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Mechanism and stereochemistry of enzymic reactions involved in porphyrin biosynthesis

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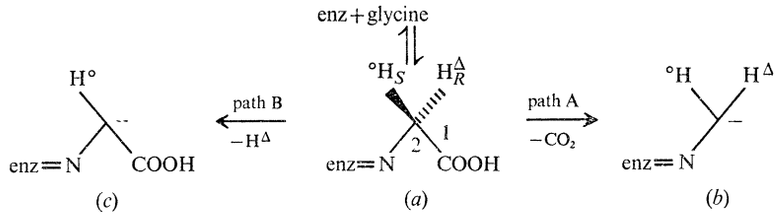
5-Aminolaevulinate synthetase catalyses the condensation of glycine and succinyl-CoA to give 5-aminolaevulinic acid. At least two broad pathways may be considered for the initial C—C bond forming step in the reaction. In pathway A the Schiff base of glycine and enzyme bound pyridoxal phosphate (*a*) undergoes decarboxylation to give the carbanion (*b*) which then condenses with succinyl-CoA with the retention of both the original C2 hydrogen atoms of glycine. In pathway B, loss of a C2 hydrogen atom gives another type of carbanion (*c*) that reacts with succinyl-CoA. Evidence has been presented to show that the initial C—C bond forming event occurs via pathway B which involves the removal of the pro *R* hydrogen atom of glycine. Subsequent mechanistic and stereochemical events occurring at the carbon atom destined to become C5 of 5-aminolaevulinate have also been delineated.



enz = enzyme-pyridoxal phosphate.

Several mechanistic alternatives for the formation of the two vinyl groups of haem from the propionate residues of the precursor, coproporphyrinogen III, have been examined.

$$-\overset{\beta}{C}H_2-\overset{\alpha}{C}H_2-COOH \longrightarrow -CH=CH_2 + CO_2.$$

It is shown that during the biosynthesis both the hydrogen atoms resident at the α positions of the propionate side chains remain undisturbed thus eliminating mechanisms which predict the involvement of acrylic acid intermediates. Biosynthetic experiments performed with precursors containing stereospecific labels have shown that the two vinyl groups of haem are formed through the loss of pro *S* hydrogen atoms from the β-positions of the propionate side chains. In the light of these results, three related mechanisms for the conversion, propionate → vinyl, have been considered.

In order to study the mechanism of porphyrinogen carboxy-lyase reaction, stereospecifically deuterated, tritiated-succinate was incorporated into the acetate residues of uroporphyrinogen III which on decarboxylation generated asymmetric methyl groups in coproporphyrinogen III and then in haem. Degradation of the latter yielded chiral acetate deriving from C and D rings of haem. Configurational analysis of this derived acetate shows that the carboxy-lyase reaction proceeds with a retention of configuration.

Mechanism of action of ALA synthetase

Introduction and background

The enzyme 5-aminolaevulinate synthetase (ALA-synthetase) is involved in the synthesis of the first unique intermediate in porphyrin and corrin biosynthesis. It catalyses the reaction according to the equation:

$$NH_2-CH_2-CO_2H + HO_2C-CH_2-CH_2-COSCoA \longrightarrow$$
$$CO_2 + CoASH + NH_2-{}^5CH_2-{}^4CO-{}^3CH_2\,{}^2CH_2-{}^1COOH$$

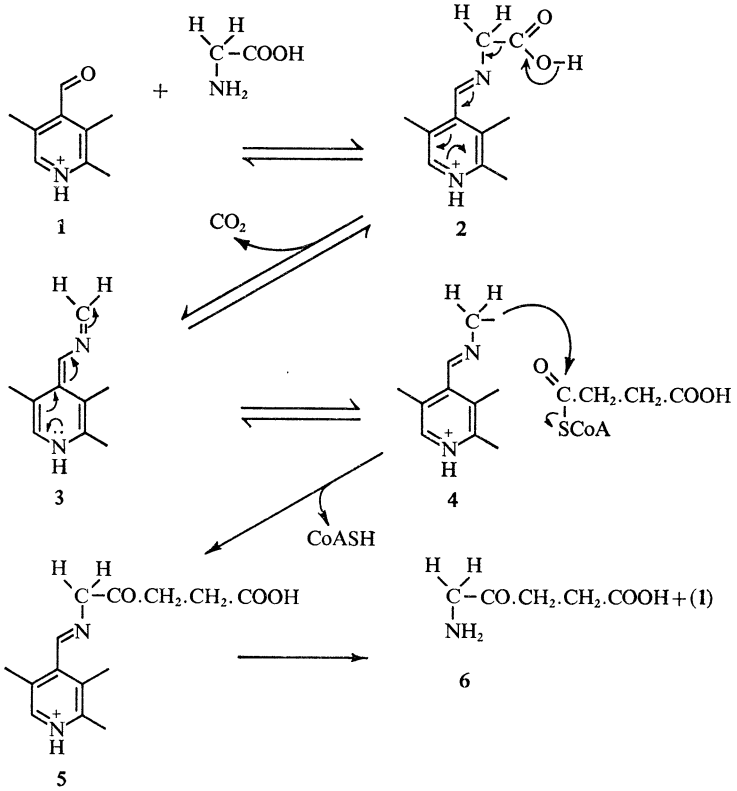
5-aminolaevulinic acid (ALA)

Early studies of Neuberger & Shemin on ALA-synthetase established a number of interesting features including the obligatory involvement of pyridoxal phosphate for the catalytic activity (for reviews see Neuberger 1961; Jordan & Shemin 1973). About six years ago we initiated a programme of research to explore the mechanistic details of the ALA-synthetase reaction (Akhtar & Jordan 1968; Zaman, Jordan & Akhtar 1973).

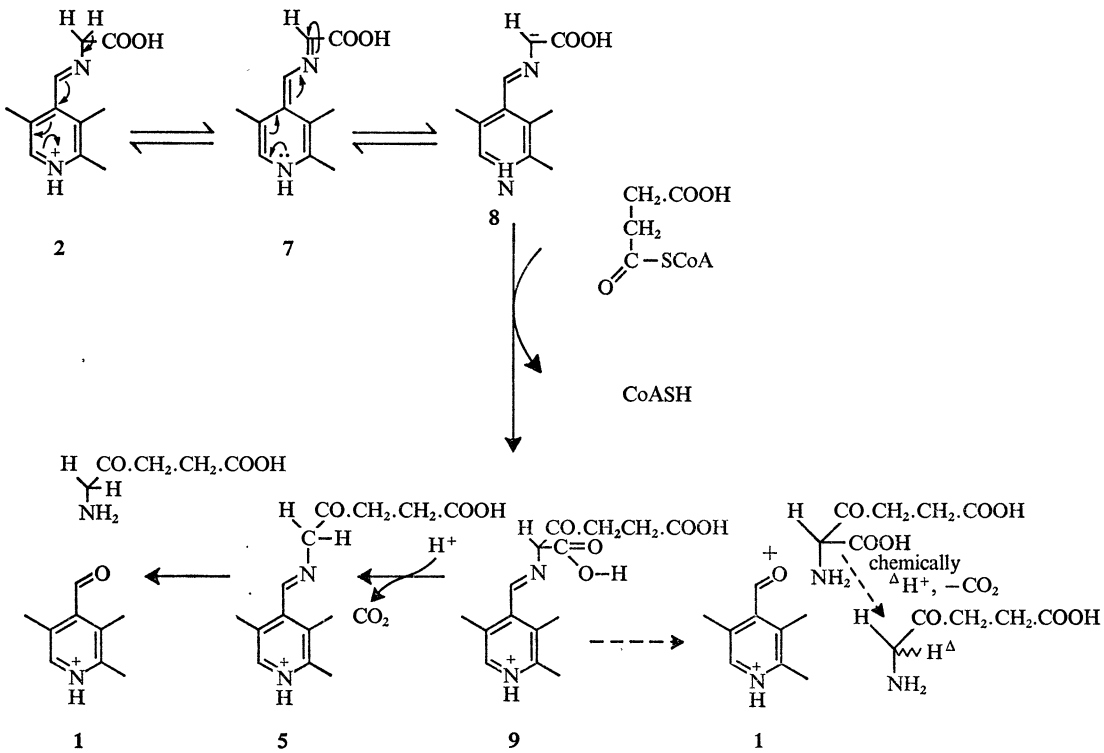
Our present-day knowledge of the way in which pyridoxal phosphate participates in a variety of diverse transformations of amino acids permits the consideration of two types of mechanisms for the biosynthesis of ALA from glycine and succinyl-CoA (schemes 1 and 2) Both mechanisms envisage that the first event in the transformation is the formation of a Schiff base intermediate between the enzyme-bound pyridoxal phosphate and glycine (**2**). The binding in this fashion dramatically alters the properties of the C—H and –COOH groups of glycine. These bonds which are unusually stable in the parent amino acids may be labilized due to the electron withdrawing properties of the pyridine nucleus. It has been assumed in this and other mechanistic conjectures on pyridoxal phosphate dependent reactions that this electron withdrawing property will be greatly improved by the presence in the enzyme active site of a suitable group able to protonate the pyridine N as shown in structure **2**. Two mechanistic courses are now available for the decomposition of the intermediate **2**. In mechanism 1, a decarboxylation reaction leads to the formation of the carbanion species (**4**) which then condenses with succinyl-CoA to give the Schiff base complex of ALA linked to the enzyme-bound pyridoxal phosphate (**5**), which upon hydrolysis furnishes free ALA (scheme 1). In mechanism 2 the first step is the removal of a proton to give another type of carbanion intermediate (**7** $\rightleftharpoons$ **8**) which reacts with succinyl-CoA. All the six carbon atoms of the two substrates are now linked as α-amino-β-ketoadipic acid to the pyridoxal phosphate–enzyme complex (**9**). Two alternatives are available for the further manipulation of this complex. In mechanism 2*a* a decarboxylation process aided by the presence of a carbonyl group at one of the β-positions and a vinylogous electron withdrawing group on the other gives ALA-pyridoxal phosphate–enzyme complex (**5**). The latter on hydrolysis gives ALA in an uneventful process. An alternative mechanism which has been considered in the literature, involves the hydrolysis of the intermediate 9 to release α-amino-β-ketoadipic acid which decarboxylates non-enzymically to ALA (shown by broken arrows, ----→, in scheme 2). The rapidity with which α-amino-β-ketoadipic acid loses CO_2 has been established by the work of Laver, Neuberger & Scott (1959).

