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Mechanism and Stereochemistry of the 5-Aminolaevulinate Synthetase Reaction*

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1. Two mechanisms for the biosynthesis of 5-aminolaevulinate from glycine and succinyl-CoA (3-carboxypropionyl-CoA) are considered. One of the mechanisms involves the retention of both the C-2 H atoms of glycine during the synthesis of 5-aminolaevulinate, whereas the other predicts the retention of only one of the C-2 H atoms of glycine. 2. Highly purified 5-aminolaevulinate synthetase from *Rhodopseudomonas spheroides* was used to show that the C-2 H atom of glycine with *R* configuration is specifically removed during the biosynthesis of 5-aminolaevulinate. 3. The mechanism of the condensation therefore differs from the analogous reaction of the biosynthesis of sphinganine from palmitoyl-CoA and serine, in which the C-2 H of serine is retained (Wiess, 1963).

The enzyme 5-aminolaevulinate synthetase is involved in the initial stage of porphyrin biosynthesis (for a review see Jordan & Shemin, 1972). It catalyses the reaction:

$$NH_2\text{–}CH_2\text{–}CO_2H + HO_2C\text{–}CH_2\text{–}CH_2\text{–}COSCoA \rightarrow CO_2 + CoASH + NH_2\text{–}CH_2\text{–}CO\text{–}CH_2\text{–}CH_2\text{–}CO_2H$$

Early studies on aminolaevulinate synthetase established a number of interesting features including the obligatory involvement of pyridoxal phosphate for the catalytic activity (Burnham & Lascelles, 1963 and references therein; Neuberger, 1961 and references therein; Kikuchi *et al.*, 1958). At least two broad mechanisms may be considered for the formation of 5-aminolaevulinate from glycine and succinyl-CoA (Scheme 1). In mechanism I, the Schiff base of glycine and pyridoxal phosphate (IV) formed initially undergoes decarboxylation to give carbanion (V), which condenses with succinyl-CoA with retention of both C-2 H atoms; subsequent hydrolysis furnishes 5-aminolaevulinate (Scheme 1).

In mechanism II, loss of a C-2 H^+ gives carbanion (VII) that reacts with succinyl-CoA to give the Schiff base of α-amino-β-oxoadipic acid and pyridoxal phosphate (VIII). The latter intermediate may then yield 5-aminolaevulinate through a number of mechanisms, i.e. either (VIII) → (IX) → 5-aminolaevulinate, or (VIII) → (X) → 5-aminolaevulinate (Scheme 1). A mechanism of the type II has been favoured by previous workers (Kikuchi *et al.*, 1958).

In the present paper we describe the use of glycine, variously tritiated in the 2 position, in the elucidation of the mechanism of action of 5-aminolaevulinate synthetase. A preliminary account of a part of this work has already been published (Akhtar & Jordan, 1968).

* It is a privilege to dedicate this paper to Professor Edgar Lederer on the occasion of his sixty-fifth birthday, which fell on 5 June 1973.

