen atom of ALA is removed by the 
L-alanine-4,5-dioxovalerate transaminase while the other \( \delta \)-hy-
drogen atom is lost through the participation of another con-
taminant enzyme.

It has already been demonstrated that in the case of ALA 
synthetase the contaminant exchange activity can be eliminated 
by extensive purification. Therefore it is proposed that the 
use of highly purified L-alanine-4,5-dioxovalerate transaminase 
may yet prove to be a convenient method for preparing ALA 
which is stereospecifically tritiated at the \( \delta \)-carbon atom.
Experimental.

Materials.

$[5-^{14}\text{C}]\text{ALA HCl}$ was purchased from NEN Chemicals, Germany. Non-radioactive ALA HCl and pyridoxal phosphate were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. The rest of the chemicals and solvents were bought from the British Drug Houses Ltd., Poole, Dorset, U.K.

Methods.

The chemical synthesis of 4,5-dioxovalerate.

a) Preparation of 3,5-dibromolaevulinic acid: 250 ml two-necked round bottom quick-fit flask, containing 10.1 ml (or 11.5 g) of laevulinic acid (0.1 mole) and 44 ml of chloroform, was fitted with a reflux condenser and a fine tipped dropping funnel containing 10.6 ml (0.2 mole) of bromine diluted with an equal volume of chloroform. While the contents of the flask were being continuously stirred at room temperature, bromine was added slowly in a dropwise manner during 40-45 min. (A pale precipitate began to appear before all the bromine had been added). After completing the addition of bromine, most of the chloroform was evaporated under vacuum below 20°C and the remainder was removed by filtration. The filtered precipitate was crystallized from chloroform to obtain fine white crystals, which had melting point between 111 and 113°C (the literature value 113-114°C). The yield of dibromolaevulinic acid was 6 g (52%).
b) The preparation of 4,5-dioxovalerate. 10 g of 3,5-dibromo-
levulinic acid dissolved in 600 ml of water was heated on a boiling
water bath for 6 hr. After cooling, an aliquot of the solution was
titrated with NaOH to determine the amount of HBr and then a
calculated amount of AgNO₃ (12.4 g) was added. The AgBr pre-
cipitate was filtered off and the filtrate was evaporated under
vacuum on a rotatory film evaporator at a temperature below 35°.
The residue, a solid film, was dried overnight over KOH and
P₂O₅ in an evacuated desiccator. The dried material was dis-
solved in the minimum amount of absolute ethyl acetate and pre-
cipitated by addition of absolute ether. The precipitate was dried
over KOH and P₂O₅ in an evacuated desiccator. The 3,5-dioxo-
valerate (1 g) preparation was a fine white powder which had
melting point of 120°.

\[
\begin{align*}
\text{Br-CH}_2\text{-CO-CHBr-CH}_2\text{-COOH} & \xrightarrow{\text{H}_2\text{O}} \text{OH-CO-CH}_2\text{-CH}_2\text{-COOH} + 2\text{HBr} \\
\end{align*}
\]

The partial purification of L-alanine-4,5-dioxovalerate
transaminase (126).

Conditions for growth and harvesting of Rhodopseudomonas
spheroides have been given on p.60.

Crude extract: 60-70 g wet weight of Rhodopseudomonas spheroides
cells were suspended in 70 ml of 0.1 M potassium phosphate
buffer (pH 7.0) and disrupted for 20-25 min by means of an MSE
ultrasonicator. The broken cells were centrifuged at 105,000 g
for 100 min. The clear supernatant was pooled, diluted with
buffer to 100 ml and dialysed overnight against a large volume
of 0.1 M potassium phosphate buffer containing 0.1 mM β-mercapto-
Pleat treatment: To 100 ml of the crude extract were added 5 ml of 0.25 M MgSO₄, 5 ml of 2.5 M sodium pyruvate, and 5 ml of 2.5 mM pyridoxal phosphate. The mixture was heated to 50°C and maintained at this temperature for 10 min and then rapidly cooled. The precipitated material was discarded after centrifugation. Since pyruvate is an inhibitor of the enzyme, the aliquot of the enzyme solution retained at this stage was dialysed before assaying.

Protamine sulphate: To 100 ml of heat treated supernatant was added, at 0°C, 20 ml of 2% (w/v) protamine sulphate with stirring during 25-30 min at 4°C. The stirring was continued for another 10 min. The precipitated material was discarded.

Fractionation with (NH₄)₂SO₄: To 100 ml of protamine sulphate-treated supernatant at 25°C, 19.35 g (30% saturation) of (NH₄)₂SO₄ was gradually added (during 15 min) with stirring. After equilibration for 10 min at 25°C, the precipitate was discarded. Then a further 7.37 g (40% saturation) of (NH₄)₂SO₄ was slowly added at 0°C during 30 min. After equilibration for another 30 min, the precipitated protein was retained, dissolved in 5 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 mM β-mercaptoethanol and dialysed against 2 l. of 0.05 M potassium phosphate buffer (pH 7.0) containing 0.1 mM β-mercaptoethanol. Any precipitate was discarded. The enzyme solution was stored at -18°C.

This preparation was stable in water solution containing 0.1 mM β-mercaptoethanol up to four weeks.
The partial purification of
L-alanine-4,5-dioxovalerate transaminase.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml)</th>
<th>Total Protein (mg)</th>
<th>Sp. Activity (units/mg)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>100</td>
<td>500</td>
<td>0.0197</td>
<td>1</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>100</td>
<td>100</td>
<td>0.09</td>
<td>4.5</td>
</tr>
<tr>
<td>((NH_4)_2SO_4)</td>
<td>5</td>
<td>50</td>
<td>0.24</td>
<td>12.2</td>
</tr>
</tbody>
</table>

**Incubation conditions:** The reaction mixtures contained L-alanine, (30 μmoles); potassium phosphate buffer (pH 7.0), (45 μmoles); enzyme, (0.1 ml); and 4,5-dioxovalerate, (1 μmole) in a total volume of 0.75 ml. The reactions were started by the addition of 4,5-dioxovalerate and carried out for 15 min at 37°. The incubations were terminated by the addition of 0.25 ml of trichloroacetic acid and 0.2 ml of freshly made 0.01 M iodoacetic acid (to alkylate β-mercaptoethanol which would interfere with the colorimetric assay of the aminoketone). The precipitated protein was removed by centrifugation.

**The colorimetric assay for ALA:** 1.0 ml of the deproteinized incubation mixture was added to 1.0 ml of 2 M sodium acetate buffer (pH 4.6) and heated with 0.1 ml of acetylacetone at 100° for 10 min. After cooling to room temperature, 2.0 ml of modified Ehrlich's reagent (1 g of p-dimethylamino benzaldehyde, 10 ml of 60% perchloric acid and 40 ml of glacial acetic acid) was added. The colour was allowed to develop for 15 min and then measured against a blank at 553 μm.
Unit: One unit of enzyme activity is defined as the amount of enzyme which catalysed the synthesis of 1 µmole of ALA in 15 min at 37°.

The incorporation of \(^3\)H at the \(\delta\)-carbon atom of ALA.

The reaction mixture contained potassium phosphate buffer, (pH 7.4), (75 µmoles); ALA, (10 µmoles); \(T_2O\) (20 m Curie to give specific activity of \(22.2 \times 10^6\) cpm/µg atom of hydrogen); active enzyme or boiled enzyme, (0.72 units) in a total volume of 1.15 ml. The incubation was carried out for 1 hr at 37°. At the end of incubation period the incubation mixture was freeze dried thoroughly to remove \(T_2O\). The residue was dissolved in \(H_2O\) and ALA degraded to obtain the formaldehyde dimeredone derivative (tables 3.3, 3.4) as described on p64.

The determination of exchange of \(^3\)H located at the \(\delta\)-carbon atom of \([\beta,\delta^{3}H_4]ALA\).

In this case the incubation mixtures contained \([\beta,\delta^{3}H_4;5^{14}C]\)-ALA, (0.8 µmole, 75% tritium was located at the \(\delta\)-carbon atom and \(14C=6 \times 10^6\) cpm); potassium phosphate buffer, (pH 7.0), (120 µmoles); active enzyme or boiled enzyme (1.2 units) in a total volume of 2.0 ml, (table 3.5). The incubation was carried out at 37° and aliquots were removed at various time intervals during the incubation period of 1 hr.

Aliquots from above were added into a 50 ml flask containing 3 mg of nonradioactive ALA and 6 mg of sodium borohydride at 0°. After 30 min the contents of the flask were acidified with
50% acetic acid to pH 6.0, treated with a saturated solution sodium bicarbonate (10 ml), sodium metaperiodate (1 ml from freshly made 100 mg/ml solution) and formaldehyde (10 mg) for 8 hrs in complete darkness and finally processed to obtain the formaldehyde dimedone derivative as described on p.64.

For table 3.5, the percentage exchange was calculated from the differences in $^3$H:$^{14}$C ratios of the formaldehyde dimedone derivatives at zero-time and at other times shown in the table.
CHAPTER 4

MECHANISM AND STEREOCHEMISTRY OF

VINYL GROUP FORMATION IN HAEM BIOSYNTHESIS.
Introduction.

Protoporphyrinogen-IX is formed by the stepwise oxidative decarboxylation of coproporphyrinogen-III (see below). This reaction is catalysed by the enzyme coproporphyrinogenase. Only in Rhodopseudomonas spheroides and Chromatium strain D does this enzyme not require molecular oxygen (83) for, in vitro, conversion of coproporphyrinogen-III to protophyrinogen-IX. In all other systems (aerobic and anaerobic alike) studied so far, this reaction requires molecular oxygen for the oxidative decarboxylation and no hydrogen acceptor has been found capable of replacing oxygen.

Several mechanisms have been suggested to explain the reaction. Granick and Sano (126) proposed that a hydride ion is removed from the β-carbon atom of the propionic acid side chain with simultaneous decarboxylation to yield a vinyl group (fig. 4.1).
This mechanism may be prevalent in anaerobic organism, however it fails to account for the oxygen requirement in aerobic systems. Porra et al (84) found that the conversion of coproporphyrinogen-III to protoporphyrinogen-IX by ox liver mitochondrial extracts went through a protein-bound stage involving a linkage that was presumably of the thioether type, for it was split by silver salts but not by acid treatment. Furthermore, since porphyrins with vinyl or hydroxyethyl groups combine readily with thiol compounds (127) such as cysteine or cysteine-containing peptides, the coproporphyrinogenase reaction was temptingly envisaged to proceed via an initial unsaturation of 2- and 4- propionic acid side chains, followed by combination with a thiol group of protein to form the thioether intermediate, from which CO$_2$ could readily be lost by decarboxylation (fig. 4.2). Against this proposed mechanism is the fact that 2,4-trans-diacyclic acid deuteroporphyrinogen-IX (structure 4.1), in vitro, is not converted to protoporphyrinogen-IX by beef liver mitochondria (81). Batlle et al (82) have suggested
that the real intermediate might be 2,4-cis-diacrylate deuteroporphyrinogen-IX. Supporting this view was the evidence that maleic acid, but not fumaric acid, inhibited the coproporphyrinogenase activity and French et al (128) have isolated, from calf and foal meconium, a porphyrin which has cis-acrylic acid at position 2,4,6 or 7 (it is most likely to be 2 or 4). However, it is not yet known if this compound is an intermediate in the porphyrin biosynthesis.