Synthesis of chiral glycines

We envisaged that the mechanistic alternatives considered above may be evaluated by following the fate of the two methylene hydrogen atoms of glycine during their incorporation into



SCHEME 1. Mechanism 1 for the ALA-synthetase reaction. In all illustrations structure **1** represents enzyme bound pyridoxal phosphate.



SCHEME 2. Mechanisms 2a (⟶) and 2b (- - - -→) for the ALA-synthetase reaction.

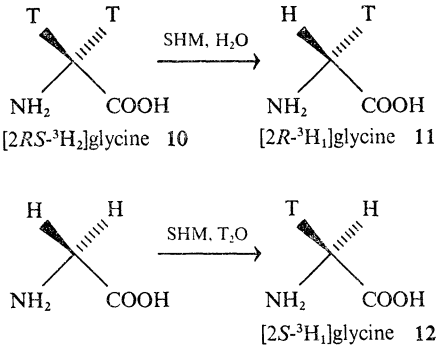
the 5 position of ALA. For investigating this facet we needed not only the conventional species of tritiated glycine in which the two hydrogen atoms at the α-carbon were equally replaced by tritium but also chiral glycines containing tritium stereospecifically located in either of the two orientations as shown in structures **11** and **12**, scheme 3. Our recognition that the enzyme serine transhydroxymethylase which is normally involved in the tetrahydrofolate-dependent interconversion of glycine and serine also catalyses a stereospecific exchange of one of the α-hydrogen atom of glycine with the protons of the medium provided the basis for the preparation of highly desirable species of *R* and *S* glycines **11** and **12** (Akhtar & Jordan 1968, 1969; Jordan & Akhtar 1970; Akhtar, El-Obeid & Jordan 1975). Table 1 shows that when [2*RS*-$^3H_2$]glycine was incubated with either mitochondrial or cytoplasmic serine transhydroxymethylase there was a slow

TABLE 1. EXCHANGE OF ONE OF THE HYDROGEN ATOMS OF GLYCINE BY MITOCHONDRIAL AND CYTOPLASMIC SERINE HYDROXYMETHYLASES

(Enzyme, 0.1 unit, was incubated at 37 °C for 2 h under N_2 in a final volume of 1 ml with [2*RS*-^{3}H:2^{14}C]glycine (18.6 μmol containing 1.5×10^5 count/min of ^{14}C, T/C ratio 4.67), pyridoxal phosphate (0.03 μmol) potassium phosphate buffer pH 7.2 (75 μmol) and DL-tetrahydrofolic acid (2 μmol). Glycine was isolated after the incubation as its benzyloxycarbonyl derivative.)

	T/C ratio of recovered glycine	loss of T (%)
mitochondrial enzyme		
boiled enzyme	4.67	—
complete system	2.26	51.60
tetrahydrofolate absent	4.52	3.30
cytoplasmic enzyme		
boiled enzyme	4.67	—
complete system	2.32	50.32
tetrahydrofolate absent	4.54	2.57

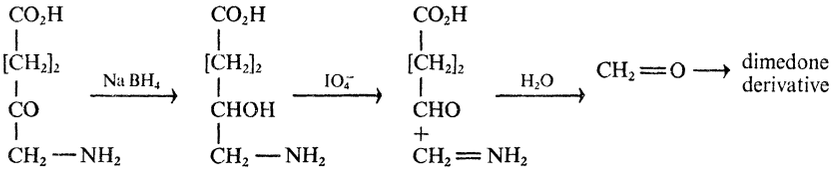
release of tritium into the medium. In the presence of tetrahydrofolate the rate of this release dramatically increased and ceased after 50 % of the original radioactivity of [2*RS*-$^3H_2$]glycine had been lost. It has been proved elsewhere that the resulting glycine contains tritium in the pro-*R* position and may be formulated as [2*R*$^3H_1$]glycine (**11**) (Akhtar & Jordan 1969; Jordan & Akhtar 1970; Zaman *et al.* 1973). The other enantiomer of glycine containing tritium in the pro-*S* position (**12**) was prepared by the incubation of glycine with serine transhydroxymethylase in the presence of T_2O. The preparation of stereospecifically labelled glycines has also been achieved in other laboratories (Besmer & Arigoni 1969; Wellner 1970; Palekar, Tate & Meister 1970).



SCHEME 3. Synthesis of stereospecifically tritiated glycine. SHM = serine-transhydroxy methylase.

Enzymic and non-enzymic exchange of C5 hydrogen atoms of ALA

Before these three samples of glycines could be used for studying the mechanism of ALA synthetase reaction, two types of problems needed exploration. One of these was foreseen but the other was unexpected. It is known that the hydrogen atoms attached to C5 of ALA undergo relatively facile exchange with the protons of the medium through an enolization mechanism. In a systematic study we noted with some relief that the chemical exchange was minimal at pH 7.0 which fortunately happens to be the optimal pH for the synthesis of ALA. However, for the determination of radioactivity, the biosynthetic ALA needed to be rigorously purified by chromatography which is normally performed under either acidic or alkaline pH values. In order to avoid exchange of C5 hydrogen atoms under these conditions, the biosynthetic ALA was reduced with $NaBH_4$, and then isolated and purified as its dihydro derivative. The latter, after treatment with periodate gave formaldehyde which was converted into the dimedone derivative (scheme 4) for the measurement of radioactivity (Zaman *et al.* 1973). It was proved that during these chemical manipulations the integrity of the C5 hydrogen atoms of ALA was maintained.



Scheme 4. Isolation of the C5 atom of 5-aminolaevulinate.

The stage was now set for performing the biosynthetic experiments when the progress was interrupted by the unexpected discovery that the extracts of *Rhodopseudomonas spheroides*, the source of ALA synthetase, contained an enzymic activity which catalysed the exchange of C5 hydrogen atoms of ALA with the protons of the medium. Therefore, before the preparation of ALA synthetase free from this unwanted contaminant could be attempted, a method for the measurement of the exchange activity was needed. For this purpose ALA containing tritium at the 3 and 5 position was prepared by heating the unlabelled material with T_2O. The sample was admixed with [5-^{14}C]ALA, then treated with $NaBH_4$ and carried through the steps of scheme 4 for the isolation of C5 as formaldehyde dimedone derivative and the determination of its T/^{14}C ratio. A decrease in the T/^{14}C ratio of formaldehyde dimedione, isolated from an incubation of the doubly labelled ALA with an extract, was taken as a measure of the exchange activity (Zaman 1973 *et al.*). Figure 1 shows that whereas the crude extracts of *Rhodopseudomonas spheroides* showed a high rate of exchange, the activity was virtually absent in 600-fold purified enzyme.

Enzymic synthesis of ALA from variously tritiated glycines

By means of this enzyme [2*RS*-$^3H_2$]-, [2*R*-$^3H_1$]- and [2*S*-$^3H_1$]glycine (**10**, **11** and **12** respectively) in tris buffer pH 7.5 were separately converted into ALA which was isolated in 2.5% radiochemical yield. Results in table 2 show that, whereas in the conversion of the randomly tritiated sample of glycine (**10**) into ALA 50% of the original radioactivity was lost, with [2*R*-$^3H_1$]- and [2*S*-$^3H_1$]glycine respectively either complete or no loss of radioactivity occurred (Zaman *et al.* 1973). These results showing that the formation of ALA is accompanied by the

loss of a single hydrogen atom with pro-*R* stereochemistry eliminate mechanism 1, and prove that the biosynthesis occurs through the sequence of reactions shown either in the pathway of mechanism 2*a* or 2*b*. The non-involvement of mechanism 1 for the biosynthesis of ALA contrasts with another enzymic reaction in the sphingolipid biosynthesis pathway which results in formation of 3-oxosphingamine from palmitoyl-CoA and serine. In this particular case the formation of the new C—C bond occurs without the labilization of the C2H of serine, thus implicating the participation of a mechanism of the type 1 (Weiss 1963). It would therefore appear that both modes of condensation predicted by mechanisms 1 and 2 operate in biological systems.