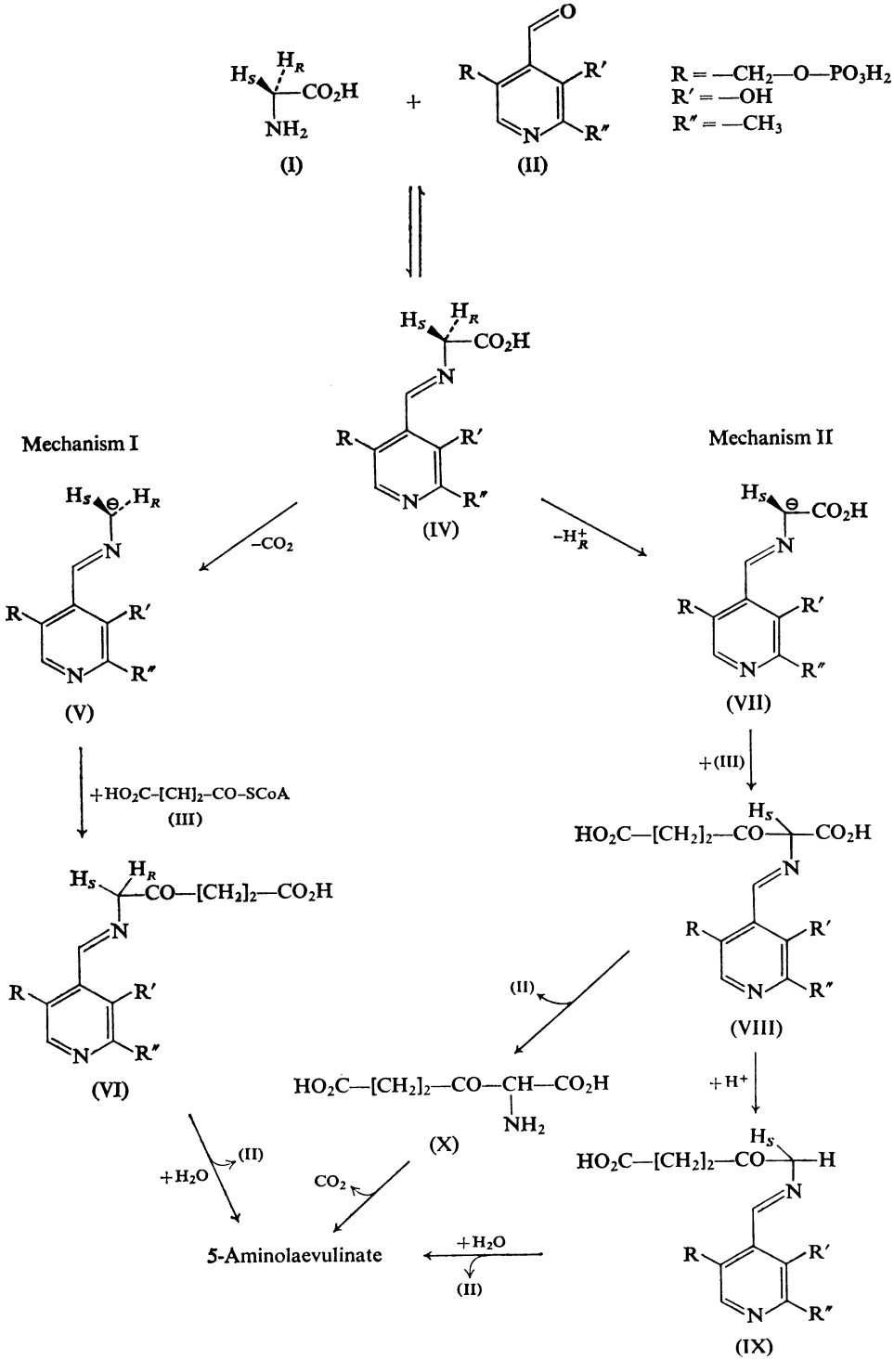
Experimental

Materials

[2-$^3H_2$]Glycine, [2-^{14}C]glycine and ^3H_2O were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 5-Amino[5-^{14}C]laevulinic acid hydrochloride was purchased from NEN Chemicals, Germany. Non-radioactive 5-aminolaevulinic acid hydrochloride, pyridoxal phosphate and ATP were obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. CoA and Trizma base (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. Other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Purification of 5-aminolaevulinate synthetase. (*a*) Crude enzyme. *Rhodopseudomonas spheroides* cells (2–4g wet wt., N.C.I.B. 8253) were suspended in 10ml of 0.04M-potassium phosphate buffer (pH7) and disrupted at 0°C for 10–15min, in an MSE ultrasonicator. The disrupted cells were centrifuged at 105000*g* for 1.5h. The clear upper portion of the supernatant was used in experimental work within 4h of centrifugation. The specific activity of the enzyme was always between 0.04 and 0.06 unit/mg.



Scheme 1. *Mechanisms of formation of 5-aminolaevulinate from glycine and succinyl-CoA*

For details see the text.

(*b*) The 33-fold purified enzyme. The method of Tuboi *et al.* (1970*a*,*b*) was followed up to the stage of Sephadex G-200. The specific activity at this stage was 1.86 units/mg of protein. The difference in specific activities between their preparation and ours may be explained by the fact that our assay method was different and also that in our column chromatography we pooled eluant fractions from both sides of the peak of enzyme activity, whereas Tuboi *et al.* (1970*a*,*b*) collected only the fractions with maximal activity.