Sano (129) has reported that 20-22% of the synthetic 2,4-bis (β-hydroxypropionate) deuteroporphyrinogen-IX (structure 4,II) can be transformed enzymically to protoporphyrinogen-IX under anaerobic conditions. This would suggest that the hydroxy compound is either a direct intermediate (route a→b, fig. 4,3) or may be converted into one by one of many routes: it may go either through cis-acrylic acid intermediate (route c→d→b or a→e→f), or via a ketonic intermediate (a→g→h→i) to yield protoporphyrinogen-IX.
For our purpose we simplified the fig. 4.3 into two broad mechanisms. In mechanism I, the decarboxylation of the propionate residue is envisaged to be facilitated through the participation of a ketonic intermediate, as shown in the sequence
(A) → (B) → (C). The species (C) is then converted to a vinyl group by a multistep process. This mechanism requires that in the conversion (A) → (D), both β-hydrogen atoms of the propionic acid side chain are removed.

**Mechanism I**

\[
\begin{align*}
\text{Pyr} & \quad \beta \text{CH}_2 \quad \alpha \text{CH}_2 \quad \text{COOH} \rightarrow \text{Pyr} \quad \beta \quad \alpha \text{CH}_2 \quad \text{COOH} \\
\text{(A)} & \quad \text{(B)} \\
\text{Pyr} & \quad \beta \quad \alpha \text{CH}_3 \rightarrow \rightarrow \rightarrow \quad \text{Pyr} \quad \beta \quad \alpha \text{CH} = \text{CH}_2 \\
\text{(C)} & \quad \text{(D)}
\end{align*}
\]

In the mechanism II, the decarboxylation reaction is proposed to take place through the loss of a β-substituent leading directly to the formation of a vinyl group. This mechanism predicts that only one of the two original β-hydrogen atoms of (A) is retained in the vinyl group.

**Mechanism II**

\[
\begin{align*}
\text{Pyr} & \quad \beta \quad \text{CH} \quad \alpha \text{CH}_2 \quad \text{COOH} \rightarrow \text{Pyr} \quad \beta \quad \alpha \text{CH} \quad \alpha \text{CH}_2 \quad \text{CO} \\
\text{Pyr} & \quad \beta \quad \alpha \text{CH} = \text{CH}_2 \\
\text{X} & = \text{H, OH or a good leaving group.}
\end{align*}
\]

A careful examination of the porphyrin biosynthetic pathway reveals that the β-carbon atoms of the propionate residues in haem originates from β-carbon atom of ALA (which itself arises from the β-carbon atom of 2-oxoglutarate) and that the vinyl groups (rings A and B) and intact propionate residues (rings C and D) are present within
the same molecule of haem (fig. 4.4). Therefore to study the mechanism of the vinyl group formation, we decided to convert \([\beta,\delta-^3\text{H}_4,\delta-^{14}\text{C}]\text{ALA}\) biologically into haem which after conversion to protoporphyrinogen-IX followed by reduction to mesoporphyrin-IX was degraded to give ethylmethyl maleimide (originating from the rings A and B) and haematinic acid (emanating from the rings C and D) (fig. 4.5). The interesting feature of the design of this experiment is that \(^{14}\text{C}\) activity is equally distributed in ethylmethyl maleimide and haematinic acid and neither of these compounds contains tritium located at \(\delta\)-carbon atom of ALA. Therefore, any difference in the tritium contents (i.e. difference in \(^3\text{H}/^{14}\text{C}\) ratios) of ethylmethyl maleimide and haematinic acid must reflect changes taking place during the conversion of propionate residues to vinyl groups (assuming, of course, that \(\beta\)-hydrogen atoms of propionates do not participate in any other reaction).

The biosynthetic system employed during the present course of study was haemolysed preparations of avian (duck or phenyl hydrazine-treated chick) red blood cells which mainly consist of immature nucleated red cells, young reticulocytes and some non-nucleated mature cells (the treatment with phenyl hydrazine or periodic bleeding induces and/or invigorates the production of nucleated and reticulocyte type cells). The reason for using a system rich in nucleated red cells and reticulocytes is that only these cells have the capacity (although reticulocytes less than nucleated cells) to synthesise protein (mainly globin in the case of reticulocytes) and haem and incorporate it into haemoglobin.
Fig. 4.4
Fig. 4.5 Degrading of haem.
They also contain, inter alia, the complete enzyme system for the tricarboxylic acid cycle which provides succinyl CoA for haem biosynthesis. The mature red cells on the other hand have lost all the biosynthetic capacity (table 4.1).

**Table 4.1**

**Metabolic characteristics of red cell at various stages of its development.**

<table>
<thead>
<tr>
<th></th>
<th>Nucleated cells</th>
<th>Reticulocytes</th>
<th>Mature red cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DNA synthesis</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RNA synthesis</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RNA present</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lipid synthesis</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Haem synthesis</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>T.C.A. cycle</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Glycolytic pathway</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pentose phosphate pathway</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* T.C.A. = Tricarboxylic acid
Results.

The distribution of $^3$H in a molecule of tritiated ALA.

The evaluation of the two proposed mechanisms required the presence of tritium only at the $\beta$-carbon atom of ALA, it was necessary to show that the tritiated ALA which was to be used for haem biosynthesis had no tritium at the $\alpha$ carbon atom. (It will be remembered that the tritium at the $\delta$-carbon atom of ALA is not carried into the degradation products of haem).

A mixture of tritiated ALA, prepared by autoclaving ALA in the present of $T_2O$ (Experimental p.43), and $[^\delta^{14}C]$ALA with $^3H/^{14}C$ ratio of 21.2 was degraded with periodic acid to succinic acid (Experimental p.43) which had $^3H/^{14}C$ ratio of 11.95 suggesting that 56.5% of the total tritium radioactivity in ALA was located in the fragment consisting of carbon atoms 1-4 (the remaining 43.5% being at the C-5 carbon atom).

\[
\begin{align*}
\text{ALA} & \quad \to \quad \text{Succinic acid} \\
1\text{COOH} & \quad (\alpha) \quad 2\text{CH}_2 \\
& \quad (\beta) \quad 3\text{CH}_2 \\
& \quad (\gamma) \quad 4\text{CO} \\
& \quad (\delta) \quad 5\text{CH}_2 \text{NH}_2 \\
& \quad \text{IO}_4^- \\
1\text{COOH} & \quad (\alpha) \quad 2\text{CH}_2 \\
& \quad (\beta) \quad 3\text{CH}_2 \\
& \quad (\gamma) \quad 4\text{COOH} \\
& \quad (\delta) \quad 5\text{CH}_2 \text{O} \\
& \quad \text{Formaldehyde}
\end{align*}
\]

Considering the structure of ALA it was reasonable to expect that only C-3 and C-5, which are adjacent to a carbonyl group, would be tritiated. Still the possibility of some of the 56.5% tritium being located at the C-2 could not be discounted.
In order to investigate this possibility, deuterated ALA was prepared under various conditions and degraded to succinic anhydride (Experimental p. 145), which was analysed for its deuterium contents.

The analysis of the mass spectrum of succinic anhydrides.

From the mass spectrum of undeuterated succinic anhydride, taken under the same experimental conditions, it was found that for the molecular fragment \((\text{CH}_2\text{CO})_2\text{O}\) of mass 100, the peak height \((P_{100})\) had the following relation to the adjacent peaks:

\[
P_{99} = 0, \quad P_{100} = 1.0, \quad P_{101} = 0.077, \quad P_{102} = 0,
\]

\[
P_{103} = 0, \quad P_{104} = 0.
\]

This distribution was then used to correct each of the observed peak heights \((P)\) of deuterated succinic anhydrides from masses 100 to 104 to the corrected height \((p)\) which would have been observed without overlapping from other species. The correction involved the solution of these equations:

Corrected peak height at mass 100 \((p_{100})\) = \(P_{100}\)

Corrected peak height at mass 101 \((p_{101})\) = \(P_{101} - 0.077P_{100}\)

Corrected peak height at mass 102 \((p_{102})\) = \(P_{102} - 0.077P_{101}\)

Corrected peak height at mass 103 \((p_{103})\) = \(P_{103} - 0.077P_{102}\)

Corrected peak height at mass 104 \((p_{104})\) = \(P_{104} - 0.077P_{103}\)

After correcting the peak heights (see table below), the relative percentage of the deuterated species (table 4.2) showed that when ALA was autoclaved with \(D_2\text{O}\) alone for 1 hr, it gave only monodeuterated (45%) and dideuterated (39%) species of
<table>
<thead>
<tr>
<th>Peaks at masses</th>
<th>15 min autoclaving in D₂O &amp; DCl₂</th>
<th>1 hr autoclaving in D₂O &amp; DCl</th>
<th>1 hr autoclaving in D₂O</th>
<th>2 hr autoclaving in D₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>p</td>
<td>P</td>
<td>p</td>
</tr>
<tr>
<td>100</td>
<td>0.0</td>
<td>0.00</td>
<td>0.0</td>
<td>0.00</td>
</tr>
<tr>
<td>101</td>
<td>1.5</td>
<td>1.50</td>
<td>5.5</td>
<td>5.50</td>
</tr>
<tr>
<td>102</td>
<td>13.5</td>
<td>13.38</td>
<td>12.0</td>
<td>11.75</td>
</tr>
<tr>
<td>103</td>
<td>5.5</td>
<td>4.46</td>
<td>8.5</td>
<td>7.60</td>
</tr>
<tr>
<td>104</td>
<td>2.5</td>
<td>2.16</td>
<td>4.0</td>
<td>3.40</td>
</tr>
</tbody>
</table>

succinic anhydride thus indicating that hydrogen atoms from only one of the methylene groups (probably β-methylene of the fragment consisting of 1 to 4 carbon atoms) of ALA had exchanged under these conditions of deuteration.

That the absence of trideuterated and tetradeuterated species of succinic anhydride was not due to the loss of deuterium atoms during the degradation of ALA was shown by the results (Table 2) of experiments A and B. In these experiments where deuteration was carried out under much more rigorous conditions (D₂O and DCl), the derived succinic anhydride contained trideuterated (D₃, 27%) tetradeuterated (D₄, 12%) as well as monodeuterated (D₁, 19.6%) and diddeuterated (D₂, 41.8%) species (table 4.2, experiment B). Therefore under these conditions the hydrogen atoms from both α- and β-methylene groups of ALA must have been replaced by deuterium atoms.