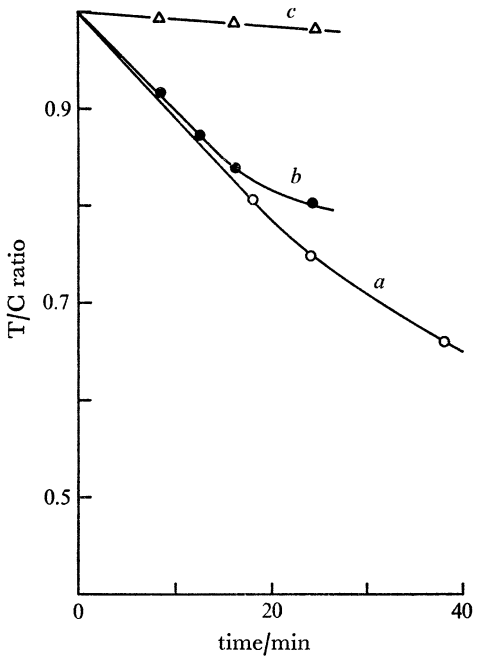


FIGURE 1. Exchange of the C5 hydrogen atoms of 5-aminolaevulinic acid under incubation conditions for the biosynthesis of 5-aminolaevulinic acid. Samples of 5-amino-[3,5-$^3H_4$]laevulinate (56–80 μg) containing [5-^{14}C]aminolaevulinate (2.6×10^6 count/min) were incubated at 37 °C for the times indicated with enzyme preparations A, B or C together with all components of the 5-aminolaevulinate synthetase system except glycine (table 2). Curve *a* (crude enzyme preparation), curve *b* (33-fold enzyme preparation), curve *c* (600-fold enzyme preparation). Incubations were terminated by addition of 3 mg 5-aminolaevulinate and 6 mg of $NaBH_4$ at 0 °C. The C5 atom of 5-aminolaevulinate was isolated as formaldehyde dimedone. T/C ratios have been normalized to unity for comparison.

The stereochemistry of the reactions involved in the elaboration of C5 of ALA and the amino methyl carbon of porphobilinogen

We may now consider features which distinguish mechanism 2*a* from 2*b*. In mechanism 2*a* all bond-breaking and bond-forming processes involved in the creation of the C5 of ALA should be stereospecific since these occur while the intermediates are bound to the enzyme. On the other hand, in mechanism 2*b*, because of the participation of a non-enzymic decarboxylation reaction, the C5 H bonds of ALA will be formed in a non-stereospecific fashion (scheme 2, broken arrows). Our projected approach for investigating the stereochemistry of the process was based on the following rationale. If we consider a hypothetical experiment for the biosynthesis of ALA from [2*RS*-$^3H_2$]glycine and assume that both the stages in the overall reaction

depicted by steps 1 and 2 in scheme 5 occur with the retention of configuration then the oxidative degradation of the biosynthetic ALA will furnish [2*R*-$^3H_1$]glycine (scheme 5, sequence A). The chirality of glycine expected from other combinations of retention and inversion processes are shown in sequence B → D (scheme 5). In real practice, however, the direct oxidation of ALA under conditions which maintained the chirality of C5 was not possible and an

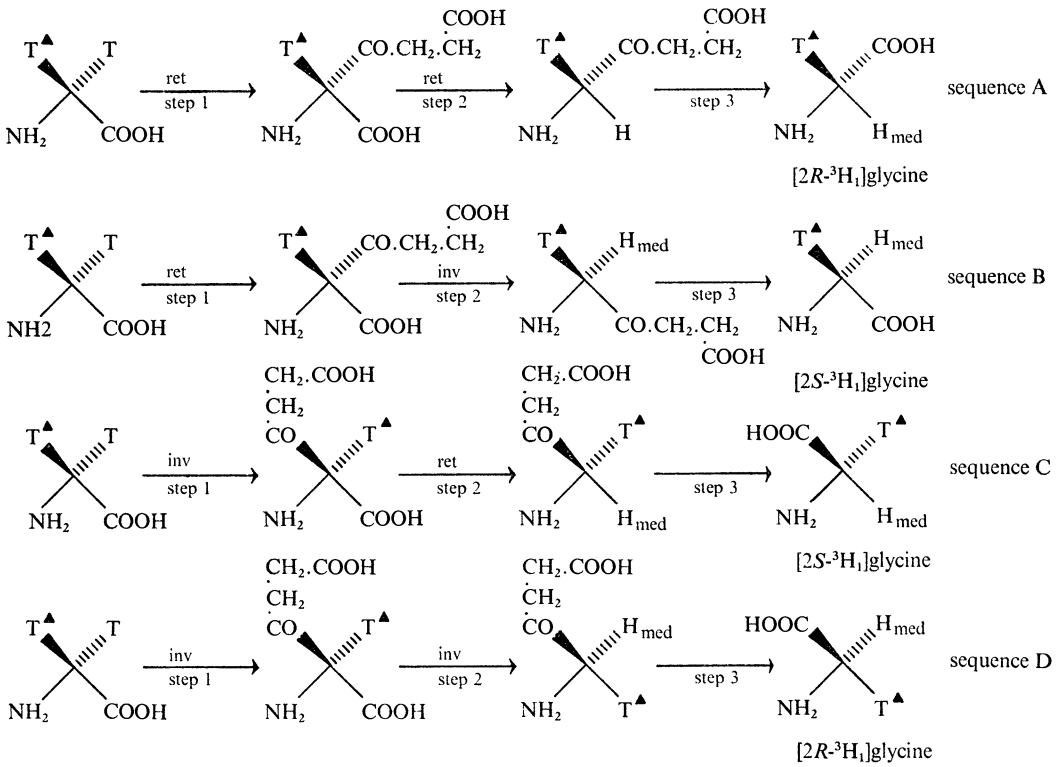
TABLE 2. STEREOCHEMISTRY OF PROTON ELIMINATION DURING ENZYMIC SYNTHESIS OF 5-AMINOLAEVULINIC ACID FROM LABELLED GLYCINE

Radioactive glycines (see below) containing 1.5×10^6 count/min in 56 μmol were incubated at 37 °C for 10 min in a final volume of 1.5 ml with succinyl CoA (2 μmol), $MgSO_4$ (10 μmol), $MnSO_4$ (0.025 μmol), pyridoxal phosphate (0.2 umol), β-mercaptoethanol (1 μmol), tris-HCl buffer pH 7.5 (75 μmol) and enzyme (600-fold purified, 8.2 units/0.5 ml).

Incubations were terminated by addition of 3 mg 5-aminolaevulinate and 6 mg $NaBH_4$ at 0 °C. The C5 of 5-aminolaevulinic acid was isolated as formaldehyde-dimedone.

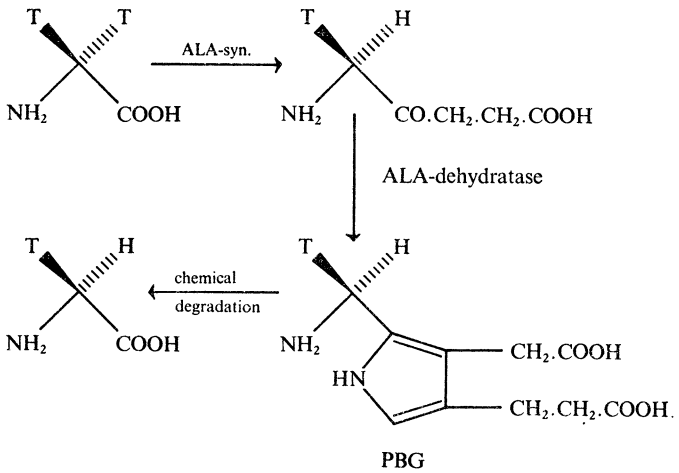
initial glycine incubated	T/C ratio	T/C ratio of 5-amino-laevulinic acid†	tritium lost in 5-aminolaevulinate biosynthesis (%) predicted	found
[2*R*-^{3}H:2-^{14}C]glycine	1.55	0.04	100	97
[2*RS*-$^3H_2$:2-^{14}C]glycine	4.01	1.90	50	53
[2*S*-^{3}H:2-^{14}C]glycine	5.80	5.80	0	0

† As formaldehyde dimedone.



SCHEME 5. Sequence of stereochemical events involved in the biosynthesis of ALA. Step 1, replacement of the pro-*R* hydrogen atom of glycine by succinyl moiety; step 2, decarboxylation followed by incorporation of a medium derived proton (H^+_{med}); step 3, a hypothetical oxidation reaction. ret, retention of configuration; inv, inversion of configuration.

indirect procedure was therefore developed. The first stage of this involved the synthesis of porphobilinogen from [2*RS*-$^3H_2$]glycine by ALA synthetase and ALA dehydratase (scheme 6). In order to accomplish our final objective we needed the conversion, glycine ⟶ porphobilinogen, to occur in good yield, and in view of the low conversion of glycine into ALA normally obtained, a considerable improvement in the efficiency of this reaction was needed. We were fortunate to make the observation that when the ALA synthetase reaction was carried out in phosphate instead of tris buffer a fivefold higher conversion of glycine into ALA was achieved. Less-efficient conversion of glycine ⟶ ALA in tris buffer may be attributed to the inhibition of ALA synthetase reaction by tris, owing to its resemblance to glycine.



Scheme 6. Biosynthesis and degradation of porphobilinogen (PBG).

Incubation of [2*RS*-$^3H_2$]glycine with highly purified preparations of ALA synthetase and ALA dehydratase, which were shown to be free from the exchange activity (cf. figure 1) produced porphobilinogen (PBG) in about 2% yield. After separation of the biosynthetic material by cellulose t.l.c., the porphobilinogen band was eluted with water under mild conditions and rechromatographed to give 6×10^5 count/min of PBG. For the isolation of glycine from PBG the following degradation procedure was developed. PBG was acetylated by treatment with acetic anhydride in saturated $NaHCO_3$ for 5 min at 0 °C. The acetyl porphobilinogen was oxidized with $KMnO_4$ at pH 7.5 for $2\frac{1}{2}$ h at 60 °C. The products of oxidation were extracted into ethylacetate and glycine was subsequently isolated by hydrolysis of the amide with 6 M HCl and purification on cellulose t.l.c. In model experiments the overall yield in the degradation was 7–10%.