(*c*) The 600-fold purified enzyme. The method employed was essentially that of Warnick & Burnham (1971) up to and including the calcium phosphate gel stage. Thereafter a modification was made as follows. The enzyme from the calcium phosphate gel step (5ml, sp. activity 4 units/mg of protein) was dialysed against 0.25M-potassium phosphate buffer (pH7) containing 0.1M-2-mercaptoethanol and 10% (v/v) glycerol and then applied to a DEAE-Sephadex A-25 column (1.5cm×20cm) previously equilibrated with the same buffer. The enzyme, which was largely unabsorbed, was collected in a total volume of 8ml containing 60% of the total activity applied to the column. This stage gave a further 10-fold purification of the enzyme, which now had a specific activity of 33 units/mg of protein. The different specific activities relative to those of Warnick & Burnham (1971) are due to the different nature of our assay method.

Unit of the enzyme activity. This is defined as 1 μmol of 5-aminolaevulinate formed/h at 37°C.

Enzyme assay. The assay mixture contained glycine (50mM), pyridoxal phosphate (0.1mM), potassium phosphate buffer, pH6.9 (50mM), EDTA (3mM), bovine serum albumin (5%, w/v), enzyme (0.1ml) and succinyl-CoA (2μmol) in a total volume of 0.375ml. After incubation for 10min, the reaction was stopped by addition of 0.2ml of 20% (w/v) trichloroacetic acid. The sample was centrifuged and the clear supernatant was assayed as follows.

Supernatant (0.5ml) was mixed in a test tube with 0.2ml of acetylacetone and 9.3ml of 1M-sodium acetate buffer (pH4.6). The tube was then heated in a boiling-water bath for 10min. After cooling, 2ml of this solution were added to 2ml of modified Ehrlich reagent (Mauzerall & Granick, 1956) and the E_{553} was measured after 15min.

Preparation of succinyl-CoA. Succinyl-CoA was freshly prepared by a modification of the method of Simon & Shemin (1953) in which succinic anhydride (1mg), $NaHCO_3$ (4.5mg) and CoA (8mg) were stirred in 1ml of water for 30min at 0°C.

Preparation of stereospecifically tritiated glycine. The [2*R*-^{3}H]- and [2*S*-^{3}H]-glycine were prepared as previously described (Jordan & Akhtar, 1970) by an exchange reaction with highly purified serine hydroxymethylase [kindly provided by our colleague H. A. El-Obeid and prepared by the method of Akhtar & El-Obeid (1972)]. The stereochemical purity of [2*R*-^{3}H]- and [2*S*-^{3}H]-glycine was at least 98%.

Isolation of the C-5 atom of biosynthesized 5-aminolaevulinate as a formaldehyde dimedone derivative. Incubation mixtures or samples therefrom were transferred to a 50ml flask containing 3mg of non-radioactive 5-aminolaevulinate and 6mg of $NaBH_4$ and the reaction was allowed to proceed in ice for 30min. The contents of the flask were then transferred to a centrifuge tube and the flask was washed with 2–3ml of methanol. The washing was added to the centrifuge tube. After centrifugation, the supernatant was evaporated to a small volume below 40°C. The sample was then spotted on a silica gel thick-layer plate (1mm thick) and developed in methanol–acetone–aq. NH_3 (sp.gr. 0.88) (20:10:3, by vol.). After drying thoroughly in a current of air, a small strip of the plate was sprayed with 0.5% (w/v) ninhydrin in acetone. The bands of glycine (R_F 0.27) and 5-amino-4-hydroxypentanoic acid (R_F 0.59) were removed and thoroughly eluted with aq. 10% (v/v) NH_3 and methanol respectively. The methanolic solution of 5-amino-4-hydroxypentanoic acid was evaporated to a small volume below 40°C and rechromatographed and eluted with methanol. The methanolic solution was evaporated to dryness and treated with saturated $NaHCO_3$ (10ml), sodium metaperiodate (1ml from a 100mg/ml solution) and formaldehyde (10mg, 0.025ml from a 40% solution of formaldehyde) and the reaction mixture was left in complete darkness for 8h. After this any precipitate was removed by filtration and the pH of the contents of the flask was adjusted to about 6 by dropwise addition of 50% (v/v) acetic acid.