These results therefore demonstrated, indirectly, that the
For experiments A and B, deuterated ALA was prepared by autoclaving ALA (100 mg) with 0.25 ml of D$_2$O (99.8% D) and 0.25 ml of DCl (20% D). For experiments C and D, deuteration of ALA was performed by autoclaving ALA (100 mg) with 0.5 ml of D$_2$O (99.8% D). The deuterated ALA was degraded to succinic anhydride (SA) and the percentage of non-deuterated (D$_0$), monodeuterated (D$_1$), dideuterated (D$_2$), trideuterated (D$_3$) and tetradeuterated (D$_4$) species were found as described in the text.

<table>
<thead>
<tr>
<th>ALA autoclaved with</th>
<th>% D$_0$ SA</th>
<th>% D$_1$ SA</th>
<th>% D$_2$ SA</th>
<th>% D$_3$ SA</th>
<th>% D$_4$ SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>D$_2$O &amp; DCl for 15 min (A)</td>
<td>0.00</td>
<td>7.00</td>
<td>62.25</td>
<td>20.75</td>
<td>10.00</td>
</tr>
<tr>
<td>D$_2$O &amp; DCl for 1 hr (B)</td>
<td>0.00</td>
<td>19.60</td>
<td>41.80</td>
<td>27.55</td>
<td>12.00</td>
</tr>
<tr>
<td>D$_2$O for 1 hr (C)</td>
<td>15.50</td>
<td>45.30</td>
<td>39.20</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>D$_2$O for 2 hrs (D)</td>
<td>2.56</td>
<td>31.00</td>
<td>61.5</td>
<td>2.95</td>
<td>0.00</td>
</tr>
</tbody>
</table>
tritiated ALA prepared by autoclaving ALA with tritiated water for a short period would contain tritium atoms only at the β- and δ-carbon atoms.

The in vitro biosynthesis of haem by duck and phenyl hydrazine-treated chick blood.

The purpose of these experiments was to compare the haem biosynthesising capacity of phenyl hydrazine-treated chick blood (which will be referred to as anaemic blood) with that of duck blood and at the same time establish the ALA incorporation into haem.

$[4-{\text{14}}^C]ALA \ (2 \times 10^6 \text{ cpm})$ was incubated with equal volumes of haemolysed preparations of duck and anaemic chick blood as described in table 4.3. The results show that the duck blood is slightly more efficient than the anaemic chick blood* in biosynthesising the haem from ALA, (about 10-11% of which was incorporated into the haem during 15 hrs).

However, when injections of phenyl hydrazine were continued for 12 days† the incorporation of ALA in the haem was increased to 18-22%.

These results demonstrated that either system (i.e. duck blood or anaemic chick blood) could be used successfully for subsequent experimental work. However, we decided to use the anaemic chick blood owing to its easier availability.

* The normal period of phenyl hydrazine injections was 7 days.
The in vitro biosynthesis of haem from

$[4-{^{14}}C]ALA$ by haemolysed preparations of duck blood

and anaemic chick blood.

$[4-{^{14}}C]ALA$, (18 µmoles containing $2 \times 10^6$ cpm); FeSO$_4\cdot$7H$_2$O, (0.72 µmoles/1 ml blood); 0.15 M KH$_2$PO$_4$/Na$_2$HPO$_4$ buffer in 0.483 M KCl, pH 7.4, (5.5 ml); penicillin and streptomycin, (1.5 mg of each); and haemolysed blood preparation (fresh blood, 25 ml) in a total volume of 38 ml. The incubations were carried out at 37°. A 5 ml aliquot was removed from each incubation after 20 min. The incubation was terminated after 15 hr by the addition of a large excess of acetone (6 x the volume of incubation mixture) and then the isolated haem was measured for radioactivity as described in the experimental section.

<table>
<thead>
<tr>
<th></th>
<th>haemolysed duck blood</th>
<th>haemolysed phenyl hydrazine-treated chick blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 min</td>
<td>15 hr</td>
</tr>
<tr>
<td>Total $^{14}$C in haem (cpm).</td>
<td>22,600</td>
<td>218,000</td>
</tr>
<tr>
<td>% incorporation of ALA.</td>
<td>1.13</td>
<td>10.9</td>
</tr>
</tbody>
</table>
The degradation of $^{14}$C-haem to haematinic acid and ethylmethyl maleimide.

In order to determine that the procedure for degradation of haem (Experimental, p.150) could give products which could be measured satisfactorily, 10 mg of $^{14}$C-haem prepared from $[4-^{14}$C]ALA (50,000 cpm) were subjected to the removal of iron to give protoporphyrin-IX. After addition of carrier protoporphyrin-IX (50 mg) the latter was reduced to mesoporphyrin-IX which was oxidised with chromium trioxide ($CrO_3$). The reaction mixture was separated into acidic and neutral fractions and sublimed to obtain haematinic acid and ethylmethyl maleimide. The results showed that 28% of the total haem radioactivity was recoverable in haematinic acid and 30-33% in ethylmethyl maleimide, thus establishing that if a sufficient amount of isotopic activity were present in haem, workable quantities of it could be recovered in the degradation products.

The in vitro biosynthesis of haem from $[\beta,\delta-^3H_4]$ALA by haemolysed preparation of anaemic chick blood.

Having ensured that our biosynthetic system and the degradation procedure were satisfactory, 30 µmoles of $[\beta,\delta-^3H_4]$-ALA ($2.17 \times 10^6$ cpm/µmole) were incubated at 37° for 15 hrs with a haemolysed preparation of anaemic chick blood (25 ml of fresh blood). $3.35 \times 10^6$ cpm of tritium were incorporated into haem. 20 mg of this haem (60,000 cpm of $^3H$) were mixed with another sample of haem biosynthesised from $[4-^{14}$C]ALA to
give $^3\text{H}/^{14}\text{C}$ ratio of about 1.0 and were then converted to protoporphyrin-IX. The latter, after dilution to 60 mg with non-radioactive protoporphyrin-IX was degraded to haematinic acid originating from the propionate side chain-containing rings C and D and ethylmethyl maleimide derived from the vinyl group-containing rings A and B. The $^3\text{H}/^{14}\text{C}$ ratios of haematinic acid and ethylmethyl maleimide were found to be 1.0 and 0.58 (table 4.4) respectively. After purifying these compounds by gas-liquid chromatography, these values were changed to 1.0 and 0.59 respectively, suggesting that the purification of haematinic acid and ethylmethyl maleimide could not be improved by gas-liquid chromatography.

These results were construed to mean that during the formation of the vinyl group, 40-42% of the tritium located at the $\beta$-carbon atom of the propionic acid residue was lost. This value is adequately close to the theoretical loss of 50% if the vinyl group formation occurs through the mechanism of the type II, which requires the loss of only one of the two $\beta$ hydrogen atoms of the propionate side chain during the vinyl group formation. The mechanism I requires that the ethylmethyl maleimide should be devoid of the tritium radioactivity.

The in vitro biosynthesis of haem from [3-$^3\text{H}_2$]2-oxoglutarate.

The presence of an enzyme system, in the nucleated red cells and reticulocytes, for conversion of 2-oxoglutarate into ALA (table 4.1 and equation below) suggested an additional approach for studying the mechanism of vinyl group formation.
The biosynthesis of haem from $[^{3}H]ALA$.

The incubation mixtures contained ALA, (30 μmoles, $6.52 \times 10^6$ cpm $^3H$); FeSO$_4$7H$_2$O, (0.72 μmoles/ml blood); 0.15 m KH$_2$PO$_4$/Na$_2$HPO$_4$ buffer in 0.483 M KCl, pH 7.4, (5.5 ml); penicillin and streptomycin (1.5 mg of each); and haemolysed blood preparation (from 25 ml blood) in a total volume of 38 ml. The incubation was carried out at 37° for 15 hr and terminated by the addition of 230 ml of acetone. After isolation of haem, the latter was degraded to haematinic acid (HA) and ethylmethyl maleimide (EMM) which were counted for radioactivity.

<table>
<thead>
<tr>
<th></th>
<th>$^3H/^{14}C$ ratio</th>
<th>$^3H$ loss</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
<td>Predicted</td>
<td>Found</td>
</tr>
<tr>
<td>HA</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>EMM</td>
<td>0.58</td>
<td>0.5</td>
<td>42</td>
</tr>
</tbody>
</table>

The attraction of this approach lay in the fact that it was thought to provide not only a method for confirming the previous results but that it also made available a possible route for establishing the stereochemistry of the vinyl group formation since procedures for the synthesis of \([3RS-^3\text{H}_2]\), \([3R-^3\text{H}]\) and \([3S-^3\text{H}]\)-2-oxoglutarate are well documented (Experimental, p.145).

\[
\begin{align*}
\text{1COOH} & \quad + \text{CoASH} \quad - \text{CO}_2 \\
(\alpha) & \quad 2\text{CH}_2 \\
(\beta) & \quad 3\text{CH}_2 \\
(\gamma) & \quad 4\text{CO} \\
(\delta) & \quad 5\text{CO}_2\text{H}
\end{align*}
\begin{align*}
\text{Glycine} & \quad \text{1COOH} \\
(\alpha) & \quad 2\text{CH}_2 \\
(\beta) & \quad 3\text{CH}_2 \\
(\gamma) & \quad 4\text{CO} \\
(\delta) & \quad 5\text{CH}_2\text{NH}_2 \quad \text{derived from glycine}
\end{align*}
\begin{align*}
\text{2-oxoglutarate} & \quad \text{succinyl CoA} \\
& \quad \text{ALA}
\end{align*}
\]

Since 2-oxoglutarate is a metabolite for the tricarboxylic acid cycle, it was thought that the tritium label in \([3-^3\text{H}_2]\)2-oxoglutarate might be randomized due to its metabolism via this cycle. However, the stereochemistry of the reaction involved (fig. 4.6) revealed that after passing through the cycle once both hydrogen atoms located at the C-3 of 2-oxoglutarate were lost. Since this particular problem was ruled out, \([3-^3\text{H}_2]\)2-oxoglutarate \((210 \times 10^6 \text{ cpm } ^3\text{H})\) was incubated with haemolysed preparation of anaemic chick blood in the presence of glycine which served as the substrate for the equation above, malate to provide energy and malonate to minimize the metabolism of 2-oxoglutarate via the tricarboxylic acid cycle. The haem \((30 \text{ mg containing } 38,010 \text{ cpm } ^3\text{H})\) was mixed with 37,000 cpm of \(^{14}\text{C}\).
Fig. 4.6 Randomization of Label in 2-oxoglutarate via the tricarboxylic acid cycle.
haem (prepared from [4-\(^{14}\)C]ALA to act as internal reference) and then degraded, as described earlier, to obtain haematinic acid and ethylmethyl maleimide which had \(^{3}\text{H}/^{14}\text{C}\) ratios of 1.0 and 0.67 respectively (table 4.5). The reduction of only 33% in the tritium radioactivity of ethylmethyl maleimide compared to haematinic acid was disappointingly lower than the expected value of 50% indicated by the results of the biosynthetic experiments performed with [\(\beta,\delta-^{3}\text{H}_2\)]ALA.