Now biosynthetic PBG (1.5×10^5 count/min) was oxidized by the above method to furnish glycine which after extensive purification contained 5×10^3 count/min of tritium. The glycine was then mixed with [2-^{14}C]glycine and the doubly labelled sample was processed for the determination of the stereochemistry of the tritium by using the enzyme, serine transhydroxymethylase. In a parallel control experiment [2*RS*-$^3H_2$]- and [^{14}C]glycine were mixed to produce a reference sample which had total number of counts as well as T/C ratio similar to those of the experimental sample. From both incubations samples were removed at zero time and after the addition of carrier, glycine was isolated as its benzoyloxycarbonyl derivative and used for the measurement of the initial T/^{14}C ratios. The two samples were then allowed to incubate for

2 h at 37 °C and glycine was isolated as above and counted. Results in table 3 show that 87 % of tritium radioactivity present in glycine obtained from the degradation of biosynthetic PBG was lost during incubation with the serine transhydroxymethylase. In the control experiment with [2*RS*-$^3H_2$:2-^{14}C]glycine, however, as expected, only 50 % of tritium activity was removed under identical conditions. Since it is well known that serine transhydroxymethylase stereospecifically exchanges the pro-*S* hydrogen atom of glycine, therefore the loss of 87 % of tritium in experiment 1 of table 3 shows that the α position of the glycine and hence by implication the aminomethyl carbon atom of PBG and C5 of ALA all have the *S*-chirality (Abboud, Jordan & Akhtar 1974).

TABLE 3. DETERMINATION OF THE STEREOCHEMISTRY OF TRITIUM IN GLYCINE (OBTAINED FROM THE DEGRADATION OF BIOSYNTHETIC PORPHOBILINOGEN) BY MEANS OF THE EXCHANGE REACTION WITH SERINE TRANSHYDROXYMETHYLASE

Incubation conditions were as described in table 1 except that glycine containing 1000 count/min of ^{14}C was used (initial T/C ratio as indicated below) glycine was isolated after incubation as its benzyloxycarbonyl derivative.

initial glycine incubated	T/C ratio of initial glycine	T/C ratio of recovered glycine	loss of tritium (%)
[2-^{3}H:2-^{14}C]glycine (from porphobilinogen degradation)	$\frac{1510}{480} = 3.70$	$\frac{210}{427} = 0.49$	87.0
[2*RS*-$^3H_2$:2-^{14}C]glycine (control)	$\frac{1674}{471} = 3.65$	$\frac{806}{444} = 1.82$	50.0

Mechanism of action and the stereochemistry of the ALA-synthetase reaction

The formation of a chiral centre at C5 of ALA biosynthetized from [2*RS*-$^3H_2$]glycine eliminates the participation of the pathway, shown in mechanism 2*a*, involving the hydrolysis of the intermediate (**9**) to α-amino-β-ketoadipic acid with subsequent non-enzymic decarboxylation to ALA.

The cumulative evidence presented above may be interpreted to suggest that an early event in the biosynthesis of ALA involves the removal of the pro-*R* hydrogen atom from the glycine-pyridoxal phosphate-enzyme complex (**13**, scheme 7) to give the carbanion species (**14**) which condenses with succinyl-CoA to form **15**. Further support for the formation of the carbanion species (**14**) in the catalysis has been provided by the recent unpublished work of Jordan and Laghai, who have shown that incubation of highly purified ALA synthetase with [2*RS*-$^3H_2$]-glycine, but in the absence of succinyl-CoA results in the stereospecific exchange of the pro-*R* hydrogen atom to give [2*S*-$^3H_1$]glycine. Thus the hydrogen atom of glycine exchanged in the partial reaction is the same which is removed in overall biosynthesis of ALA.

Results summarized in table 3 demonstrate that the pro-*S* hydrogen atom of the precursor glycine is retained in the biosynthesis and occupies the pro-*S* position at the 5-position of ALA. This information when considered in conjunction with the analysis presented in scheme 5 proves that of the two crucial bond forming events one of them proceeds by an inversion and the other by a retention of configuration† either via the route depicted by sequence B or C (scheme 5). An unambiguous choice between these two possibilities is not yet possible. However, on intuitive grounds one may argue that in view of the preponderance of retention mechanisms in biological reactions involving the replacement of C—H bonds by C—C bonds, the first stage in the ALA

† Similar conclusion has been drawn from independent studies carried out by Professor D. Arigoni (E.T.H. Zurich).

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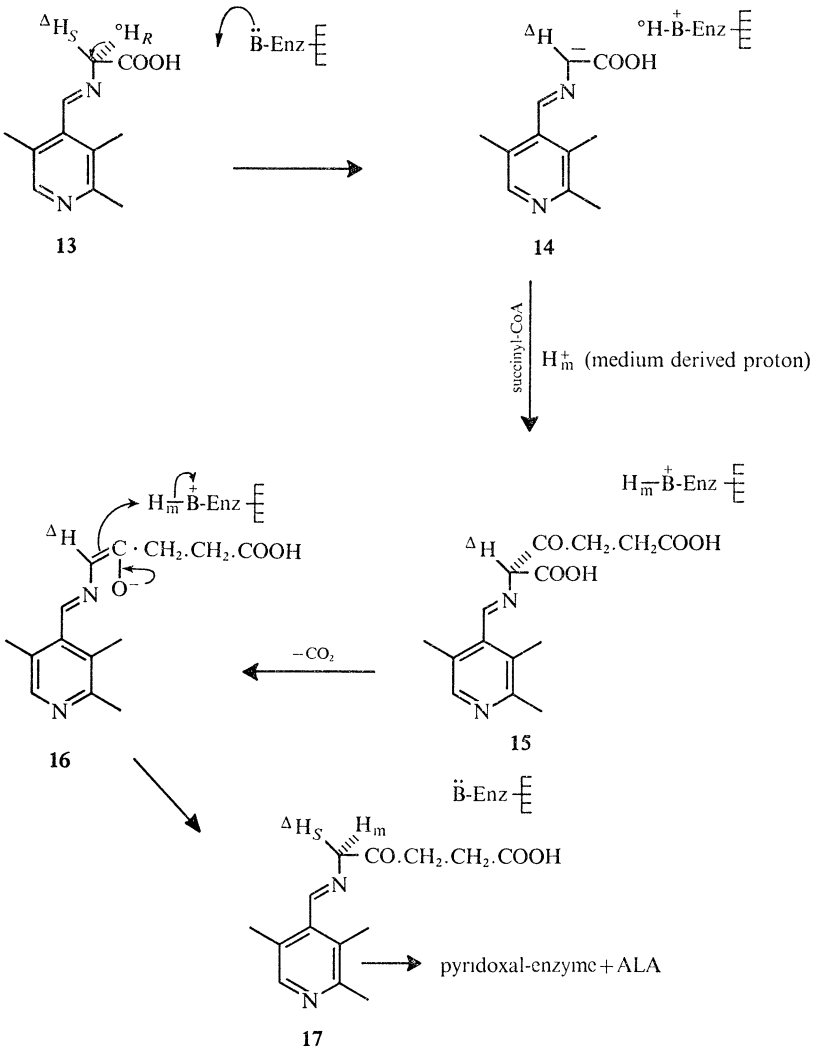
synthesis may also occur by retention. The intermediate enol (**16**) formed by the decarboxylation of **15** may then be economically protonated by using the same group on the enzyme which initially removed the pro-*R* hydrogen atom of glycine thus resulting in an inversion in this step (scheme 7). In this connexion it is interesting to note that the stereochemistry of the *R* hydrogen atom originally removed in the reaction **13** → **14** is the same as the medium derived proton H^+ which is added in the conversion **16** → **17**.



13 14 15 16 17

Scheme 7. Postulated mechanism and stereochemistry of ALA-synthetase. It is assumed that in the conversion **14** ⟶ **15** the hydrogen (H·) abstracted by the basic group on the enzyme exchanges with the proton of the medium.

Studies on the formation of vinyl groups in haem biosynthesis

Introduction

In the preceding section we have focused attention entirely on the mechanism of the formation of the amino-methyl group of ALA which originates from the α-position of glycine. The C2 and C3 of ALA are derived from the two methylene carbon atoms of succinate. During

the biosynthesis of porphyrins these two carbon atoms, C2 and C3, of ALA occupy the α and β positions respectively, in the four propionate side chains of uro- and coproporphyrinogen III (**24**) (scheme 11). The penultimate stage in haem biosynthesis then involves the oxidative decarboxylation of the propionate residues into vinyl groups. We envisaged that the mechanism of this process may be elucidated by tracing the fate of hydrogen atoms located at the α and β position of propionate side chains during their conversion into vinyl groups (Zaman, Abboud & Akhtar 1972; Zaman & Akhtar 1975*a*, *b*).

In this section we describe an account of the work directed to the synthesis of samples of ALA, stereospecifically labelled with tritium at the C2 and C3. These samples were used to produce PBG which was converted into haem. The latter was then degraded to provide information on the mechanism of vinyl group formation (Abboud 1975).