Dimedone reagent (10ml) was added and the precipitation of the formaldehyde dimedone was allowed to proceed for 1h in ice. The precipitate was filtered, washed with 100ml of chilled water and crystallized slowly from hot methanol. The crystals were washed with 100ml of chilled water and dried at 100°C (m.p. 182°C).

Dimedone reagent. The reagent was made by dissolving, on a steam bath, 0.71g of $NaH_2PO_4,2H_2O$, 0.93g of Na_2HPO_4 and 1g of dimedone (which had been recrystallized twice from acetone) in 100ml of water.

Preparation of 5-amino[3,5-$^3H_4$]*laevulinate.* 5-Aminolaevulinate (50mg), 0.15ml of ^3H_2O (1Ci/ml) and 0.15ml of conc. HCl were autoclaved in an evacuated and sealed tube for 1h at 100kPa (15lb/in^2). After this the contents of the tube were freeze-dried. The residue was dissolved in 2ml of H_2O and again freeze-dried. This was repeated twice; 58% of the total ^{3}H incorporated into 5-aminolaevulinate was shown to be located at the C-5 atom by the following method. Tritiated 5-aminolaevulinate (0.5mg) and 1μCi of 5-amino[5-^{14}C]laevulinate were dissolved in 0.5ml of water; 0.25ml of this solution (1–1.5mg of

5-aminolaevulinate) was reduced with $NaBH_4$ (3mg) at 0°C for 30min, then 0.1ml of this solution was chromatographed, only once, to separate 5-amino-4-hydroxypentanoic acid (as described above). Another 0.1ml of the $NaBH_4$-treated solution was degraded to obtain the C-5 atom as the formaldehyde dimedone derivative. The amount of ^3H located at the C-5 atom of 5-aminolaevulinate was estimated by comparing the $^3H/^{14}C$ ratio of 5-amino-4-hydroxypentanoic acid with that of the formaldehyde dimedone derivative. This method of analysis showed that 58% of the original radioactivity was located at C-5. We have assumed that the remaining ^3H is located at the C-3 atom of 5-aminolaevulinate since H atoms at this position, being adjacent to a carbonyl group, are more likely to be labilized than those at the C-2 position. However, since in the biosynthetic and the exchange studies we isolated specifically the C-5 atom, the knowledge of ^3H at any other position in the molecule of 5-aminolaevulinate was not required.

Determination of exchange of ^3H located at C-5 of 5-amino[3,5-$^3H_4$]laevulinate. To study the exchange of ^3H atoms located at C-5 atom of 5-amino[3,5-$^3H_4$]-laevulinate, aminolaevulinate synthetase (0.41 unit of crude, 7.9 units of 33-fold-purified or 8.2 units of 600-fold-purified enzyme) was incubated at 37°C with all the constituents necessary for the biosynthesis of 5-aminolaevulinate except glycine, in the presence of 80μg of 5-amino[3,5-$^3H_4$]laevulinate for curve *A*, (Fig. 2), 50μg of 5-amino[3,5-$^3H_4$]laevulinate for curve *B* and 50μg of 5-amino[3,5-$^3H_4$,5-^{14}C]laevulinate ($^{14}C = 2.6\times10^6$c.p.m.) for curve *C*.

Samples of the incubation mixtures were taken at various time-intervals and added into a flask containing 3mg of non-radioactive 5-aminolaevulinate and 6mg of $NaBH_4$ at 0°C. After 30min, the contents of the flask were acidified to pH6 with 50% (v/v) acetic acid and treated with a saturated solution of $NaHCO_3$ (10ml), $NaIO_4$ (1ml from a 100mg/ml solution) and formaldehyde (10mg) for 8h in complete darkness. The contents of each flask were processed (as described above) to obtain the formaldehyde dimedone derivatives. A known weight of each sample of the dimedone derivative was measured for radioactivity.