A plausible explanation for the lower than the expected loss of tritium in the vinyl group formation during the haem biosynthesis from [3-\(^{3}\text{H}_2\)]2-oxoglutarate was thought to lie in the possibility of an enzyme catalysed randomization of the carbon skeleton at the succinyl CoA stage as shown in a series of reactions below.

\[
\begin{align*}
\text{HOOC} \cdot \text{CH}_2 \cdot \text{CT}_2 \cdot \overset{\circ}{\text{CO}} \cdot \text{COOH} & \xrightarrow{\text{CoASH}} \text{HOOC} \cdot \text{CH}_2 \cdot \text{CT}_2 \cdot \overset{\circ}{\text{O}} \text{SCoA} + \text{CO}_2 \\
\text{HOOC} \cdot \text{CT}_2 \cdot \overset{\circ}{\text{O}} \text{SCoA} & \xrightarrow{\text{equation 2}} \text{HOOC} \cdot \text{CT}_2 \cdot \text{CT}_2 \cdot \overset{\circ}{\text{COOH}} \\
\text{HOOC} \cdot \text{CT}_2 \cdot \text{CT}_2 \cdot \overset{\circ}{\text{COOH}} & \xrightarrow{\text{equation 3}} \text{HOOC} \cdot \text{CT}_2 \cdot \text{CT}_2 \cdot \overset{\circ}{\text{O}} \text{SCoA}
\end{align*}
\]

It was envisaged that after its formation from [3-\(^{3}\text{H}_2\)]2-oxoglutarate (equation 1), succinyl CoA was hydrolysed to give succinic acid (equation 2). The latter being a symmetric molecule could then be reconverted to succinyl CoA through the participation of either of its carboxylic groups (equation 3) thus completely randomizing the 2-oxoglutarate carbon skeleton. The conversion of this new species of succinyl CoA \(\text{HOOC} \cdot \text{CT}_2 \cdot \text{CT}_2 \cdot \overset{\circ}{\text{O}} \text{SCoA}\) to haem followed by the degradation of the
Table 4.5

The biosynthesis of haem from $[3^{-3}_H]2$-oxoglutarate.

The incubation mixtures contained $[3^{-3}_H]2$-oxoglutarate, 
(34 μmoles, $210 \times 10^6$ cpm $^3H$); glycine, (13.4 μmoles/ml 
blood); neutralized L-malate, (1.12 mmoles); disodium malonate, 
(169 μmoles); FeSO$_4$$H_2$O, (0.72 μmoles/ml blood); penicillin 
and streptomycin, (1.5 mg of each); and haemolysed blood 
preparation (from 25 ml of blood) in a total volume of 38 ml. 
The incubations were performed at 37° for 15 hrs, terminated 
by the addition of 230 ml of acetone and then processed to 
 obtain $^3H$-haem.

30 mg of $^3H$-haem (38,010 cpm), 10 mg of $^{14}C$-haem 
prepared from $4^{-14}C$ ALA containing 37,050 cpm were converted 
to protoporphyrin-IX which, after dilution with 20 mg of cold 
protoporphyrin-IX, was degraded to haematinic acid (HA) and 
ethylmethyl maleimide (EMM) as described in the experimental 
section.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$^3H/^{14}C$ ratio in HA</th>
<th>$^3H/^{14}C$ ratio in EMM</th>
<th>$^3H$ loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[3^{-3}_H]2$-oxoglutarate</td>
<td>1.0</td>
<td>0.67</td>
<td>33</td>
</tr>
<tr>
<td>$[\beta,^{-3}_H_4]ALA$</td>
<td>1.0</td>
<td>0.58</td>
<td>42</td>
</tr>
</tbody>
</table>
latter would have the following distribution of radioactivity in ethylmethyl maleimide (A) and haematinic acid (B) (fig. 4.7).

This pattern, of course, would be expected to be completely different from that when there was no randomization of the 2-oxoglutarate carbon skeleton (fig. 4.8).

The feature that becomes apparent from the comparison between fig. 4.7 and fig. 4.8 is that with $[3-^3\text{H}_2]$2-oxoglutarate as a substrate for haem biosynthesis, any contribution due to randomization would lead to a lesser tritium loss than when there was no randomization at all.
The in vitro biosynthesis of haem from $[\beta^3H_2]ALA$.

It was considered that the problem of randomization of the carbon skeleton of 2-oxoglutarate could be avoided by first converting this compound to ALA and then using the latter as the substrate for haem. Therefore, our colleague, M.M. Abboud, incubated $[3^3H_2]2$-oxoglutarate with 2-oxoglutarate dehydrogenase (partially purified from Escherichia coli) and ALA synthetase (isolated from Rhodopseudomonas spheroides), in situ, to obtain supposedly $[\beta^3H_2]ALA$ which was then used (without its isolation from the incubation mixture) to biosynthesise haem in the usual way (table 4.6). The tritiated haem (20 mg containing $48 \times 10^3$ cpm $^3$H) thus obtained, after mixing with $^{14}$C-haem (40,000 cpm $^{14}$C prepared from $[4^{14}$C]ALA) to act as internal reference, was degraded to haematinic acid and ethylmethylmaleimide which had $^{3}$H/$^{14}$C ratios of 1.0 and 0.69 respectively (table 4.6). These results, which were unchanged even after purification of the samples by gas-liquid chromatography, were not different from those obtained by direct incubation of $[3^3H_2]2$-oxoglutarate with the anaemic blood preparations (table 4.6). It was therefore deduced that the problem of randomization of 2-oxoglutarate carbon skeleton had not been circumvented.

The determination of magnitude of randomization of 2-oxoglutarate carbon skeleton.

Since we had apparently failed to obviate the randomization, it was decided to diminish and determine the extent of it by biosynthesising haem from $[5^{14}$C]2-oxoglutarate in the presence of excess non-
Table 4.6
The biosynthesis of haem from $[\beta - ^3H_2]ALA$.

4 μmoles of $[3-^3H_2]2$-oxoglutarate ($^3H = 61 \times 10^6$ cpm/μmole) were incubated (by M.M. Abboud) with 2-oxoglutarate dehydrogenase (partially purified from Escherichia coli) and ALA synthetase (isolated from Rhodopseudomonas spheroides) in situ.

1 μmole of supposedly $[\beta - ^3H_2]ALA$ synthesised by this system was incubated with a haemolysed preparation of anaemic chick blood (fresh blood 25 ml) as described in table 4.4 to biosynthesise tritiated haem. The $^3H$-haem (20 mg $48 \times 10^3$ cpm) was mixed with $^{14}C$-haem (40,000 cpm) and degraded to haematinic acid (HA) and ethylmethyl maleimide (EMM).

<table>
<thead>
<tr>
<th>No.</th>
<th>$^3H/^{14}C$ ratio in HA</th>
<th>$^3H/^{14}C$ ratio in EMM</th>
<th>$^3H$ loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
<td>Predicted</td>
<td>Found</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.69</td>
<td>0.50</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>0.69</td>
<td>0.50</td>
</tr>
</tbody>
</table>
radioactive succinate. The latter was expected to dilute any radioactive succinic acid generated through equation 2 (p.113) and, hence diminish its contribution to the porphyrin biosynthetic pathway.

The advantage of using $[5-^{14}\text{C}]2$-oxoglutarate was that in the absence of randomization of label, the degradation of the haem would yield all the radioactivity in haematinic acid and none at all in ethylmethyl maleimide. This point is fully illustrated in fig. 4.9. The presence of radioactivity (if any) in ethylmethyl maleimide could be used, therefore, to estimate the extent of randomization.

Bearing this in mind, $[5-^{14}\text{C}]2$-oxoglutarate ($40 \times 10^6$ cpm contained in 68 μmoles) were incubated with haemolysed preparations of anaemic chick blood in the presence of disodium succinate (310 μmoles) as described in table 4.5. On degradation of haem (25 mg) biosynthesised in these experiments, the ratio of the specific activities of haematinic (A) acid and ethylmethyl maleimide (B) was found to be 6.4:1 ($\frac{945}{147}$ cpm). The distribution of radioactivity in (A) and (B) is shown below.

The conclusion was drawn, therefore, that the molecules of succinyl-CoA incorporated into haem as $\text{HOOC-CH}_2-\text{CH}_2-\text{GOSCoA}$
Fig. 4.9 Letters A-D refer to pyrrolic rings in haem molecule.
and $^{14}$C-CH$_2$-CH$_2$-COSCoA were in the ratio of 9.2:1.

This would give about 9% randomization of the carbon skeleton of $[^{14}$C]2-oxoglutarate (see above).

When haem biosynthesized from $[3^{3}$H$_2]$2-oxoglutarate under these conditions of minimal randomization, was mixed with $^{14}$C-haem and degraded, haematinic acid and ethylmethyl maleimide were found to have $^{3}$H/$^{14}$C ratios of 1.0 and 0.61 respectively.

When one allows for 9% contribution made by randomization of the label, the theoretical $^{3}$H/$^{14}$C ratios of 1.0 and 0.59, (illustrated in fig. 4.10) predicted by the mechanism of the type II, for haematinic acid and ethylmethyl maleimide were in close agreement with the experimentally determined values.

The enzymic preparation of $[3R-^{3}$H]- and $[3S-^{3}$H]-2-oxoglutarate.

In order to determine which of the two $\beta$-hydrogen atoms of the propionic acid side chains was removed during the vinyl group formation in the biosynthesis of haem, samples of $[3R-^{3}$H]- and $[3S-^{3}$H]-2-oxoglutarate were prepared by means of an exchange reaction involving NADPH-dependent isocitrate dehydrogenase.