The biosynthesis of stereospecifically tritiated PBG

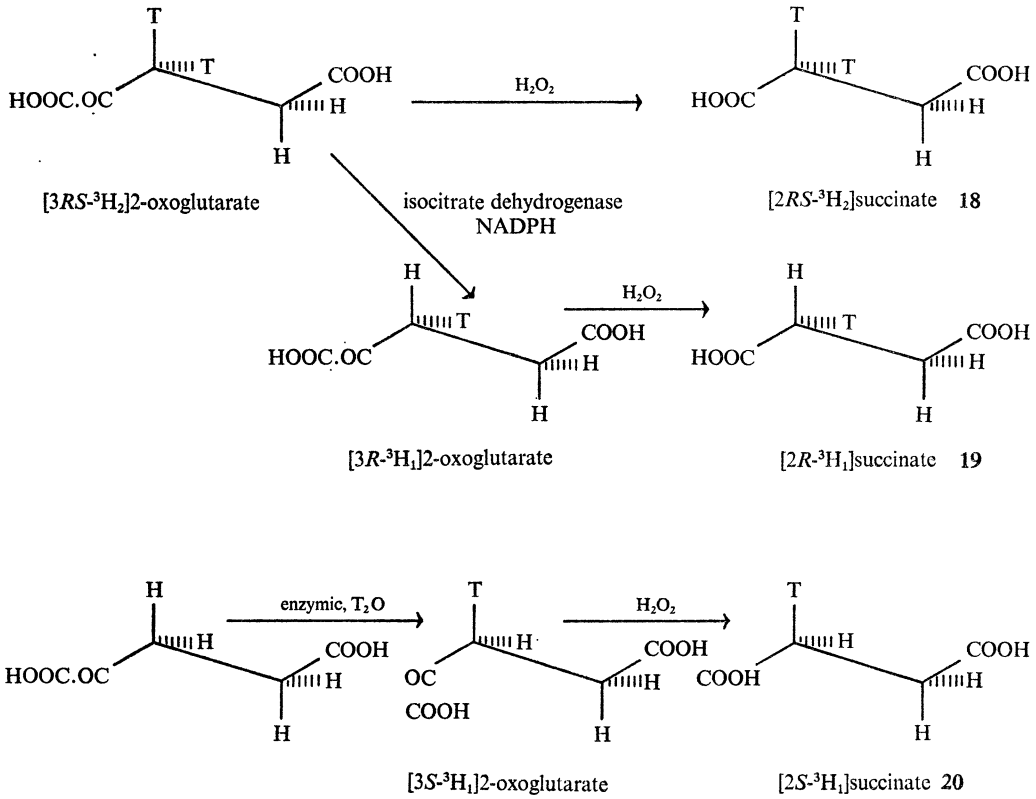
The two samples of chiral succinate **19** and **20** needed as starting material for the present investigation were prepared (scheme 8) by the method already available in the literature (Englard & Hanson 1969; Rose 1960). Thus 2-oxoglutarate when heated in a sealed tube with T_2O gave [3*RS*-$^3H_2$]2-oxoglutarate. A portion of this was treated with H_2O_2 to give [2*RS*-$^3H_2$]-succinate (**18**). The remaining [3*RS*-$^3H_2$]2-oxoglutarate was incubated with isocitrate dehydrogenase in the presence of NADPH, when a time-dependent release of tritium in the medium occurred which ceased after 50% of the original radioactivity had been lost. The resulting sample which has previously been shown to contain all the tritium in the 3*R* position may be formulated as [3*R*-$^3H_1$]2-oxoglutarate (Englard & Hanson 1969; Rose 1960; Leinhard & Rose 1964). The latter was converted into [2*R*-$^3H_1$]succinate by reaction with H_2O_2. [2*S*-$^3H_1$]-succinate was prepared using the same principle as elaborated above for the preparation of the *R*-enantiomer. With the *S*-sample, however, greater manipulative skill and caution was needed, since in this case the tritium is incorporated during the course of an enzymic exchange which is performed in relatively dilute solution (52 μmol of 2-oxoglutarate in 10 ml medium) thus necessitating the use of a large amount of T_2O in order to incorporate sufficient radioactivity into 2-oxoglutarate. In our experiments the incubations were performed with 10 Ci of T_2O in 5–10 ml of incubation medium to give after oxidation with H_2O_2 2–3 × 10^6 (count/min)/μmol of [2*S*-$^3H_1$]succinate.

These three tritiated samples of succinate needed to be converted into succinyl-CoA for incorporation into ALA. We accidently made the observation, however, that partially purified preparations of ALA synthetase contained an activity which catalysed an extremely rapid exchange between succinate and succinyl-CoA as shown:

$$HO_2C.CH_2.CH_2.COSCoA + HO_2C.\overset{\Delta}{C}H_2.CH_2.CO_2H$$
$$\Downarrow\Uparrow$$
$$HO_2C.CH_2.CH_2.CO_2H + HO_2C.\overset{\Delta}{C}H_2.CH_2.COSCoA + HO_2C.CH_2.\overset{\Delta}{C}H_2.COSoA$$

Thus the laborious and wasteful conversion of those precious samples of chiral succinate into their respective CoA derivatives was no longer necessary. It was found that an incubation reaction containing non-radioactive succinyl-CoA (enzymically produced from α-ketoglutarate dehydrogenase, NAD, CoASH and 2-oxoglutarate) when mixed with another system containing partially purified ALA synthetase (Zaman *et al.* 1973; Abboud 1975), glycine and tritiated succinate resulted in the formation of labelled ALA. The biosynthetic material was isolated by

applying the incubation medium to a column of Dowex-1-Cl (130 mm × 6.5 mm) and ALA was conveniently eluted with water. The radiochemical yield of tritiated ALA from [^{3}H]succinate was usually 15–30 %. Three samples of ALA biosynthesized from [2*RS*-$^3H_2$]-, [2*R*-$^3H_1$]- and [2*S*-$^3H_1$]-succinate were incubated (table 4) with ALA dehydratase (Heyningen & Shemin 1971) to produce [*RS*-^{3}H]-, [*R*-^{3}H]- and [*S*-^{3}H]PBG respectively (scheme 9, structures **21–23**).



Scheme 8. Synthesis of stereospecifically tritiated succinate.

Table 4. Labelling of porphobilinogen from tritiated succinate

[2*RS*-$^3H_4$]succinate (3×10^7 count/min in 4 μmol) was incubated for 30 min with a combined α-ketoglutarate dehydrogenase and 5-aminolaevulinate synthetase system. The 5-amino [2-*RS*-$^3H_4$]laevulinic acid after isolation on Dowex-1 Cl^- was incubated with a 5-aminolaevulinate dehydratase system. After incubation, the porphobilinogen was purified on Dowex-2 acetate.

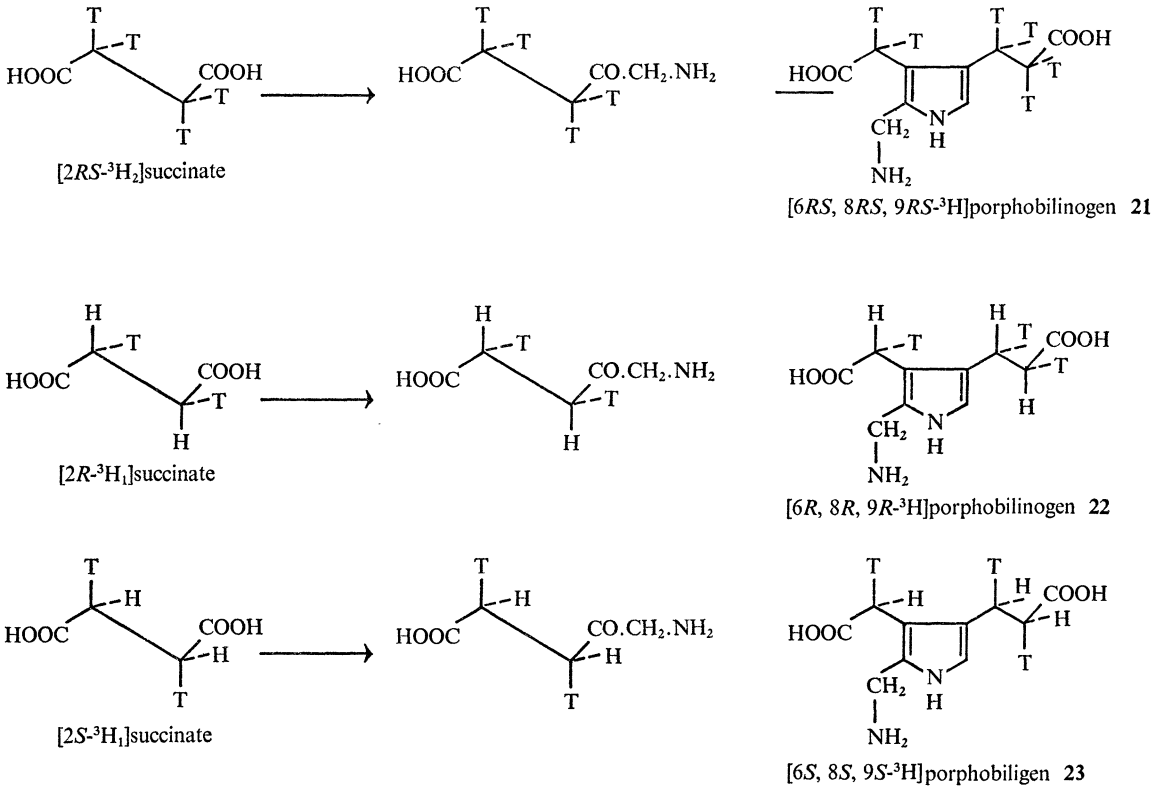
product isolated	net quantity synthesized / μg	radioactivity / count/min	% yield (colorimetric)	% radioactive yield (from succinate)
5-aminolaevulinic acid	200	4.6×10^6	20	15
porphobilinogen	140	1.75×10^6	7	6

Biosynthesis and degradation of haem

These three tritiated samples of PBG were converted into haem by using a haemolysate obtained from anaemic chicken blood (Zaman *et al.* 1972; Zaman & Akhtar 1975*a*, *b*; Zaman 1973). The biosynthetic samples of haem were subjected to the sequence of reactions shown in

scheme 10 (Shemin 1957). The degradation converted the vinyl group containing rings A and B of haem into ethylmethyl maleimide while the rings C and D with their intact propionate residues gave haematinic acid.