The percentage exchange for curves *A* and *B* was calculated from the differences in specific radioactivities (c.p.m./mg) of the dimedone derivatives at zero time and at other respective times. For curve *C*, the percentage exchange was calculated from differences in $^3H/^{14}C$ ratios of the dimedone derivatives at zero time and at other times shown in Fig. 2.

Isolation of enzymically synthesized 5-aminolaevulinate. After incubation the remaining radioactive glycine was diluted with a large excess of non-radioactive glycine and the solution was freeze-dried immediately. The freeze-dried material was extracted at 0°C for 1h with 5ml of ice-cold methanol that had been saturated with glycine. Acetone (5ml) was then added to the methanol solution to precipitate most of the glycine. The precipitate was removed by centrifugation and the supernatant was evaporated below 40°C to a small volume, which was spotted on a thick-layer plate. The latter was developed in butanol–acetic acid–water (4:1:1, by vol.) and after drying 5-aminolaevulinate was detected by spraying a small strip of the plate with 0.5% ninhydrin solution in acetone (w/v). The 5-aminolaevulinate band (R_F 0.27) was eluted from the silica with methanol, which was then evaporated to leave solid 5-aminolaevulinate; 75% of the biosynthetic 5-aminolaevulinate can be recovered by this method.

Results

Enzymic synthesis of labelled 5-aminolaevulinate and the isolation of its C-5 atom as formaldehyde dimedone

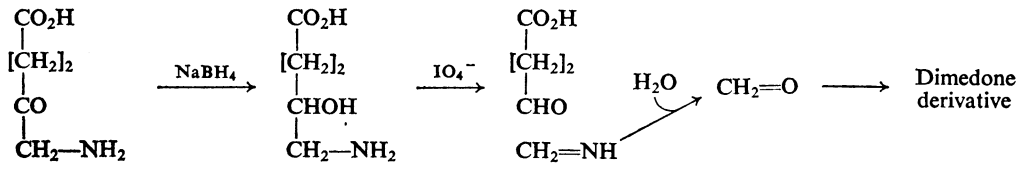
[2-^{14}C]Glycine was incorporated into 5-aminolaevulinate by using the crude enzyme preparation as described in Table 1. The 5-aminolaevulinate was reduced with $NaBH_4$ to 5-amino-4-hydroxypentanoic acid, which was separated from residual glycine and oxidized with periodate. The C-5 atom of 5-aminolaevulinate was liberated as formaldehyde and was isolated as its crystalline dimedone derivative (Scheme 2).

The results in Table 1 show that 25000c.p.m. were incorporated into the dimedone derivative, representing 4.25% conversion of glycine into 5-aminolaevulinate. A similar non-radioactive incubation, carried out simultaneously, on colorimetric assay indicated 5.3% conversion of glycine into 5-aminolaevulinate. The radiochemical yield of formaldehyde based on the colorimetric assay was thus in excess of 80%. In

Table 1. *Incorporation of [2-^{14}C]glycine into the C-5 atom of 5-aminolaevulinate*

The incubation mixture contained [2-^{14}C]glycine (500000c.p.m., 27μmol); sodium succinate (75μmol), CoA (1.3μmol), ATP (7.5μmol), pyridoxal phosphate (0.2μmol), reduced glutathione (1.5μmol), $MgSO_4$ (10μmol), $MnSO_4$ (0.025μmol), Tris–HCl buffer, pH7.5 (75μmol), and crude enzyme preparation (0.41 unit) in a total volume of 1.5ml. Incubation at 37°C for 1h was terminated by the addition of 3mg of 5-aminolaevulinate and 6mg of $NaBH_4$ at 0°C. The samples were then processed as described under 'Methods'.

Conditions of incubation	Total ^{14}C in formaldehyde dimedone derivative (c.p.m.)
Complete system	25000
Minus CoA	730
Boiled enzyme	850



Scheme 2. *Isolation of the C-5 atom of 5-aminolaevulinate*

The details of this procedure are described under 'Methods'.

the absence of CoA no appreciable glycine radioactivity was incorporated into 5-aminolaevulinate. These results show that under our conditions the C-2 atom of glycine is efficiently incorporated into the C-5 atom of 5-aminolaevulinate.