$[3R-^{3}$H]2-oxoglutarate was prepared by incubating randomly labelled $[3-^{3}$H$_2]$2-oxoglutarate with isocitrate dehydrogenase in the presence of NADPH (table 4.7) over a long period of time. The results (fig. 4.11) showed that in the presence of complete system there was time-dependent tritium loss which ceased after 50% of the original radioactivity of $[3-^{3}$H$_2]$2-oxoglutarate had been released into the medium. Under parallel conditions but in the presence of boiled enzyme, there was less than 5% tritium loss
2-Oxoglutarate  

\[
\text{COOH} \quad \text{CH}_2 \quad \text{COOH} \\
\text{CT}_2 \quad \text{CT}_2 \quad \text{CO} \\
\text{CO} \quad \text{CO}_2 \\
\text{COOH}
\]

Randomization of carbon skeleton

\[
\text{COOH} \quad \text{CT}_2 \quad \text{CT}_2 \quad \text{CO} \\
\text{CO} \quad \text{COO} \\
\text{COOH}
\]

Succinyl CoA

\[
\text{COOH} \quad \text{CT}_2 \quad \text{CT}_2 \quad \text{COO}_2 \\
\text{CO} \quad \text{CO}_2 \\
\text{CH}_2 \text{NH}_2
\]

ALAs

\[
\text{[H]Haem} \quad \text{[14C]Haem}
\]

(i) + (ii) Degradation

\[
\text{3H/14C ratios of HA and EMM are 1.0 and 0.58 respectively.}
\]

\[
\frac{3\text{H}}{14\text{C}} = \frac{45.5 + 9 + 9}{2} = 31.75 \quad \frac{3\text{H}}{14\text{C}} = \frac{91 + 9 + 9}{2} = 54.5
\]

Fig. 4.10 In this representation it is assumed that the vinyl group formation occurs through mechanism II (p 97). Numerical figures indicate tritium radioactivity at those carbon atoms. * indicates $^{14}$C radioactivity originating from [4-$^{14}$C]ALA.
The enzymic preparation of $[3^3\text{H}]2$-oxoglutarate.

The incubation mixtures contained $[3\text{RS}^3\text{H}_2]2$-oxoglutarate disodium salt, (52 µmole, $4.26 \times 10^6$ cpm); NADPH, (6 µmole); MgCl$_2$, (6 µmole); triethanolamine-HCl buffer, pH 7.4, (1 mmole) and isocitrate dehydrogenase (25 units) in a total volume of 10 ml. The control incubations contained either no NADPH or boiled enzyme. The incubations were carried out at 30°. At intervals 0.1 ml aliquots were removed, diluted to 0.5 ml with H$_2$O and applied to Dowex-1-Cl column (50 x 6.5 mm). The column was washed with water (10 ml). 0.1 ml of the eluent was counted to determine the free tritium in the medium.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Minus NADPH</th>
<th>Boiled Enzyme</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^3\text{H}$ in medium (cpm)</td>
<td>% $^3\text{H}$ loss</td>
<td>$^3\text{H}$ in medium (cpm)</td>
</tr>
<tr>
<td>0</td>
<td>0.91x10$^6$</td>
<td>0.21</td>
<td>0.90x10$^6$</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>9.5x10$^6$</td>
<td>2.2</td>
<td>10x10$^6$</td>
</tr>
<tr>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.0</td>
<td>20x10$^6$</td>
<td>4.7</td>
<td>20.5x10$^6$</td>
</tr>
</tbody>
</table>

More enzyme and NADPH were added at this stage to ensure that reduction in the rate of tritium loss from $[3^3\text{H}_2]2$-oxoglutarate was not due to the lack of these components.
Chemical and enzymatic degradation of $\Delta^2$-[3-3H2]-oxobutirate.

Figure 4.11

Chemical

Enzymatic

Time (hr)

Loss $H_3^3\%$

0 10 20 30 40 50
from $[3^{-3}\text{H}_2]2$-oxoglutarate.

In view of the findings of Rose (130) and Rose (131) this sample, which had specific activity of $4.12 \times 10^6$ cpm/µmole and was used without its isolation from the incubation mixture, should contain more than 95% of the tritium in 3R configuration and may be represented as shown below:

\[
\begin{align*}
\text{HOOC-} & \quad \text{H} \\
& \quad \text{S} \\
& \quad \text{3R} \\
\text{CH}_2\text{COOH}
\end{align*}
\]

The stereoisomer with the opposite configuration (i.e. $[3S^{-3}\text{H}]2$-oxoglutarate) was made by incubating non-radioactive disodium 2-oxoglutarate with isocitrate dehydrogenase and NADPH in the presence of tritiated water (table 4.8). After 6 hrs, the incubation mixture was freeze dried to recover T$_2$O and after dissolving the residue in water, it was applied to Dowex-1-Cl column which was washed free of tritium with water. The tritiated 2-oxoglutarate was eluted with 0.05N HCl. The solid compound, which had specific activity of $5.5 \times 10^6$ cpm per µmole was obtained after evaporating HCl by freeze drying. Under identical conditions non-enzymic incorporation of tritium into 2-oxoglutarate was found to be less than 0.5%.

The stereochemistry of proton elimination during the vinyl group formation in the haem biosynthesis.

To determine the stereochemistry of the proton eliminated during the vinyl group formation $[3R^{-3}\text{H}]$-, $[3S^{-3}\text{H}]$- and $[3RS^{-3}\text{H}_2]$-2-oxoglutarate were converted to haem as described previously.
The preparation of $[3S-^3H]2$-oxoglutarate.

The constitution of the incubation mixture was identical to that described in table 4.7 except that non-radioactive disodium 2-oxoglutarate was used and the incubation mixture contained 10 curies of tritiated water ($66.6 \times 10^8$ cpm/$\mu$g atom of hydrogen). After 6 hr the incubation mixture was freeze dried and the residue, after dissolving in 10 ml $H_2O$, was applied to Dowex-1-Cl column (130 x 6.5 mm) and washed with water until no significant tritium could be detected in the eluent. Finally the 2-oxoglutarate was eluted with 0.05N HCl, 0.1 ml of which was counted to determine incorporation of tritium into 2-oxoglutarate.

<table>
<thead>
<tr>
<th></th>
<th>Total $^3H$</th>
<th>$^3H$ cpm/$\mu$mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>complete system</td>
<td>$285 \times 10^6$</td>
<td>$5.5 \times 10^6$</td>
</tr>
<tr>
<td>minus enzyme</td>
<td>$1.32 \times 10^6$</td>
<td>$2.5 \times 10^4$</td>
</tr>
</tbody>
</table>
After mixing this tritiated haem with $^{14}$C-haem, it was degraded to haematinic acid and ethylmethyl maleimide. The results (table 4.9) indicated that haematinic acid and ethylmethyl maleimide obtained from haem, which was biosynthesised from $[3R-^3H]2$-oxoglutarate, had $^3H/^{14}C$ ratios of 1.0 and 0.92 respectively (table 4.9). On the other hand, haem biosynthesised from $[3S-^3H]2$-oxoglutarate gave haematinic acid and ethylmethyl maleimide which had $^3H/^{14}C$ ratios of 1.0 and 0.61 respectively. Since in these experiments one expected a complete loss and complete retention of tritium during the vinyl group formation, the presence of more than 90% tritium radioactivity in ethylmethyl maleimide obtained from $[3R-^3H]2$-oxoglutarate was clearly in agreement with the mechanism of the type II.

However, in view of the close similarity between the results obtained from haems biosynthesised from $[3RS-^3H_2]$- and $[3S-^3H]$-2-oxoglutarate, it was envisaged that probably the label in the latter had racemized to give $[3RS-^3H_2]2$-oxoglutarate. This possibility seemed particularly feasible when it was considered that the isolation of $[3S-^3H]2$-oxoglutarate involved prolonged treatment with HCl.

In order to investigate this possibility of racemization, part of the $[3S-^3H]2$-oxoglutarate, used in the biosynthesis of haem above, was incubated with isocitrate dehydrogenase and NADPH. If racemization had not occurred one would expect a time-dependent loss of all the tritium from this compound. However the results (table 4.10) indicated that there was only
Table 4.9

The biosynthesis of haem from [3R-\(^3\)H]-, [3S-\(^3\)H]- and [3RS-\(^3\)H\(_2\)]-2-oxoglutarate.

[3R-\(^3\)H]2-oxoglutarate, (107.5 x 10\(^6\) cpm as made in table 4.7); [3S-\(^3\)H]2-oxoglutarate, (142.5 x 10\(^6\) cpm, as made in table 4.8); and [3RS-\(^3\)H\(_2\)]2-oxoglutarate, (130 x 10\(^6\) cpm) were converted to haem as described in table 4.5.

30,000 cpm of each type of \(^3\)H haem were degraded to haematinic acid (HA) and ethylmethyl maleimide (EMM) as described in Experimental.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(^3)H/(^14)C in HA</th>
<th>(^3)H/(^14)C in EMM</th>
<th>% (^3)H retention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
<td>† Predicted</td>
<td>Found</td>
</tr>
<tr>
<td>[3R-(^3)H]2-oxoglutarate</td>
<td>1.0</td>
<td>0.92</td>
<td>1.0</td>
</tr>
<tr>
<td>[3S-(^3)H]2-oxoglutarate</td>
<td>1.0</td>
<td>0.62</td>
<td>0.16</td>
</tr>
<tr>
<td>[3RS-(^3)H(_2)]2-oxoglutarate</td>
<td>1.0</td>
<td>0.61</td>
<td>0.58</td>
</tr>
</tbody>
</table>

† Predicted after accounting for 9% randomization of carbon skeleton of 2-oxoglutarate.
50% enzyme-catalysed $^3$H loss from this sample identical to the loss from an authentic $[3RS-^3H_2]2$-oxoglutarate (fig. 4.12). The results demonstrated clearly that the label in $[3S-^3H]-2$-oxoglutarate had racemised.


Owing to our inability to prepare $[3S-^3H]$2-oxoglutarate, it was felt necessary to substantiate previous results by using stable stereoisomers. We decided to employ succinic acid which has the advantage not only of being stable but also of having a carbon skeleton which, unlike that of 2-oxoglutarate, does not undergo randomization.

The samples of succinic acid required for the biosynthetic work were prepared as follows. Treatment of $[3RS-^3H_2]2$-oxoglutarate with hydrogen peroxide gave $[2R-^3H]_2$succinic acid. The incubation of $[3RS-^3H_2]2$-oxoglutarate with isocitrate dehydrogenase in the presence of NADPH led to the release of 50% of the original tritium in the medium (p. 123 and table 4.7). After treatment of the incubation mixture with hydrogen peroxide, $[2R-^3H]$succinic acid was obtained. The recommended procedure (132) for the preparation of $[2S-^3H]$succinic acid proved unsatisfactory in our hands. Therefore this stereoisomer was prepared by incubating disodium 2-oxoglutarate with isocitrate dehydrogen-
Table 4.10

The detritiation of \( [3RS-^3H_2] \) and \( [3S-^3H] \) 2-oxoglutarate
by isocitrate dehydrogenase.