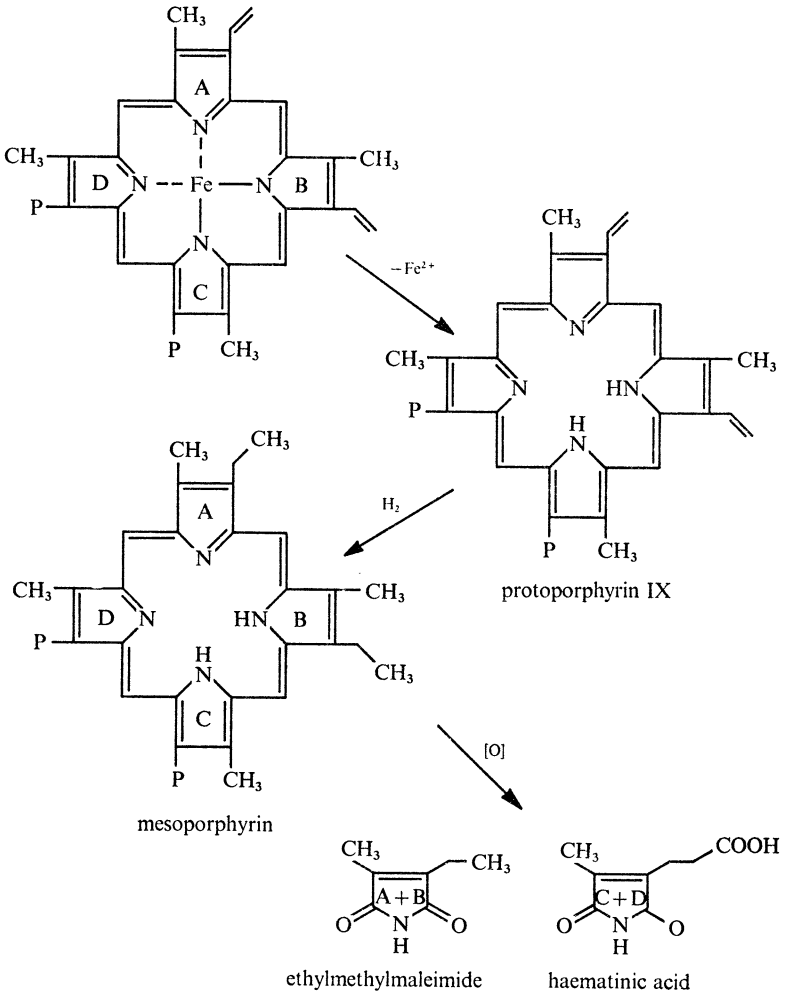
The comparison of the profile of tritium atoms in haematinic acid with that of ethylmethyl maleimide gave direct information on the nature of the molecular events occurring during the conversion of the propionate residues into vinyl groups. It is interesting to point out that the presence of vinyl side chains as well as propionate residues within the haem molecule obliterated the need for the isolation and determination of radioactivity on coproporphyrinogen III, which would have been a formidable task.



SCHEME 9. Synthesis of stereospecifically tritiated porphobilinogen. All the samples of succinate were tritiated only at C2, however, since it is a symmetrical molecule, the biosynthetic products will contain radioactivity at carbon atoms originally derived from C2 as well as C3 of succinate.

It was found that a doubly labelled sample of haem, in which tritium originated from [*RS*-^{3}H]PBG (**21**) and ^{14}C from [4-^{14}C]ALA, on degradation gave haematinic acid and ethylmethyl maleimide which had T:^{14}C ratios of 1.0 and 0.85 respectively. This radiochemical data shows that for six tritium atoms present in haematinic acid, ethylmethyl maleimide contains only five, one of the tritium atoms therefore must have been lost during the vinyl group formation. When haem biosynthesized from [*R*-^{3}H]PBG (**22**) was similarly processed haematinic acid and ethylmethyl maleimide with respective T:^{14}C ratios of 1.0 and 0.96 were obtained, thus showing that none of the pro-*R* hydrogen atoms present in the acetic and propionate side-chains of the precursors were disturbed during the formation of the vinyl groups. In contrast when haem biosynthesized from [*S*-^{3}H]PBG (**23**) was subjected to the degradation sequence,

haematinic acid was found to have T:^{14}C ratio of 1.0 and ethylmethyl maleimide of 0.70 which established that only one of the two pro-*S* hydrogen atoms from each propionate side chain was removed during haem biosynthesis (scheme 11, **24** → **25**). For the sake of clarity, in scheme 11, we have traced the path of tritium during the biosynthesis and degradation of haem using only [*S*-^{3}H]PBG (**23**).



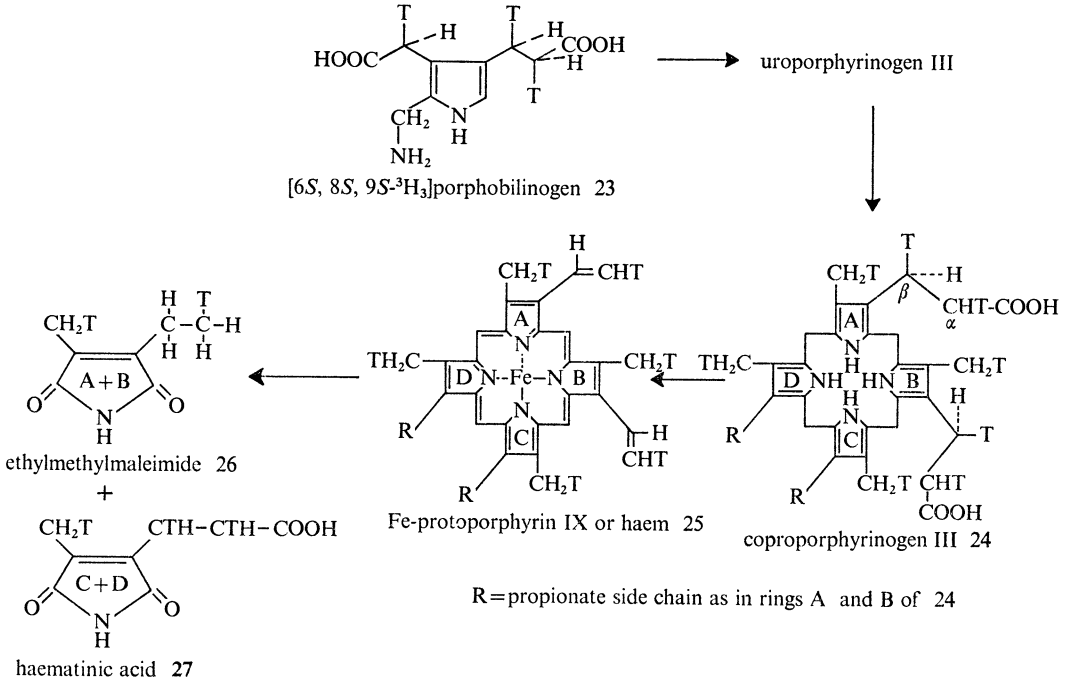
Scheme 10. Degradation of haem.

Table 5. The biosynthesis of haem from labelled porphobilinogen

Haemolysed chicken blood was incubated separately with [*RS*-^{3}H]porphobilinogen, [*R*-^{3}H]porphobilinogen or [*S*-^{3}H]porphobilinogen. The biosynthetic haem was mixed with ^{14}C haem and degraded to ethylmethylmaleimide (rings A+B), and haematinic acid (rings C+D). The loss of tritium was assessed by comparison of the T/C ratios of ethylmethylmaleimide with the T/C ratio of haematinic acid.

porphobilinogen incubated	T/C ratio rings A+B	T/C ratio rings C+D	loss of tritium from rings A+B (%) expected†	found
[*RS*-^{3}H]porphobilinogen (**21**)	0.84	1.0	**17**	16
[*R*-^{3}H]porphobilinogen (**22**)	0.95	1.0	0	5
[*S*-^{3}H]porphobilinogen (**23**)	0.65	1.0	**33**	35

† Assuming loss of 1 hydrogen from the propionate side chain.



SCHEME 11. Elimination of the pro-*S* hydrogen atoms in the formation of the vinyl groups.

The mechanism and stereochemistry of vinyl group formation

A number of competing mechanisms for the conversion of the two propionate residues of coproporphyrinogen III (**24**) into vinyl groups of protoporphyrin IX (structure **25** but without Fe) can either be envisaged or have been considered in the literature. Mechanisms involving the intermediacy of β-keto acids as shown by the sequence