Enzymic synthesis of 5-aminolaevulinate from [2RS-$^3H_2$,2-^{14}C]glycine

To study the mechanism of 5-aminolaevulinate biosynthesis [2*RS*-$^3H_2$,2-^{14}C]glycine ($^3H/^{14}C = 2.0$) was incubated with the crude 5-aminolaevulinate synthetase preparation for various times (Fig. 1) and the C-5 atom of 5-aminolaevulinate was in each case isolated as the formaldehyde dimedone derivative. On extrapolation of the dimedone curve to zero time, the $^3H/^{14}C$ ratio of 1.0 was obtained. This value, when compared with $^3H/^{14}C$ ratio of 2.0 for the precursor glycine, represents a 50% loss of the ^3H present in the original sample of glycine. The extent of this ^3H loss agrees with the predicted loss of 50% required by mechanism II and strongly suggests that one of the C-2 H atoms of glycine is lost during the biosynthesis of 5-aminolaevulinate.

Exchange of the C-5 atom(s) of 5-aminolaevulinate under the incubation conditions

The possibility was considered that the loss of one of the H atoms under discussion occurred, not during the biosynthesis of 5-aminolaevulinate, but in a subsequent event in which another enzyme catalysed the stereospecific exchange of one of the C-5 H atoms of biosynthesized 5-aminolaevulinate with protons of the medium. We checked this by incubating samples of 5-amino[3,5-$^3H_4$]laevulinate with the crude enzyme preparation (used for the biosynthetic studies described above) in the presence of non-radioactive glycine. The results in Fig. 2, curve *A*, show that during 40 min of incubation in the presence of crude enzyme extracts there was an approximately linear loss of the ^3H located at C-5 due to exchange with the protons of the medium. The extent of the exchange, though not invalidating the conclusions regarding the loss of an H atom drawn from the experiments of Fig. 1, highlighted the need for a more refined approach. Accordingly, purification of

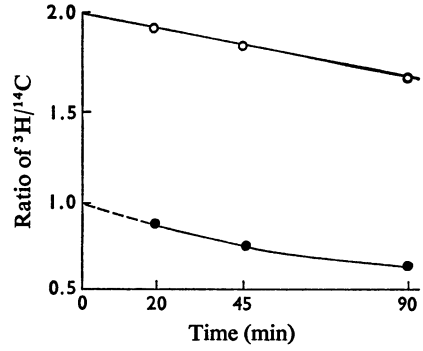


Fig. 1. *Enzymic synthesis of 5-aminolaevulinate from [2RS-$^3H_2$,2-^{14}C]glycine*

Samples of doubly labelled glycine were incubated at 37°C with the crude enzyme system as described in Table 1 for the times shown below, after which the reaction was terminated by addition to 3 mg of 5-aminolaevulinate and 6 mg of $NaBH_4$ at 0°C to furnish, after chromatography, glycine and 5-amino-4-hydroxypentanoic acid (see 'Methods'). The latter was then degraded to formaldehyde which was isolated as the dimedone derivative as described under 'Methods'. ●, Glycine; ○, 5-aminolaevulinate.

5-aminolaevulinate synthetase was undertaken and two preparations, a 33-fold-purified and a 600-fold-purified enzyme were obtained, and used to determine the extent of the exchange reaction. Fig. 2 shows that the enzymic activity catalysing the unwanted exchange of the C-5 C–H bond of 5-aminolaevulinate was greatly diminished in the 600-fold-purified enzyme.

Stereochemistry of proton elimination from glycine during 5-aminolaevulinate synthesis with 600-fold-purified 5-aminolaevulinate synthetase

Further experiments with [2*RS*-^3H,2-^{14}C]glycine and 600-fold-purified enzyme showed that biosynthesis of 5-aminolaevulinate involved a loss of 52% of the original radioactivity. Next the stereochemistry of the elimination reaction was studied. [2*R*-^3H, 2-^{14}C]Glycine and [2*S*-^3H,2-^{14}C]glycine prepared by

Table 2. *Stereochemistry of proton elimination from glycine during enzymic synthesis of 5-aminolaevulinate*