Triethanolamine-HCl buffer, pH 7.1, (100 μmoles), MgCl₂,
(0.6 μmole); NADPH, (1.3 μmole); isocitrate dehydrogenase,
(10 units); and \( [3RS-^3H_2] \) 2-oxoglutarate, (7 μmole, 24 x 10^6
cpm) or \( [3S-^3H] \) 2-oxoglutarate, (7 μmole, 5.5 x 10^6 cpm)
were incubated in a total volume of 1 ml at 30°. The \(^3\)H
loss into the medium was estimated as described in
table 4.7.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>([3S-^3H] ) 2-oxoglutarate</th>
<th>Authentic ([3RS-^3H_2] ) 2-oxoglutarate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (^3)H in medium</td>
<td>% of the total (^3)H in medium (ie % (^3)H loss)</td>
</tr>
<tr>
<td></td>
<td>cpm</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>1.8x10³</td>
<td>3.29</td>
</tr>
<tr>
<td>0.50</td>
<td>1.8x10⁶</td>
<td>32.90</td>
</tr>
<tr>
<td>1.5</td>
<td>2.47x10⁶</td>
<td>45.00</td>
</tr>
<tr>
<td>3.5</td>
<td>2.72x10⁶</td>
<td>49.40</td>
</tr>
<tr>
<td>6.5</td>
<td>2.8x10⁶</td>
<td>51.00</td>
</tr>
</tbody>
</table>

\( \dagger \) 2 units of enzyme were added.
and [3RS-3\text{-H}\text{\textsuperscript{2}}\text{-oxoglutarate}]

determination of [3S\text{-H}\text{\textsuperscript{2}}\text{-oxoglutarate}]

Fig. 4.12
ase in the presence of $T_2O$ and NADPH followed by treatment with hydrogen peroxide to $[2S-^3H]$succinic acid. The non-enzymic incorporation of tritium in succinic acid under these conditions was found to be less than 0.5%.

In order to facilitate the understanding of the work described below, attention is again drawn to the fact that succinic acid is a symmetrical compound. Therefore the label introduced at only one of the methylene groups may be considered to be equally distributed between both methylene groups. Consequently $[2R-^3H]$succinic acid may be represented by the structure in fig. 4.13. For the sake of clarity the path of the labelled tritium during the biosynthesis and degradation is traced, in fig. 4.13 and fig. 4.14, using only $[2R-^3H]$succinic acid.

In parallel incubations the three samples of tritiated succinic acid prepared above were biologically converted to haem which was mixed with $^{14}C$-haem (prepared from $[4-^{14}C]$-ALA) to act as internal reference. The degradation of haem biosynthesised from $[2RS-^3H_2]$succinic acid gave haematinic acid and ethylmethyl maleimide which had $^3H/^{14}C$ ratios of 1.00 and 0.85 respectively (table 4.11). These values were
rationalized by assuming that for the six $^3\text{H}$ atoms present in haematinic acid, ethylmethyl maleimide contained only five (fig. 4.12). The haem biosynthesised from $[2R-^3\text{H}]$succinic acid gave $^3\text{H}/^{14}\text{C}$ ratios of 1.0 and 0.94 - 0.96 respectively in haematinic acid and ethylmethyl maleimide, thus showing that the hydrogen atom with R configuration was not eliminated during the vinyl group formation. However, when haem synthesised from $[2S-^3\text{H}]$-succinic acid was degraded, haematinic acid and ethylmethyl maleimide had $^3\text{H}/^{14}\text{C}$ ratios of 1.0 and 0.70 respectively, thus establishing the loss of the $S$ hydrogen atom during the vinyl group formation in haem biosynthesis. The remarkable feature of the results described above was the close agreement between the experimental and predicted values (table 4.11).

Discussion.

Since the mature mammalian erythrocytes lack the protein and haem biosynthesising capacity (table 4.1), in studies of haem biosynthesis it has always been necessary to employ either bone marrow, avian blood or blood from anaemic animals. All these systems contain nucleated red cells and reticulocytes and therefore possess the full apparatus for the biosynthesis of haem. In laboratories anaemia is often induced by periodic bleeding or by injections of haemolytic agents like phenylhydrazine. Though the mechanism of action of this compound is still obscure, it may be surmised that it can somehow abolish (or over-stimulate) the erythropoietic control system and thus cause a
Table 4.11

$[2RS-^3H_2]$succinic acid, $(215 \times 10^6 \text{ cpm})$; $[2R-^3H]$succinic acid, $(108 \times 10^6 \text{ cpm})$; and $[2S-^3H]$succinic acid, $(45 \times 10^6 \text{ cpm})$ all contained in 85 $\mu$moles, were converted to haem as described in table 4.5 except that no more non-radioactive succinic acid was added to the incubation mixtures.

10 mg of haem (110,000 cpm) made from $[2RS-^3H_2]$-succinic acid, 8 mg of haem (24,000 cpm) synthesised from $[2R-^3H]$succinic acid and 12 mg of haem (30,000 cpm) prepared from $[2S-^3H]$succinic acid were degraded to haematinic acid (HA) and ethylmethyl maleimide (EMM).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$^3H/\text{^14C}$ ratio in HA</th>
<th>$^3H/\text{^14C}$ ratio in EMM</th>
<th>Found</th>
<th>Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[2RS-^3H_2]$succinic acid</td>
<td>1.00</td>
<td>0.85</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>$[2R-^3H]$succinic acid</td>
<td>1.00</td>
<td>0.94 - 0.96</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>$[2S-^3H]$succinic acid</td>
<td>1.00</td>
<td>0.70</td>
<td>0.67</td>
<td></td>
</tr>
</tbody>
</table>
[2RS-$^3$H$_2$]succinic acid

$T = ^3$H

[2S-$^3$H]succinic acid

[2R-$^3$H]succinic acid

HA = Haematinic Acid

EMM = Ethylmethyl maleimide
release of immature red cells into the circulation.

Previous workers (133-136) had established that whole blood cells were permeable to glycine, succinate and ALA but not to citrate, 2-oxoglutarate or any intermediate past the ALA stage in the biosynthesis of haem and that haemolysis did not diminish the haem biosynthesising capacity of the blood. We found that haemolysed preparations of duck blood and phenylhydrazine-treated chick blood incorporated about 10% ALA into haem (table 4.3). However, if the injections of phenylhydrazine were continued beyond the normal one-week period, haemolysed chick blood preparations could produce up to 22% incorporation of ALA into haem. These results were in agreement with those of Vogel et al. (137) who found that haem biosynthesis was approximately proportional to the percentage of reticulocytes in duck blood.

In contrast to early steps in haem biosynthesis, i.e. glycine to coproporphyrinogen-III, which are anaerobic reactions, conversion of coproporphyrinogen-III to protoporphyrin-IX is an aerobic reaction in all systems so far studied except Rhodopseudomonas spheroides and Chromatium strain D (83). A similar oxygen requiring system has been demonstrated in the biosynthesis of long chain fatty acid biosynthesis. Bloch (138) showed that the co-enzyme A esters of long chain fatty acids were converted to the corresponding olefinic acids by a particulate preparation from yeast only in the presence of oxygen and NADPH. Oxygen appeared to be the primary electron acceptor and was not replaceable by any other electron acceptor.
Since the reaction exhibited the characteristic dual requirement of an oxygenase (hydroxylase), the fatty acid desaturation was considered to involve hydroxylation or some other type of oxygenation of the substrate in the primary step (this was in spite of the fact that 9- or 10-hydroxystearate, 9-ketostearate, 9,10-cis-epoxy stearate and 9,10-dihydroxystearate have proven totally inactive as precursors of oleate in the yeast system).

In view of this analogy between the fatty acid and haem biosynthesis Sano prepared 2,4'-bis-(p-hydroxy propionate) deuteroporphyrinogen-IX and showed that it could be converted to protoporphyrin-IX under anaerobic conditions. Although these results explained the participation of oxygen in the reaction, they failed to explain the mechanism of the reaction, because the hydroxy intermediate could be converted to or derived from a number of intermediates. This point has been sufficiently emphasised in fig. 4.3.

In order to elucidate the mechanism of the vinyl group formation we proposed two broad mechanisms, which not only embraced a hydroxy intermediate of the type proposed by Sano but could also accommodate other proposed mechanisms (see introduction to this section). In mechanism I (p.97), the decarboxylation of the propionate leading to vinyl group formation was visualised to involve the formation of a ketonic intermediate. In this mechanism both β-hydrogen atoms of the propionic acid side chain would be removed during this reaction. In mechanism II (p.97), the decarboxylation was proposed to proceed through the
loss of a β-substituent (of propionate) leading directly to the formation of the vinyl group. This mechanism predicted that only one of the two original β-hydrogen atoms of the propionate residue would be lost.

Availability in our laboratory of ALA tritiated at β- and δ-carbon atoms (table 4.2) enabled us to perform experiments which led us to decide between these two mechanisms. When haem biosynthesised, by haemolysed preparations of phenylhydrazine-treated chick blood, from [β,δ-3H₄]ALA was mixed with ¹⁴C-haem (prepared from [4-¹⁴C]ALA) to act as an internal reference and then degraded to haematinic acid and ethylmethyl maleimide, they were found to have ³H/¹⁴C ratios of 1.0 and 0.58 (table 4.4) respectively. The loss of more than 40% of the tritium in ethylmethyl maleimide was sufficiently close to the theoretical loss of 50%, predicted by mechanism II, to suggest that the vinyl group formation occurred by a mechanism of the type II. The mechanism I would have required ethylmethyl maleimide to be completely devoid of tritium radioactivity.

Attempts to verify these results using [3-³H₂]2-oxoglutarate as direct substrate or after its prior conversion to ALA for haem biosynthesis at first were not successful (table 4.5 and table 4.6) because of the enzyme-catalysed randomization of the label in 2-oxoglutarate at the succinyl CoA stage (p.113). However, using [5-¹⁴C]2-oxoglutarate, it was shown that in the presence of excess succinate this randomization could be minimised to 9% (p.116). Therefore, when haem biosynthesised from [3-³H₂]2-oxoglutarate under these conditions of minimal randomization was
degraded and allowance was made for 9% randomization of label, the $^{3}\text{H}/^{14}\text{C}$ ratio of 1.0 for haematinic acid and 0.61 for ethylmethyl maleimide ($^{3}\text{H}/^{14}\text{C}$ ratio for ethylmethyl maleimide predicted by mechanism II is 0.59) confirmed our previous finding that the vinyl group formation occurs through mechanism of the type II.

In order to study the stereochemistry of the proton elimination during the vinyl group formation, samples of $[3\text{R}^{-3}\text{H}]-$ and $[3\text{S}^{-3}\text{H}]-2$-oxoglutarate were prepared by NADPH- and isocitrate dehydrogenase-dependent détritiation of $[3\text{RS}^{-3}\text{H}_2]^{-}$ 2-oxoglutarate (table 4.7) and tritiation of disodium 2-oxoglutarate in presence of $\text{T}_2\text{O}$ (table 4.8) respectively. Although we failed to prepare $[3\text{S}^{-3}\text{H}]2$-oxoglutarate, the conversion of $[3\text{R}^{-3}\text{H}]-2$-oxoglutarate to haem and subsequent degradation (after mixing with haem made from $[4^{-14}\text{C}]\text{ALA}$) of the latter to haematinic acid and ethylmethyl maleimide revealed that 92% of the original tritium radioactivity was retained in ethylmethyl maleimide thus showing that $\beta$-hydrogen atom of propionic acid residue with R configuration was retained during the vinyl group formation (table 4.9). This stereochemical data was further substantiated by biosynthesising haem from $[2\text{R}^{-3}\text{H}]-$, $[2\text{S}^{-3}\text{H}]-$ and $[2\text{RS}^{-3}\text{H}_2]^{-}\text{succinate}$ (these stereoisomers were prepared by, in situ, decarboxylation (with $\text{H}_2\text{O}_2$) of their 2-oxoglutarate precursors).