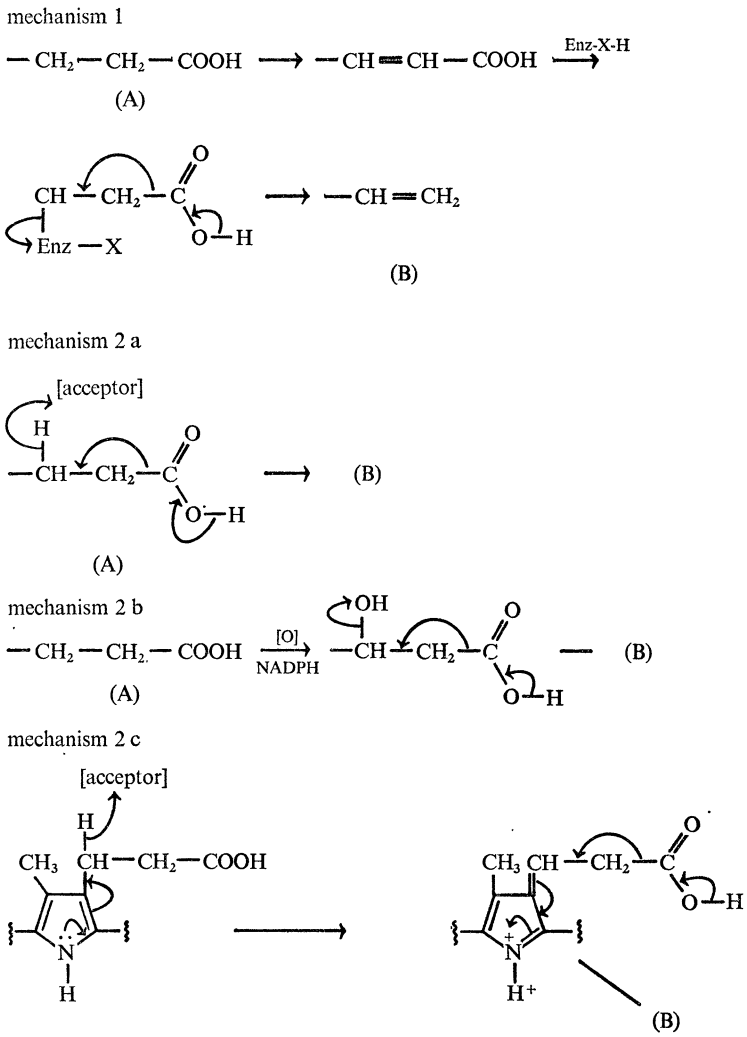
$$\underset{(A)}{-\overset{\beta}{C}H_2-\overset{\alpha}{C}H_2\text{-}COOH} \longrightarrow -CO-CH_2-COOH \longrightarrow -CO\,.\,CH_3 \rightarrow\rightarrow\rightarrow \underset{(B)}{-CH=CH_2}$$

have already been eliminated by the demonstration that only one of the two β hydrogen atoms of the propionate moiety is removed in vinyl group formation (Zaman *et al.* 1972; Zaman & Akhtar 1975; Battersby *et al.* 1972). In this and subsequent mechanistic illustrations the propionate side chain and the vinyl group are shown by the partial structures (A) and (B) respectively.

On the basis of studies carried out by Porra & Falk (1964) on the conversion of coproporphyrinogen III (**24**) into protoporphyrin IX (structure **25**, but without Fe) by ox liver mitochondrial preparations it was speculated that the reaction may occur via the initial formation of an acrylate side chain followed by attack of a nucleophile to give an intermediate from which CO_2 is lost by a decarboxylation-elimination process as shown in the sequence of mechanism 1 (scheme 12). Against this proposal was the observation that 2,4-*trans*-diacrylate deuteroporphyrinogen IX was not converted to protoporphyrinogen IX by ox liver mitochondria (Sano & Granick 1961). Battle, Benson & Rimington (1965), however, suggested that the real intermediate might be 2,4-*cis* diacrylate deuteroporphyrinogen IX. Support for this view came from two types of experiments. First it was found that maleic acid but not fumaric acid inhibited coproporphyrinogenase activity (Battle, Benson & Rimington 1965), and secondly French, Nicholson &

Rimington (1970) isolated from calf foal meconium a porphyrin containing a *cis*-acrylate side chain at position 2, 4, 6 or 7. We have, however, in the present study shown that of the four C—H bonds present in each of the propionate side-chains three are retained in the vinyl group. That the only hydrogen atom removed in the process must be originally located at the β position follows from the results described elsewhere (Zaman *et al.* 1972; Zaman & Akhtar 1975; Battersby *et al.* 1972). Thus these results showing that neither of the two α hydrogen atoms are eliminated in the vinyl group formation exclude the participation of an acrylic acid intermediate in the conversion (A) → (B) (mechanism 1, scheme 12). The non-involvement of the α hydrogen atoms in the formation of vinyl groups during the biosynthesis of haem by duck blood and of protoporphyrin IX by *Euglena gracilis* (Battersby *et al.* 1972) has also been established by an entirely different approach.

To return to the sterochemical aspect, our previous work has indicated that the β hydrogen atoms with pro-*R* configuration are retained in the formation of the two vinyl groups. The present study directly shows that both vinyl groups are formed through the loss of pro-*S*



Scheme 12. Possible mechanisms for the formation of vinyl groups in haem biosynthesis.

hydrogen atoms, for if hydrogen atoms with opposite stereochemistry were eliminated in the synthesis of the two vinyl groups, the radiochemical data in experiments 2 and 3 of table 5 would be identical whether haem had originated from 2*S* or 2*R* tritiated succinate.

The cumulative evidence presented in this paper and elsewhere (Zaman *et al.* 1972; Zaman & Akhtar 1975*a*, *b*) is therefore compatible with three closely related mechanistic alternatives shown in mechanisms 2*a*, *b* and *c* of scheme 12. In mechanism 2*a*, which was originally considered by Granick & Sano (1961), a hydride ion is removed from the β carbon atom with simultaneous decarboxylation to vinyl group.

The mechanism 2*b* proceeds via an oxygen dependent hydroxylation process in which a hydroxyl group introduced at the β carbon atom facilitates decarboxylation to give the vinyl group. Support for a β hydroxy intermediate has been provided by the work of Sano (1966), who achieved the conversion of 2,4-bis-(β-hydroxy propionate) deuteroporphyrinogen IX into protoporphyrinogen IX under anaerobic conditions using beef liver mitochondria. This work should be viewed with caution, however, since there was a non-enzymic conversion of 2,3-bis-(β-hydroxy propionate) deuteroporphyrinogen IX into protoporphyrin(ogen) IX. Furthermore, recent studies of Cavaleiro, Kenner & Smith (1974) showing that the vinyl groups in rings A and B of haem are formed in a sequential fashion makes it mandatory that if hydroxylated compounds are involved as physiological intermediates these should be 2(β-hydroxy propionate-4 (propionate) deuteroporphyrinogen IX and 2-vinyl-4(β-hydroxy propionate) deuteroporphyrinogen IX.

Finally the mechanism 2*c* predicts an oxidative reaction involving the β hydrogen atom and the pyrrole N—H bond to give the conjugated internal Schiff base intermediate, which owing to its electron withdrawing property aids decarboxylation to give the vinyl group. The last mechanism is similar to several biological decarboxylation reactions catalysed by pyridoxal phosphate and thiamine pyrophosphate linked enzymes. Although at present a choice between these three mechanisms (2*a*, *b* and *c*) cannot be made, it is, however, clear the mechanism 2*b* predicting O_2 dependent formation of the β hydrodylated derivative is unlikely to be involved in haem biosynthesis in anaerobic organisms.

Stereochemistry of methyl group formation in haem biosynthesis

Introduction

The fact that C2 and C3 of ALA give rise to the α- and β-positions in the four propionate side chains of uro- and coproporhyrinogens III (**24**) enables a study of the vinyl group formation as detailed in the previous section. The C2 of ALA is also destined for the methylene carbon in the four acetyl side chains of uroporphyrinogen III, this feature may be exploited to study the stereochemistry of the decarboxylation reaction, uroporphyrinogen III → coproporphyrinogen III, in haem biosynthesis (scheme 13).

It was envisaged that if a sample of uroporphyrinogen III of known stereochemistry could be generated such that the decarboxylation reaction produced chiral methyl residues in the product, then the isolation of these methyl residues as acetic acid for determination of its chirality would shed light on the mechanism and stereochemistry of the decarboxylation process.

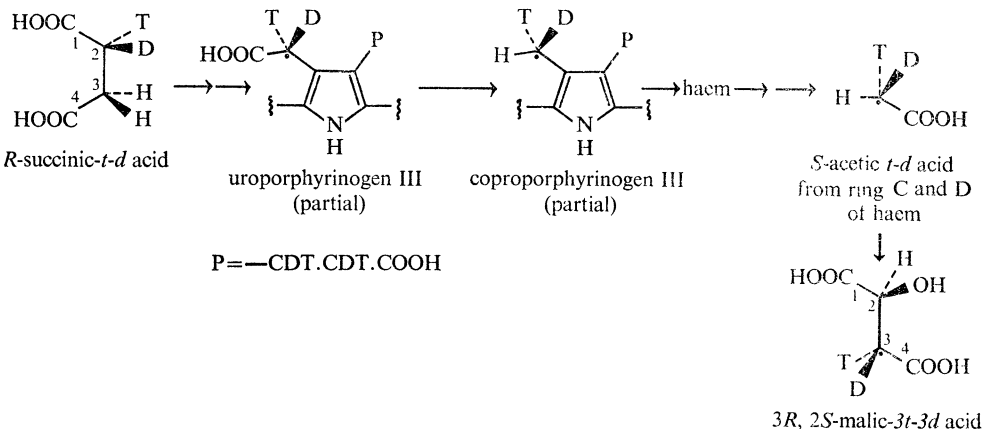
Stereospecific introduction of deuterium and tritium into precursors

Our projected approach for investigating the stereochemistry depended on the availability of samples of chiral succinate in which one of the two methylene groups was stereospecifically labelled with both tritium and deuterium. This was achieved by incubating [3-$^3H_2$]2-oxoglutarate in D_2O with NADPH and isocitrate dehydrogenase (NADP dependent) when 3*R*-2-oxoglutaric acid was produced. The latter on treatment with H_2O_2 furnished *R*-succinic-*t*-*d* acid, 24 μmol of which containing approximately 15×10^6 disintegrations min^{-1} μmol^{-1} when incubated with the haemolysed erythrocyte preparation gave stereospecifically tritiated, deuterated haem (50 mg; approximately, 40 000 disintegrations min^{-1} mg^{-1}).