The incubation mixtures contained [2*RS*-$^3H_2$,2-^{14}C]glycine (Expt. 2), [2*R*-^3H,2-^{14}C]glycine (Expt. 1) or [2*S*-^3H,2-^{14}C]glycine (Expt. 3) ($^{14}C = 1.5 \times 10^6$ c.p.m. in 56μmol), succinyl-CoA (2μmol), $MgSO_4$ (10μmol), $MnSO_4$ (0.025μmol), pyridoxal phosphate (0.2μmol), mercaptoethanol (1μmol), Tris–HCl buffer, pH7.5 (75μmol), and enzyme (600-fold purified, 8.2 units in 0.5ml) in a total volume of 1.5ml. The incubation was carried out at 37°C for 10min and then terminated by addition of 3mg of 5-aminolaevulinate and 6mg of $NaBH_4$ at 0°C. The samples were then processed as described under 'Methods' to give the formaldehyde dimedone derivatives.

Expt. no.	$^3H/^{14}C$ ratio of initial glycine	$^3H/^{14}C$ ratio of formaldehyde dimedone derivative	% of ^3H lost in 5-aminolaevulinate biosynthesis: Found	Predicted
1	1.55	0.04	97	100
2	4.01	1.90	52	50
3	5.80	5.80	0	0

the method of Jordan & Akhtar (1970) were separately incubated with the 5-aminolaevulinate synthetase for 10min (Table 2). The loss of 97% of ^3H from [2*R*-^3H]glycine and the retention of all the ^3H from [2*S*-^3H]glycine proves that the C-2 H of glycine with pro-*R* configuration is lost and that with pro-*S* configuration is retained during the biosynthesis of 5-aminolaevulinate.

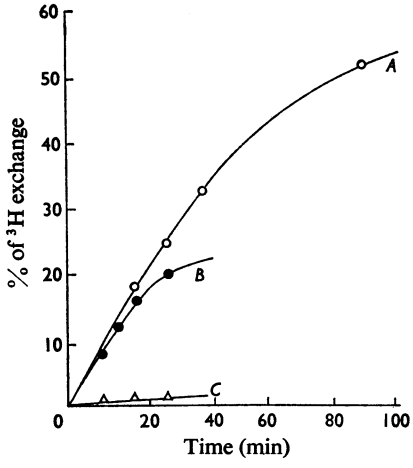


Fig. 2. *Exchange of ^3H located at C-5 of 5-amino-[3,5-$^3H_4$]laevulinate by 5-aminolaevulinate synthetase*

5-Amino[3,5-$^3H_4$]laevulinate was incubated at 37°C in the presence of the enzyme as described under 'Methods' and portions from incubation mixtures were removed at various times, reduced with $NaBH_4$ and then processed to yield the C-5 atom as formaldehyde dimedone derivatives. ○, Crude enzyme; ●, 33-fold-purified enzyme; △, 600-fold-purified enzyme.

Discussion

We have considered above two mechanisms for 5-aminolaevulinate synthetase: mechanism I requires the transfer of both the original C-2 H atoms of glycine to the C-5 atom of 5-aminolaevulinate, and mechanism II predicts the loss of only one of the glycine H atoms during biosynthesis. The present work showing the loss of a single C-2 atom of glycine establishes mechanism II for the 5-aminolaevulinate biosynthesis. This involves, in the crucial activation step, the removal of a proton with pro-*R* configuration from the enzyme–glycine–pyridoxal complex (IV) to give the carbanion species (VII), which condenses with succinyl-CoA to form (VIII).

The latter intermediate may then produce 5-aminolaevulinate either through the sequence:

(VIII) → (IX) → 5-aminolaevulinate

or via:

(VIII) → (X) → 5-aminolaevulinate

The loss of pro-*R* H of glycine in 5-aminolaevulinate synthesis should be compared with the loss of pro-*S* H previously reported for the biosynthesis of serine and threonine by the enzyme serine transhydroxymethylase (Jordan & Akhtar, 1970).

It is interesting to draw attention to another related condensation reaction, which involves the formation of 3-oxosphinganine from palmitoyl-CoA and serine (Wiess, 1963). In this particular case the formation of the new C–C bond occurs without the labilization of the C-2 H of serine, thus implicating the participation of the mechanism of the type I.

It would therefore appear that both modes of condensation predicted by mechanisms I and II operate in biological systems.

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