To understand the results of the experiments in which succinate was used for haem biosynthesis, it must be appreciated
that succinate is a symmetrical molecule and therefore label introduced at only one of the methylene groups would be equally distributed between both methylene groups (see fig. 4.13 and fig. 4.14).

The degradation of haem biosynthesised from [2RS-\(3^2\)H] succinate showed that 15% (against 17% predicted by mechanism II) of the original tritium radioactivity at the \(\beta\)-carbon atom of the propionic acid residue was lost during the vinyl group formation. When haem biosynthesised from [2R-\(3^2\)H]- and [2S-\(3^2\)H]-succinate was degraded, the results revealed 4-6% (cf. zero% predicted by mechanism II) and 30% (cf. 33% predicted by mechanism II) loss of tritium respectively (table 4.11) during the vinyl group formation (fig. 4.12a).

These and previous results allowed two main conclusions to be drawn. Firstly, both vinyl groups in a haem molecule were formed through the loss of \(\beta\)-hydrogen atoms of propionate side chains with \(S\) configuration. For if hydrogen atoms with the opposite stereochemistry were eliminated during the vinyl group formation, (i.e. if the formation of one of the vinyl groups occurred through the loss of \(S\) hydrogen and that of the other via the loss of \(R\) hydrogen) the radiochemical data in table 4.11 would be identical whether haem had originated from 2R or 2S tritiated succinate. Secondly, in all cases the relative tritium contents of haematinic acid and ethylmethyl maleimide clearly showed that hydrogen atoms at the \(\alpha\) carbon atoms of the propionate residues were not distributed during the biosynthesis of haem. This eliminates the involvement of an acrylic acid (cis or trans) intermediate. The non-
To C Hr - C O O H

[2R - $^3$H]succinate

HOOCH

HOOC—CHT.COOH

PBG

[3R - $^3$H]ALA

Coproporphyrinogen-III

T = $^3$H

M = -CHT

R$_1$ = -CHT.CHT.COOH

Protoporphyrin-IX

Fig. 4.13
Degradation of haem.

Fig. 4.14
involvement of a hydrogen atoms of propionate residues in protoporphyrin-IX biosynthesis in Euglena (139) has already been demonstrated.

The accumulative evidence presented in this chapter establishes that in anaemic chick blood the formation of both the vinyl groups in haem biosynthesis involves the loss of the β hydrogen atom with S configuration, thus suggesting the mechanistic sequence in fig. 4.15,

\[
\begin{align*}
\overset{HS}{\text{Pyr}} & \quad \overset{\text{HR}}{\text{CH}_2\text{COOH}} \quad \overset{\text{HR}}{\text{CH}_2\text{COOH}} \\
\text{Pyr} & \quad \overset{\text{H}, \text{OH or a good leaving group}}{\text{Pyr}}
\end{align*}
\]

\(X = \text{H, OH or a good leaving group}\)

\(\text{Pyr} = \text{Pyrrolic residue}\)

Fig. 4.15

or its mechanistic variant as shown in fig. 4.16 (140).

\[
\begin{align*}
\overset{\text{HR}}{\text{N}} & \quad \overset{\text{CH}_2\text{COOH}}{\text{N}} \quad \overset{\text{CH}_2\text{COOH}}{\text{N}} \\
\text{N} & \quad \overset{\text{HR}}{\text{N}} \quad \overset{\text{HR}}{\text{N}}
\end{align*}
\]

Fig. 4.16
Experimental.

Materials.

Liquid scintillation counting fluid NE 250 was purchased from Nuclear Enterprises, Edinburgh, Scotland. Sodium 2-oxoglutarate-5-$^{14}$C, δ-amino levulinic-4-$^{14}$C acid hydrochloride and T$_2$O were bought from the Radiochemical Centre, Amersham, Bucks, U.K. δ-amino levulinic-5-$^{14}$C acid hydrochloride was obtained from NEN Chemicals, Germany. Haemin, pyridoxal phosphate, palladium catalyst (10% on charcoal) were purchased from Koch-Light Laboratories, Colnbrook, Bucks, U.K. Perspex chips were obtained from Griffin and George Ltd., London, U.K. All other chemicals were bought from the British Drug Houses Ltd., Poole, Dorset, U.K.

Methods.

The preparation of $[^{3}H_4]ALA$: The tritiated ALA was prepared by autoclaving 100 mg ALA with 0.1 Curie T$_2$O contained in 0.1 ml H$_2$O (specific activity $1.1 \times 10^7$ cpm/$\mu$g atom of hydrogen) in an evacuated and sealed tube for 15 min at 15 lb/sq.in. The tritiated water was removed from ALA by freeze drying the material at least three times.

The preparation of deuterated ALA: 100 mg of ALA were autoclaved with 0.5 ml of D$_2$O (99.8% D) or 0.25 ml D$_2$O + 0.25 ml DCl (20% D) for various time intervals (see table 4.2) as described above. D$_2$O and DCl were finally removed by freeze drying three times.
The preparation of \([3-\text{H}_2]2\text{-oxoglutarate}\) (130): This compound was prepared by autoclaving in an evacuated and sealed tube 100 mg of 2-oxoglutarate with 0.25 ml of tritiated water for 15 min at 15 lb/in\(^2\). The free tritium was removed by freeze drying the sample three times. The amount of tritium radioactivity attached to (i.e. free \(^3\text{H}\)) and incorporated into 2-oxoglutarate was estimated as follows. 1 mg (5.20 \(\mu\)mole) of tritiated 2-oxoglutarate dissolved in 0.5 ml of water was applied to a Dowex-1-Cl column (50 x 6.5 mm) which was first washed with 10 ml of water (which was retained) and then with excess water. 2-oxoglutarate was eluted from the column by 0.05 N HCl (10 ml). Measurement of the radioactivity in the water gave the amount of free tritium attached to 2-oxoglutarate, whereas that in the HCl reflected the tritium incorporated into 2-oxoglutarate. It was found that 2-oxoglutarate contained \(8.3 \times 10^6\) cpm/\(\mu\)mole and only 0.2\% was free tritium.

The analysis of tritium label in ALA: This analysis essentially involved the degradation of ALA to succinic acid which was carried out as follows.

1 mg of tritiated ALA and \([4-^{14}\text{C}]\)ALA (10,000 cpm) were dissolved in 0.5 ml of \(\text{H}_2\text{O}\). 0.25 ml of this solution and 1-2 mg of non-radioactive ALA were reduced with sodium borohydride (3 mg) at 0\(^\circ\) for 30 min. 0.1 ml of the reduced solution was chromatographed, only once, to isolate \(\delta\)-amino dihydrolevulinic acid (see p.64). 0.2 ml of the original solution was treated with saturated sodium bicarbonate (10 ml), sodium metaperiodate (1 ml, from 100 mg/ml solution) and 50 mg succinic acid and the reaction mixture was stood in complete darkness for 8 hrs.
At the end of this period, the reaction mixture was filtered. The filtrate was acidified with concentrated HCl to pH 1-1.5 and then evaporated to complete dryness. Succinic acid was extracted from the residue with acetone and purified by sublimation at 100-110°/0.08 mm. The yield was about 30 mg.

By comparing the $^3$H/$^{14}$C ratios of $\delta$-amino dihydro-levulinic acid and that of succinic acid, it was found that 56.5% of the tritium radioactivity was contained in the succinic acid fragment of ALA.

The degradation of deuterated ALA to succinic anhydride: 100 mg of deuterated ALA, in a 50 or 100 ml flask, was treated with a saturated solution of sodium bicarbonate (20 ml) and 2 ml (200 mg) of sodium metaperiodate and left in the dark for 8 hrs. (No carrier succinic acid was added this time). The crude product extracted from the reaction mixture was sublimed at 100-110°/0.08 mm to give 40-45 mg of succinic acid which was then refluxed for 2 hrs with freshly distilled acetyl chloride (2.5 ml). After removal of acetyl chloride at low pressure, the residue was sublimed during 30 min at 55-60°/0.08 mm. Succinic anhydride (25 mg) thus obtained was recrystallised from anhydrous acetone and ether and then analysed for its mass spectrum.

The enzymic synthesis of [3R-$^3$H]2-oxoglutarate (131):

The reaction was carried out in a 25 ml conical flask at 30°. The incubation mixtures contained [3-$^3$H$_2$]2-oxoglutarate disodium salt (52 μmoles, 8.2 x 10^6 cpm/μmole); NADPH, (6 μmoles); MgCl$_2$, (6 μmoles); triethanolamine-HCl buffer (pH 7.1), (1 mmole) and pig-heart isocitrate dehydrogenase (25 units)
in a total volume of 10 ml. At intervals 0.1 ml aliquots were removed, diluted with water to about 0.5 ml and applied to a Dowex-1-Cl column (50 x 6.5 mm). The column was washed with water (10 ml). 0.1 ml of the eluant was counted to determine free tritium in the medium. This determination of free tritium was continued until 50% of the radioactivity in [3-\( ^3\)H]-2-oxoglutarate was released into the medium.

[3R-\( ^3\)H]2-oxoglutarate thus produced was used in haem biosynthesis (on the same day or frozen at -18° and used the following day) without isolation from the reaction mixture. The synthesis of [2R-\( ^3\)H]succinic acid (132): The incubation conditions were exactly the same as that for [3R-\( ^3\)H]2-oxoglutarate except that the pH of the buffer was 7.6. After 50% of the tritium radioactivity in [3-\( ^3\)H]-2-oxoglutarate had been released into the medium, the incubation mixture was treated with 6.6 ml of 5% hydrogen peroxide (large excess). After 45 min at 30° or room temperature, the reaction mixture was applied to a Dowex-1-Cl column (130 x 6.5 mm) which was washed with a large excess of water (to remove free tritium and hydrogen peroxide) and finally succinic acid was eluted with 0.05 N HCl (about 20 ml). Evaporation of the HCl under high vacuum furnished dry succinic acid which contained 2.52 \( \times \) 10^6 cpm of \( ^3\)H/µmole.

The enzymic synthesis of [3S-\( ^3\)H]2-oxoglutarate: The reaction was carried out at 30° in a 25 ml conical flask. The constitution of the incubation mixture was identical to that described for the synthesis of [3R-\( ^3\)H]2-oxoglutarate except that non-radioactive
disodium 2-oxoglutarate (52 μmoles) plus 10 Curies of T_2O were used (to give specific activity of 66.6 \times 10^8 cpm/μg atom of hydrogen). After 6 hrs the incubation mixture was applied to a Dowex-1-Cl chloride column (130 x 6.5 mm) and the column washed with water until no significant amount of tritium could be detected in the eluent. Finally, 2-oxoglutarate was eluted with 0.05 N HCl (25-30 ml). Solid 2-oxoglutarate (specific activity, 5.5 cpm of ^3H/μmole) was obtained by freeze drying the HCl solution.

The synthesis of [3S-^3H]succinic acid: The reaction was carried out in 100 ml round bottom quick-fit flask. The incubation conditions were the same as described for [3S-^3H]2-oxoglutarate except that the pH of the buffer was 7.5 and the incubation mixture contained only 3.3 μmoles of MgCl₂. After 2 hrs 6.6 ml of 5% hydrogen peroxide were added to the reaction mixture which was stood at 30° for 30 min and then about 20 units of catalase were added to decompose hydrogen peroxide. After 15 min the contents of the flask were freeze dried to recover T_2O. The residue was dissolved in water and succinic acid was isolated as described above. The specific activity of [2S-^3H]-succinic acid was found to be 1.07 cpm of ^3H/μmole.

The decarboxylation of 2-oxoglutarate by hydrogen peroxide:

1 mg of [3RS-^3H₂]2-oxoglutarate was dissolved in 0.5 ml of 100 mM triethanolamine-HCl buffer (pH between 7.1 and 7.6) which also contained 0.3 or 0.6 mM MgCl₂ (and sometimes 2 mg serum bovine albumin to simulate the incubation condition
for synthesis of \([2R-^3\text{H}]-\text{and [2S-}^3\text{H}]-\text{succinate}\) and then treated with 5% hydrogen peroxide (0.30 ml). After 30 min a known aliquot of the solution was spotted on a thin layer silica plate which after developing in butanol, glacial acetic acid, water and ammonia 0.88 (120: 30: 50: 5 v/v) and drying thoroughly was sprayed with 0.4% solution of bromocresol purple in 50% ethanol made alkaline to pH 10 exactly. It was found that 99.5% of the tritium radioactivity applied to the plate could be recovered in succinic acid \((R_f 0.43)\) and less than 0.05% in 2-oxoglutarate spot \((R_f 0.25)\).

**Anaemic chick blood:** Chicks were made anaemic by subcutaneous injections of phenylhydrazine (5 mg/Kg body weight) on alternate days. After one week 20-25 ml of blood was withdrawn from the wing vein and chicks allowed to recover for a week before further use. Blood was collected in a beaker containing 0.5 ml heparine.

**Haemolysis of blood (140a):** The blood was centrifuged at about 2,000 rev/min between 0-4°C for 15 min. The supernatant and the top layer of leucocytes was discarded and the red blood cells washed twice in 0.9% NaCl (isotonic saline). The washed cells were transferred to a suitable flask to which water (at 0°C) equal in volume to the original blood sample was added during manual stirring which was continued for 5 min at 0-1°C.

**The biosynthesis of haem:** The incubations were carried out in 250 ml conical flasks at 37°C for 15 hrs. The incubation mixture for haem biosynthesis from radioactive ALA contained haemolysate (prepared from 25 ml of blood); ALA, (12-30 μmoles); Ferrous
sulphate, (0.72 µmole/1 ml blood); 0.15 M \( \text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4 \) buffer (pH 7.4) in 0.483 M KCl, (5.5 ml); Penicillin, (1.5 mg); streptomycin, (1.5 mg) in a total volume of 38 ml. When haem was biosynthesized from radioactive 2-oxoglutarate, ALA was replaced by glycine, (13.4 µmole/ml blood); neutralized L-malate, (1.12 mmole); disodium malonate (169 nmole) and disodium succinate, (305 µmole). However, when radioactive succinate was substrate for haem biosynthesis the total succinate concentration was kept down to 85 µmoles.

At the end of the incubation period 230 ml of acetone were added to precipitate all the protein which was filtered and thoroughly washed with acetone. Haemin was extracted from the protein precipitate as described below.

**Isolation of haemin (141)**

A stock solution of 2% strontium chloride hexahydrate in glacial acetic acid was prepared (this required vigorous stirring for a long period). An extraction solvent composed of one part of the stock solution plus three parts of acetone was prepared immediately before use to avoid crystallization of strontium chloride. The protein precipitate (which was treated as 25 ml of blood) thoroughly wetted with water (10-15 ml, this facilitates crystallization of haemin) was added with stirring to 12 volumes of the extraction solvent. The mixture was allowed to stand for 30 min with occasional stirring. Heating to boiling point two or three times briefly during this period seemed to increase protein precipitation. The mixture was then filtered and the residue washed twice with one volume of the extraction
The combined filtrate was heated in a beaker to 100° in the presence of antibumping granules (temperature was not allowed to exceed 102°). Crystals of haemin began to appear when 60 - 70% of the solution had evaporated. The crystallization was completed when the solution was allowed to cool to room temperature. The haemin was centrifuged and washed twice with 50% acetic acid and water and once with ethanol and ether. Further crystallization did not improve the impurity of this preparation.

**Liquid Scintillation counting of Labelled Haemin:** Haemin crystals (1-2 mg) weighed in a counting vial were allowed to dissolve in 2 - 3 drops of 1M lysine hydroxide in methanol by swirling. NE 250 (Nuclear Enterprises 250, 2ml) was then added followed by the addition of 0.15 - 0.2 ml of 30% hydrogen peroxide at 60 - 70° during continuous agitation, which was sustained until the intense red colour of haemin had decolorised to a greenish-pale hue. Then the vial was stood at this temperature for 20 - 25 min to decompose hydrogen peroxide. After this further 12 ml of NE 250 were added into the vial followed by 1 drop of glacial acetic acid. The completed sample was stored in the dark at 0 - 4° for 2 - 3 hrs. and then counted in Intertechnique or Philips liquid scintillation counter (it is necessary to use a counter which refrigerates samples being counted).

The degradation of haemin to ethylmethyl maleimide and haematinic acid.

The degradation of haemin to ethylmethyl maleimide and haematinic acid involved the following steps.
of acetic acid was quickly added first and flask swirled to mix
the pyridine solution and then the rest of the acetic acid was
added). Oxygen-free nitrogen was passed through the stirred
mixture and then 4 ml of freshly made near saturated solution
of FeSO$_4$$\cdot$7H$_2$O in concentrated HCl was added followed by 3 ml
of concentrated hydrochloric acid (total water including that in
HCl must not exceed 5% v/v). While stirring the passage of
oxygen-free nitrogen was continued for 30 min. After this
period, nitrogen was stopped (but not stirring) and the mixture
diluted with excess of ether (400 ml) which had been previously
shaken with anhydrous sodium chloride (sometimes addition of
large excess of ether leads to the appearance of a white precip-
itate which can be redissolved by adding more acetic acid) and
the pH of the solution brought to about 4 by the gradual addition
of 40% solution of anhydrous sodium acetate (a white precipitate
appears and then disappears when the pH has reached about 3).
The two layered solution was transferred to a 2 l. separation
funnel and the aqueous layer removed after vigorous shaking.
After carefully neutralizing some of the excess acid in the ethereal
layer with 150 ml of 5% sodium bicarbonate (anhydrous), the
(still) acidic solution of protoporphyrin-IX was washed three times
with 150 ml of water to remove FeSO$_4$, NaHCO$_3$ and CH$_3$COO$^-$/Na$^+$
(complete neutralization of the ethereal layer must be avoided
because protoporphyrin-IX is insoluble in neutral ether). Before
evaporating to dryness in a 500 ml round bottomed quick-fit flask,
under low pressure and below 30°, a spectrum of the solution
was taken.
(b) The reduction of protoporphyrin-IX to mesoporphyrin-IX (143):

The radioactive protoporphyrin-IX was always diluted to 60 mg with non-radioactive protoporphyrin-IX made by 'macro-scale method'.

78 mg of perspex chips were dissolved in 7.2 ml of 98-100% formic acid in the flask containing 60 mg of protoporphyrin-IX (dissolution of perspex takes at least 45 min under vigorous stirring) and after addition of 45 mg of palladium catalyst (10% palladium on charcoal) the mixture was hydrogenated between 48-51° while stirring, for 1 hr. Then 400 ml of ether (previously shaken with anhydrous sodium chloride) were added to precipitate the perspex and the solution was filtered. After taking the spectrum, the solution was evaporated to dryness in a 50 ml flask. (All the ether was removed first and then the residue was transferred into a 50 ml flask). The yield was more than 90%.

(c) The oxidation of mesoporphyrin (142): 60 mg of mesoporphyrin were suspended in 4.8 ml of 20% (w/w) of sulphuric acid and stirred at room temperature. Over a period of 30 min 108 mg of CrO₃ dissolved in 0.5 ml of water were added and the mixture was stirred for another 17 hrs. After this period, the greenish solution was extracted with ether in a continuous extractor for 20-24 hrs. The filtered ether solution contained ethylmethyl maleimide and haematinic acid.

(i) The separation of ethylmethyl maleimide and haematinic acid: The above ether solution was reduced in volume to 45-50 ml and extracted once with 2.4 ml of 5% sodium bicarbonate solution and then washed twice with 2.4 ml of H₂O. The combined sodium bicarbonate extract and washings were extracted five times
with an equal volume of pure chloroform (which had been
previously washed and shaken with anhydrous sodium chloride).
The ether and chloroform solutions containing ethylmethyl
maleimide were combined. The bicarbonate solution containing
haematinic acid was acidified to about pH 2 with 2 N sulphuric
acid (1 ml) immediately after the last chloroform extraction.
The haematinic acid once acidified could be stored at 0-4°C.

(ii) The isolation of ethylmethyl maleimide:
The combined ether and chloroform solution was evaporated
to dryness under atmospheric pressure and purified by sublimation
at 0.1-0.05 mm pressure, with bath temperature between 80-90°C.
The purified material was dissolved in ether and counted for
radioactivity. Its concentration was estimated by measuring the
absorption at 290 μm (molecular extinction coefficient 400, (144).

(iii) The isolation of haematinic acid:
The acidified aqueous solution containing haematinic acid
was extracted five times with an equal volume of ethyl acetate.
The ethyl acetate solution was evaporated to dryness and the
crude haematinic acid purified by sublimation at 0.1-0.05 mm
pressure, with bath temperature between 140-150°C. The puri-
fied material was dissolved in ether and counted for radioactivity.
Its concentration was ascertained by the method described for
ethylmethyl maleimide.
REFERENCES


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