Isolation of the methyl groups of haem as chiral acetates

The biosynthesized haem was degraded to ethylmethyl maleimide and haematinic acid by the sequence haem ⟶ protoporphyrin IX ⟶ mesoporphyrin IX ⟶ ethylmethyl maleimide + haematinic acid.

The next stage in our strategy involved the development of a protocol through which haematinic acid would be oxidized to acetic acid in the absence of enolization, so that the chirality of the methyl group was maintained. After examining several approaches it was found that when haematinic acid was ozonized at pH 3.0 in the presence of H_2O_2, acetic acid was produced in a radiochemical yield which was 40 % of theoretical. In such experiments less than 1 % of the original radioactivity was associated with water. Under these conditions of minimum enolization 25 μmol of haematinic acid were converted to yield 10 μmol of acetate (scheme 13).



Scheme 13

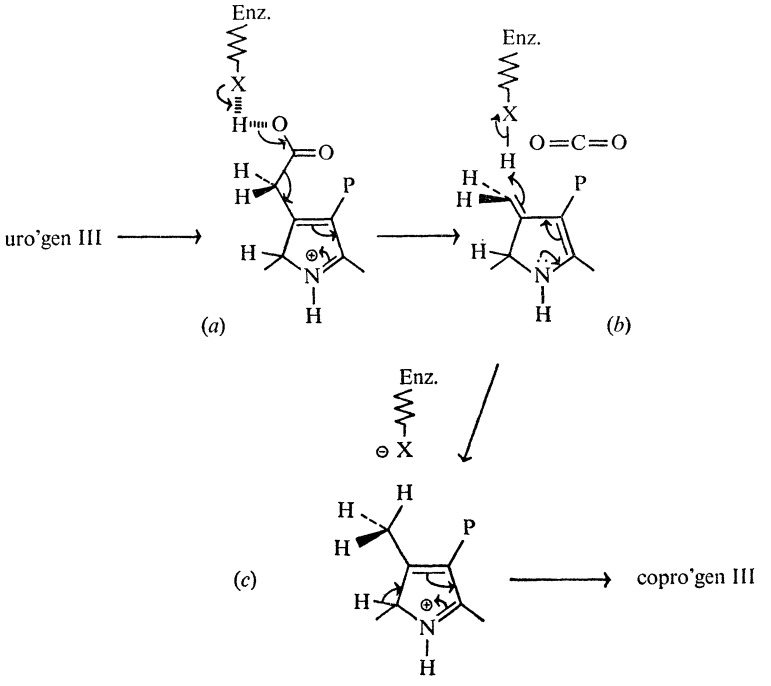
Configurational analysis and stereochemistry of porphyrinogen carboxy-lyase

In order to determine the chirality of the acetates, the elegant approach developed by Cornforth *et al.* (1969) and by Luthy, Rétey & Arigoni (1969) was adopted. Ten μmol of acetic-*t*-*d* acid was admixed with [^{14}C]acetate and incubated with acetate kinase, phosphotransacetylase and malate synthetase essentially as detailed by Rose (1970) to produce about 7 μmol of malate. It was ensured that the malate synthetase used in this experiment was free from significant fumarase activity. The biosynthesized malate was purified by elution from Dowex 1 Cl^- after initial elution of unreacted acetate and glyoxylate. One third of the malate was admixed

with non-radioactive carrier 2*S*-malate and crystallized to give malate which had a T:^{14}C ratio of 0.981. The remaining two thirds was incubated with fumarase to exchange the pro-*R* hydrogen of the biosynthesized malate with protons of the medium. The malate was recovered and recrystallized after the addition of non-radioactive carrier and had a T:^{14}C ratio of 0.302. This shows that most of the tritium in the malate biosynthesized as detailed above was in the pro-*R* (scheme 13).

It has been shown by Cornforth *et al.* (1969) that when 3*R*-2*S*-malic-3*t*-3*d* acid obtained from *S*-acetic-*t*-*d*-acid was equilibrated with fumarase 30.8 % of the ^{3}H radioactivity was retained in the equilibrated malate. Our results which are in excellent agreement with those of Cornforth *et al.* establish that the sample of acetic acid containing the methyl groups originally present in rings C and D of the biosynthesized haem has *S*-chirality. Therefore the decarboxylation reaction converting uroporphyrinogen III to coproporphyrinogen III must have occurred with a retention of configuration (scheme 13).

In the light of this stereochemical information it is tempting to speculate that the group on the enzyme involved in either removal of the hydrogen from the O—H bond of the carboxyl or in the binding of the already dissociate carboxyl may also participate in the protonation of the intermediate. (Drawn for convenience as in scheme 14 in which steps urogen ⟶ (*a*) and (*c*) ⟶ coprogen occur via enzyme catalysed protonation and deprotonation respectively.)



Scheme 14

Conclusions

The foregoing account, regarding the work carried out in our laboratories, describes the exploration of the mechanism and stereochemistry of a number of the enzymic steps in porphyrin biosynthesis. The main feature emerging from the present work is that a stereochemical approach may be successfully exploited for the elucidation of the mechanisms of enzymic

reactions involved in haem biosynthesis and also to provide stereospecifically labelled porphyrin precursors.

For instance, the synthesis of stereospecifically tritiated glycines using serine hydroxymethylase has provided the basis for the determination of the mechanism and stereochemistry of ALA synthetase. The use of stereospecifically tritiated succinate has allowed an evaluation of many of the aspects of the mechanism and stereochemistry of vinyl group formation in rings A and B of coproporphyrinogen III. Finally, the approach using hydrogen deuterium and tritium atoms at a single carbon atom has provided an approach for studying the formation of the four methyl groups at positions 1, 3, 5 and 8 of the tetrapyrrole macrocycle.

References (Akhtar *et al.*)

Abboud, M. M. 1975 Ph.D. Thesis, Southampton University.
Abboud, M. M., Jordan, P. M. & Akhtar, M. 1974 *Chem. Commun.* pp. 643–644.
Akhtar, M., El-Obeid, H. A. & Jordan, P. M. 1975 *Biochem. J.* **145**, 159–168.
Akhtar, M. & Jordan, P. M. 1968 *Chem. Commun.* pp. 1691–1692.
Akhtar, M. & Jordan, P. M. 1969 *Tetrahedron Lett.* pp. 875–878.
Battersby, A. R., Baldas, J., Collins, J., Grayson, D. H., James, K. J. & McDonald, E. 1972 *Chem. Commun.* pp, 1265–1266.
Battle, A. M. del C., Benson, A. & Rimington, C. 1965 *Biochem. J.* **97**, 731–740.
Besmer, P. & Arigoni, D. 1969 *Chimica* **23**, p. 190.
Cavaleiro, J. A. S., Kenner, G. W. & Smith, K. M. 1974 *J. chem. Soc. Perkin 1*, pp. 1188–1194.
Cornforth, J. W., Redmond, J. W., Eggerer, H., Buckel, W. & Gutschow, C. 1969 *Nature, Lond.* **221**, 1212–1213.
Englard, S. & Hanson, K. R. 1969 *Meth. Enzym.* **18**, 592–596.
French, J., Nicholson, D. C. & Rimington, C. 1970 *Biochem. J.* **120**, 393–397.
Granick, S. & Sano, S. 1961 *Fedn Proc. Fedn Am. Soc. exp. Biol.* **20**, 376.
Heyningen, S. Van & Shemin, D. 1971 *Biochemistry*, **10**, 4676–4682.
Jordan, P. M. & Akhtar, M. 1970 *Biochem. J.* **116**, 277–286.
Jordan, P. M. & Shemin, D. 1972 *Enzymes* 3rd edn, **7**, 339–356.
Laver, W. G., Neuberger, A. & Scott, J. J. 1959 *J. chem. Soc.* (*Lond.*), pp. 1483–1491.
Leinhard, G. E. & Rose, I. A. 1964 *Biochemistry* **3**, 185–190.
Luthy, J., Rétey, J. & Arigoni, D. 1969 *Nature, Lond.* **221**, 1213–1215.
Neuberger, A. 1961 *Biochem. J.* **78**, 1–10.
Palekar, A. G., Tate, S. S. & Meister, A. 1970 *Biochemistry*, **9**, 2310–2315.
Porra, R. J. & Falk, J. E. 1964 *Biochem. J.* **90**, 69–75.
Rose, I. A. 1970 *J. biol. Chem.* **245**, 6052–6056.
Rose, Z. B. 1960 *J. biol. Chem.* **235**, 928–933.
Sano, S. 1966 *J. biol. Chem.* **241**, 5276–5283.
Sano, S. & Granick, S. 1961 *J. biol. Chem.* **236**, 1173–1180.
Shemin, D. 1957 *Meth. Enzymol.* **4**, 643–646.
Wellner, D. 1970 *Biochemistry* **9**, 2307–2310.
Zaman, Z. 1973 Ph.D. Thesis, Southampton University.
Zaman, Z., Abboud, M. M. & Akhtar, M. 1972 *Chem. Commun.* pp. 1263–1264.
Zaman, Z. & Akhtar, M. 1975*a* (in preparation).
Zaman, Z. & Akhtar, M. 1975*b* (in preparation).
Zaman, Z., Jordan, P. M. & Akhtar, M. 1973 *Biochem. J.* **135**, 257–263.

Discussion

S. I. Beale (*The Rockefeller University, New York, N.Y.* 10021, *U.S.A.*). Could the solvent exchange of the C_5 hydrogens of ALA in crude enzyme preparations be due to the action of an ALA transaminase?

M. Akhtar. Although we have shown that L-alanine:4,5-dioxovalerate which is present in extracts of *R. spheroids* catalyses an exchange of C5 hydrogen atoms of ALA with the protons of the medium, some exchange activity is still present in preparations of ALA-synthetase which have no detectable activity of the transaminase.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES