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Nuclear Magnetic Resonance Studies of 5-Aminolevulinate Demonstrate Multiple Forms in Aqueous Solution[1]

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5-Aminolevulinic acid (ALA), the common precursor of heme and chlorophyll, can exist in a variety of forms at neutral pH. ^{13}C NMR studies of [3-^{13}C]ALA, [4-^{13}C]ALA, and [5-^{13}C]ALA have been used to demonstrate that the predominant species in solution under physiologic conditions is the ketone. The mole fraction of the hydrate is about 0.6%. To further substantiate the existence of the hydrate, ^{13}C NMR was used to monitor ^{18}O exchange at C_4 of [4-^{13}C]ALA with H_2 ^{18}O. Confirmation of the existence of the hydrate was achieved through direct observation by ^1H NMR. The mole fractions of the enol forms of ALA are each below 0.3%. Although direct observation of the enol forms of ALA has not been achieved, enol formation has been indirectly demonstrated by monitoring hydrogen exchange at the C_3 and C_5 methylene groups by ^1H NMR in D_2O. In neutral phosphate buffer, hydrogen exchange occurs readily at both C_3 and C_5 at a ratio of rates of 1:4. In *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid–KOH buffer the hydrogen exchange rates are more than an order of magnitude slower than in phosphate buffer, but the ratio of the exchange rates remains unchanged. The results suggest that phosphate catalyzes enolization at both C_3 and C_5. To evaluate the role of the C_5 substituent in the proton exchange reactions, levulinate and 5-chlorolevulinate (5-CLA) were also monitored for proton exchange at C_3 and C_5. For levulinate, the hydrogen exchange rates in phosphate buffer are two to three orders of magnitude slower than for ALA, and the rate of hydrogen exchange at C_5 is three times slower than hydrogen exchange at C_3. The enolization rate at C_5 of 5-CLA is identical to ALA while enolization at C_3 is about threefold slower for 5-CLA than ALA. These NMR and kinetic studies suggest that under physiologic conditions, ALA rapidly equilibrates between the ketone, the hydrate at C_4, and two or more different enols (C_3–C_4 and C_4–C_5). The alternative forms of ALA may be biologically significant as active site structures for ALA synthase, glutamate semialdehyde transaminase, or porphobilinogen synthase. These NMR studies have also elucidated the structures of condensation products of ALA which can be formed under physiologic conditions. The alternative forms of ALA, as well as the autocondensation products, may serve as the active toxin in porphyrias characterized by elevated ALA levels (e.g., lead poisoning). © 1990 Academic Press, Inc.

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[3] Abbreviations used: ALA, 5-aminolevulinate; 5-CLA, 5-chlorolevulinate; LA, levulinate; PBG, porphobilinogen; NOE, nuclear Overhauser effect; PBGS, porphobilinogen synthase; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; BES, [*N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid]; HEPES, [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid]; Mops, [3-(*N*-morpholino)propanesulfonic acid]; PIPES, [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)].

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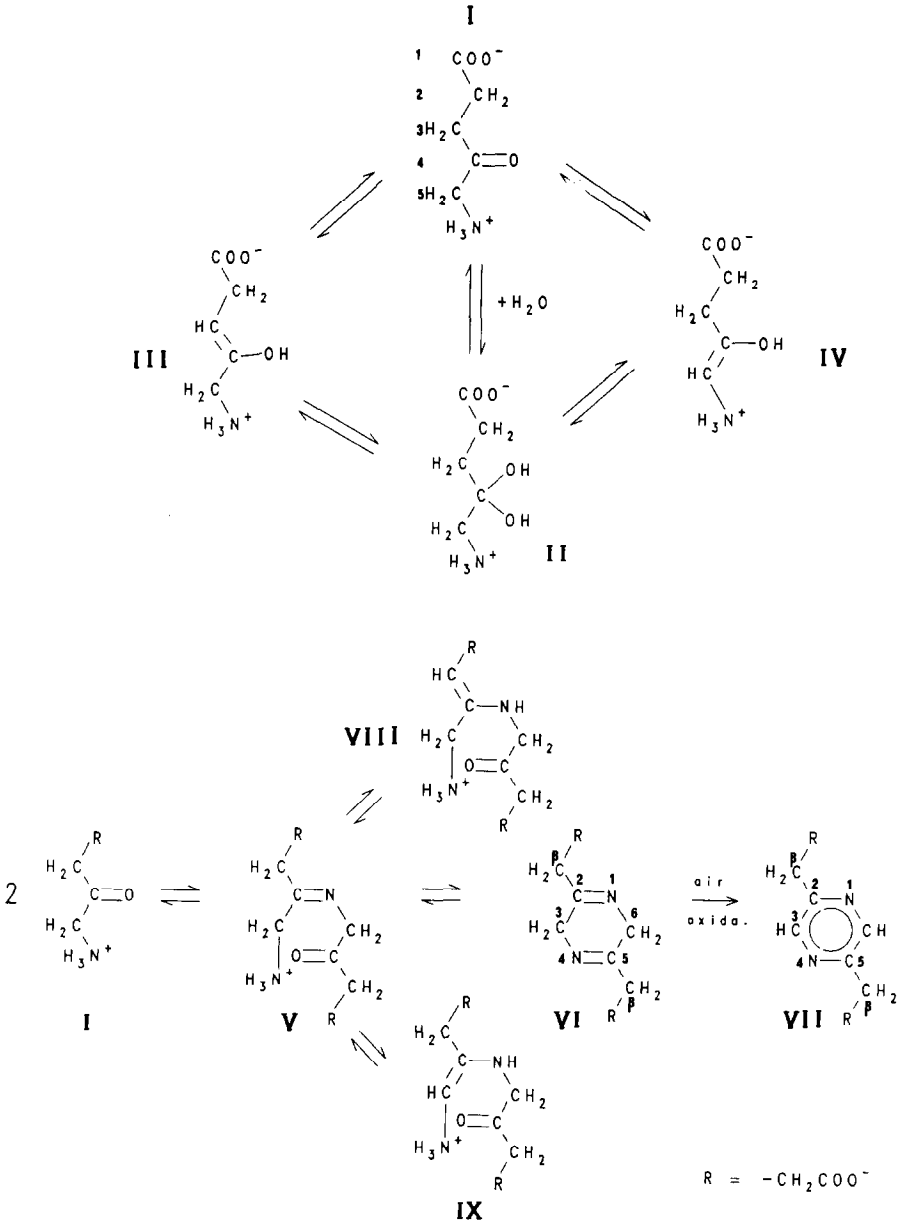
5-Aminolevulinic acid (ALA)[3] is the common precursor of all biochemical tetrapyrroles (*1*) (e.g., porphyrins, chlorophylls, vitamin B_{12}, cofactor F-430, phytochrome, and phycobilins) and is proposed as the active neurotoxin in many neuropathic porphyrias (*2*). Before one can fully understand the enzymatic or physiologic behavior of ALA, it is necessary to characterize the nonenzymatic aqueous chemistry. ALA, a 4-keto-5-amino acid, is unique in biochemistry and might exist in a number of forms in solution. Four such possible forms of ALA are illustrated in Scheme 1: the ketone **(I)**, the hydrate at C_4 **(II)**, the C_3–C_4 enol **(III)**, and the C_4–C_5 enol **(IV)**. The latter two tautomers can each exist as two stereoisomers, *E* or *Z*. These alternative structures are potentially significant in the enzymatic synthesis or utilization of ALA or as the active neurotoxin in porphyrias characterized by elevated ALA levels.

^{13}C NMR of [3-^{13}C]ALA, [4-^{13}C]ALA, and [5-^{13}C]ALA can be used to directly observe the equilibrium distribution of the forms of ALA. Supporting evidence for the existence of **II, III,** and **IV** in equilibrium with **I** can be derived through observation of oxygen and hydrogen exchange reactions. In the past, extreme conditions of acid or base have been utilized to incorporate deuterium and/or tritium into the C_3 and C_5 positions of ALA through enolization (*3–5*) but the existence of alternative isomers under physiological conditions has not previously been addressed. Hydrogen exchange reactions might also occur via enamine formation of an intermolecular Schiff base **(V)** between two ALA molecules. Therefore, to probe the role of the C_5 amino group in deuterium exchange at C_3 and C_5, deuterium exchange reactions have also been monitored for the ALA analogs levulinic acid (LA), which has no C_5 substituent, and 5-chlorolevulinic acid (5-CLA), which contains a chloro substituent in place of the amino group of ALA.

EXPERIMENTAL

Materials. ALA-HCl, LA, D_2O (99.8%, 99.96%, and >99.996%), and all buffers were purchased from Sigma Chemical Company. 5-CLA was prepared following a combination of published procedures (*6–8*). [3-^{13}C]ALA-HCl, [4-^{13}C]ALA-HCl, and [5-^{13}C]ALA-HCl (90% enriched) were purchased from Cambridge Isotope Laboratories and used without further purification. All other chemicals were reagent grade. For use in the deuterium exchange experiments, buffers in H_2O were adjusted to pH 7.0 for KP_i or pH 7.2 for TES–KOH and lyophilized in 0.5-ml aliquots. These aliquots were then lyophilized twice from 99.8% D_2O and stored dry until needed. Reported pH values are corrected for the D_2O effect (meter reading +0.4).

^{13}C NMR characterization of ALA in aqueous solution. ^{13}C NMR spectra were obtained at 75.46 MHz on a Bruker AM-300 wide-bore spectrometer with a 10-mm probe. The chemical shift reference was external dioxane set at δ 67.4. High signal-to-noise spectra of [4-^{13}C]ALA-HCl (40 mM) were obtained in 0.1 M KP_i (10% D_2O), pH 6.8. For pH titrations, 40 mM [4-^{13}C]ALA in 10 mM KP_i (10% D_2O) was used at 37°C. ^{13}C NMR spectra of [5-^{13}C]ALA and [3-^{13}C]ALA were acquired at 37°C at a concentration of 10 mM in 0.1 M KP_i, 10 μM $ZnCl_2$, 10 mM 2-



SCHEME 1. Top: Potential solution structures of ALA: **I,** the ketone; **II,** C_4 hydrate; **III,** C_3–C_4 enol (or enolate); **IV,** C_4–C_5 enol (or enolate). Bottom: Potential condensation products of ALA at mildly basic pH: **V,** mono-Schiff base of ALA; **VI,** 3,6-dihydro 2,5-pyrazinedipropanoic acid; **VII,** 2,5-pyrazinedipropanoic acid, an oxidation product of **VI; VIII,** the C_3–C_4 enamine of **V;** and **IX,** C_4–C_5 enamine of **V.** Structures **III, IV, V, VIII,** and **IX** can each exist as two stereoisomers.

mercaptoethanol (porphobilinogen synthase assay buffer). A D_2O capillary insert was used to provide the lock signal.

^1H NMR for direct observation of the hydrate. A solution of 0.9 M ALA in 10 mM KP_i was adjusted to pH 4.0. The spectra were acquired at 25°C on a Bruker AM-300 with a 5-mm ^1H probe. The H_2O signal (δ 4.8) was presaturated for 2 s before acquisition and a 7.4-s recycle time was used. When the pH was changed to 4.8 the solution became yellow, suggesting the condensation of ALA.

^{18}O exchange to probe the existence of the C_4 hydrate of ALA. [4-^{13}C]ALA-HCl (18 μmol) was added to 0.1 M KP_i in 50% $H_2{}^{18}O$, 5% D_2O (final pH 6.8; 2 ml), and incubated at room temperature for about 20 min. ^{13}C NMR spectra were obtained on a Nicolet NT-300 wide-bore spectrometer at 37°C.

Deuterium exchange to probe the existence of the enol tautomers of ALA, 5-CLA, and LA. ALA-HCl (10 μmol) was dissolved with one aliquot of lyophilized buffer (see above) in 0.5 ml of 99.96% (or >99.996%) D_2O which had been preheated to 37°C. The time of ALA dissolution was defined as zero and the sample was immediately placed in a 5-mm NMR tube and inserted into the NMR probe which had been equilibrated at 37°C. Spectra were acquired at appropriate time intervals to monitor the loss of protons at C_3 and C_5. All ^1H NMR spectra of ALA and LA were obtained on a Nicolet NT-300 spectrometer with a 5-mm proton probe; acquired with a digital resolution of 0.13 Hz/point; and processed with a 0.1-Hz Lorentzian line broadening function. For all but the first spectrum of each series, in which the sample was reequilibrating to the probe temperature, the narrow linewidths (<0.5 Hz) were an indication of stable temperature control. Deuterium exchange on 5-CLA followed the above protocol with the exception that a Bruker AM-300 spectrometer was used for data acquisition. In the case of LA, where the exchange is slow, 20 mM LA was prepared in KP_i buffer (99.8% D_2O, pH 6.8, 18 ml). Aliquots (0.5 ml) were placed in tightly sealed tubes and placed in a 37°C oven for periods of 0–40 days. After removal from the oven, aliquots were stored at −70°C until ^1H NMR spectra were obtained (probe at 25°C).

Proton exchange data analysis. Proton spectra for ALA and LA were integrated using Nicolet software at an external Data Station; for 5-CLA the spectra were processed using the program FTNMR (Hare Research, Inc) running on a VAX computer. To compensate for variations in instrument sensitivity with time, the signal intensities for the protons at C_3 and C_5 were normalized to a constant signal intensity for the protons attached to C_2 since the C_2 protons do not exchange. All proton integrals for C_3 and most of the proton integrals for C_5 include the sum of the protons from all species. In many cases, the loss of protons follows a single first-order decay for as long as the exchange reactions were monitored (Fig. 1). When decays showed more than a single rate, it was assumed that the difference was due to a secondary kinetic isotope effect resulting from deuteration and the initial rates were calculated.

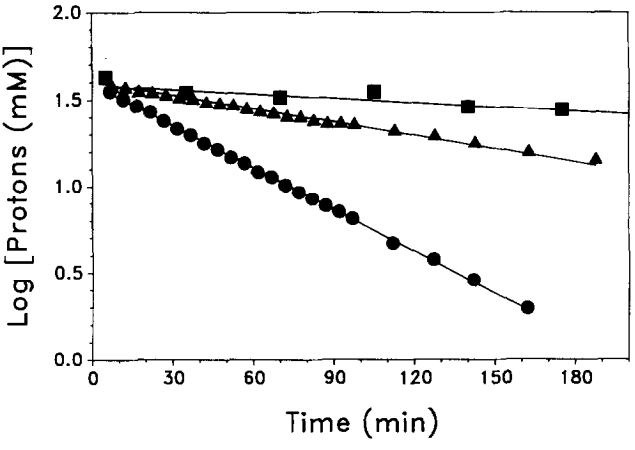


FIG. 1. Examples of pseudo-first-order proton loss through enolization of ALA in D_2O. ALA 5-CH_2 → CHD in 50 mM KP_i, pH 6.3 (●); ALA 3-CH_2 → CD_2 in 0.1 M KP_i, pH 6.8 (▲); ALA 5-CH_2 → CD_2 in TES–KOD, pH 6.8 (■). In the case of the triangles, the line reflects the initial rate (first 20 points, see Table 2 and Experimental).

RESULTS AND DISCUSSION

^{13}C NMR of [3-^{13}C]ALA, [4-^{13}C]ALA, and [5-^{13}C]ALA to Probe the Solution Behavior of ALA

Based on the chemistry of ketones, amines, and amino ketones in aqueous solutions, ALA can exist in a number of forms as shown in Scheme 1. The presence of these forms can be detected, if not completely distinguished, in the ^{13}C NMR spectra of [4-^{13}C]ALA in neutral phosphate buffer. The ketone would be expected to exhibit a chemical shift of 200–210 ppm; resonances from the enols, enamines, or Schiff bases might occur in the range 150–175 ppm; and the hydrate should appear in the range 85–100 ppm. A high signal-to-noise spectrum of [4-^{13}C]ALA in neutral 0.1 M phosphate buffer reveals eight signals at $s/n \geq 2$ when processed with a 3-Hz exponential line broadening function (Fig. 2). The signals are of comparable linewidth and can therefore be quantified by peak height. The natural abundance peak heights for C_1, C_2, C_3, and C_5 with respect to C_4, show excellent agreement with a theoretical 1.1% natural abundance and an NOE of 2 where applicable and are therefore considered quantitative. The chemical shift assignments for the predominant ketone isomer are listed in Table 1 (with coupling constants $J_{3,4}$ = 42 Hz, $J_{4,5}$ = 39 Hz). Three additional signals are observed at δ 174.0, 154.4, and 93.3 respectively at 6.5, 1.2, and 0.6% the intensity of the C_4 ketone signal. The signal at δ 93.3 is assigned to the hydrate on the basis of ^1H NMR studies described below.

The chemical shifts of δ 174.0 and 154.4 are each appropriate for any of the enol, enamine, or Schiff base forms of ALA **(III, IV, V, VI, VIII,** or **IX).** The signal at δ 154.4 was found to be insensitive to derivatization of ALA with NH_2OH and therefore does not derive from a species in equilibrium with the ketone. It has been

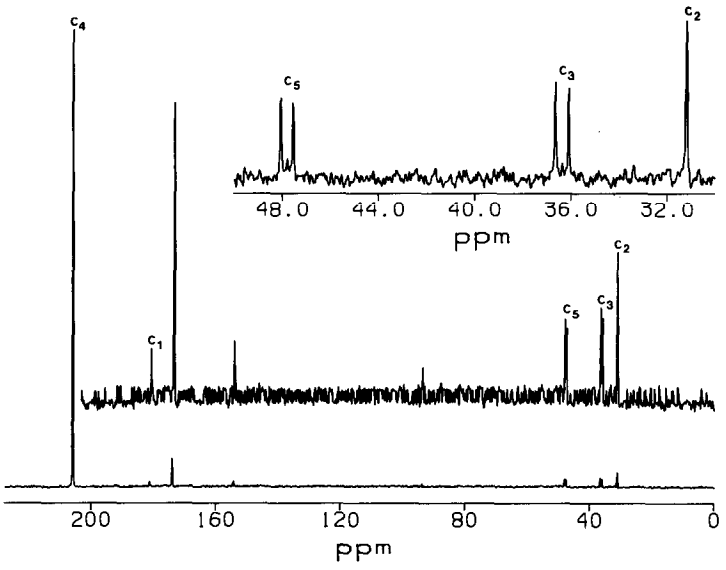


FIG. 2. ^{13}C NMR spectra of 40 mM [4-^{13}C]ALA (90% ^{13}C) in 0.1 M KP_i, pH 6.8, at 25°C. A 45° pulse angle, 4-s recycle time, and broadband ^1H decoupling were used. Bottom: C_4 ketone resonance (205.9 ppm) is the predominant signal. Middle: Tenfold vertical expansion of top spectrum, identical chemical shift scale, identifying the C_1, C_2, C_3, C_5 resonances. Top: Expanded spectrum showing the natural abundance methylene groups.

identified as a slowly accumulating oxidation product (see below). The relative intensities of δ 174.0 and 205.9 were found to be highly dependent upon pH. Below pH 6.0, the 174.0 ppm signal is not visible. At pH 8.1, the signals at 174.0 and 205.9 ppm (C_4, keto form) move to 173.3 and 206.4 ppm and are almost equal in intensity. At pH 9.5, the signal from the keto form is less than 10% of the total and moves to 213.9 ppm; at this pH the signal at 174.0 ppm shifts to 173.2 ppm.

The ^{13}C NMR spectra of [3-^{13}C]ALA and [5-^{13}C]ALA at neutral pH each contain a minor resonance, at a level of 4–6% of the ketone, which increases in intensity as the pH is raised. The chemical shifts for the minor signal derived from C_3 and C_5 are 33.8 and 51.0 ppm, respectively, at neutral pH. These signals apparently do not derive from enolic or enamine forms since the observed resonances are at least 60 ppm upfield from the chemical shifts predicted for olefinic resonances.

The remaining structures to be considered as alternative forms of ALA are the mono- and di-Schiff base dimers **(V and VI)**. For the mono-Schiff base structure **(V)** the ^{13}C NMR signals from the C_5-derived carbon α to the imino carbon are expected to be -3 to -12 ppm upfield, the C_5-derived carbon attached to the imino nitrogen will be shifted downfield from the corresponding resonance of the keto form of ALA (*9*). If the mono-Schiff base is in equilibrium with the ketone, one would expect to see three C_5-derived signals two of which must be equal in intensity. At neutral pH only two signals were observed. Therefore, the C_5-derived signal at 51.0 ppm does not arise from the mono-Schiff base structure, suggesting

TABLE 1

^{13}C and ^{1}H NMR Chemical Shifts of **I, II, VI,** amd **VII**

Compound	Carbon	Chemical shift (ppm) Neutral pH	Basic pH	Proton	Chemical shift (ppm)
I[a]	C_1	181.4		2-CH_2	2.45 (2.44)
	C_2	31.2		3-CH_2	2.75 (2.73)
	C_3	36.4	36.4	5-CH_2	4.05 (3.99)
	C_4	205.9	206.4		
	C_5	48.1	50.4		
II[b]	C_4	93.3		2-CH_2	2.34
				3-CH_2	1.95
				5-CH_2	3.02
VI[c]	C_2,C_5	174.0	173.3	3-CH_2	4.06 (4.10)
	C_3,C_6	51.0	51.3	6-CH_2	4.06 (4.10)
	β-CH_2	33.8	33.9	α-and	2.54 (2.51)
				β-CH_2	2.38 (2.39)
VII[d]	C_2,C_5	154.4			
	C_3,C_6	143.7	143.7		

[a] At neutral pH, 40 mM [4-^{13}C]ALA in 0.1 M KP_i was used to obtain the chemical shifts of all carbons. At basic pH, the corresponding ^{13}C-enriched ALA was used (pH 8.5 for [3-^{13}C] and [4-^{13}C]ALA and pH 8.1 for [4-^{13}C]ALA). ^{1}H NMR chemical shifts are reported at pH 7.0 and 8.5 (in parentheses).

[b] [4-^{13}C]ALA, 40 mM, in 0.1 M KP_i was used at pH 6.8 to obtain ^{13}C chemical shift. For ^{1}H NMR 0.9 M ALA in 10 mM KP_i at pH 4.0 was used.

[c] The ^{13}C NMR chemical shifts of **VI** were obtained using the [^{13}C]ALA indicated in parentheses: C_2/C_5 ([4-^{13}C]ALA, pH 6.8 and 8.1), C_3/C_6 ([5-^{13}C]ALA, pH 7.0 and 8.5), β-CH_2 and 8.5 (in parentheses).

[d] The ^{13}C NMR chemical shifts of **VII** were obtained as in the case of **VI.**

rather the formation of a dihydropyrazine **(VI).** Further support for the presence of a dihydropyrazine comes from the pH dependence of the C_4 chemical shift of [4-^{13}C]ALA ($\Delta\delta$ = 8.0 ppm from pH 7.0 to pH 9.5), indicating that the ketone resonance does not derive from the mono-Schiff base dimer **(V)** (*9*).

From the above data, we conclude that the signals at 174.0 ppm of [4-^{13}C]ALA, 33.8 ppm of [3-^{13}C]ALA, and 51.0 ppm of [5-^{13}C]ALA, all of which increase at basic pH, arise from the same compound in which the hybridization at both C_3 and C_5 does not change. ALA is known to form a dihydropyrazine in alkaline solutions, through an azomethine reaction characteristic of α-aminoketones (*10*). Therefore, the chemical shift values of 174.0, 51.0, and 33.8 ppm apparently arise from the C_2/C_5, C_3/C_6, and β-CH_2 of the carboxy propionyl group, of the dihydropyrazine structure, respectively (**VI,** Table 1). The position of the equilibrium between **I** and **VI** is pH dependent; equilibration is rapid with the slow and irreversible accumulation of **VII.** Compound **VII** is the species observed at δ 154.4 in the spectrum of [4-^{13}C]ALA (Table 1).

^{1}H NMR Studies to Probe the Structure of the Minor Form of ALA

A proton NMR spectrum of 10 mM ALA in 10 mM KP_i buffer at neutral pH contains three major peaks (Table 1): a singlet at 4.05 ppm from 5-CH_2, and two triplets at 2.45 and 2.75 ppm from 2-CH_2 and 3-CH_2, respectively. A second set of

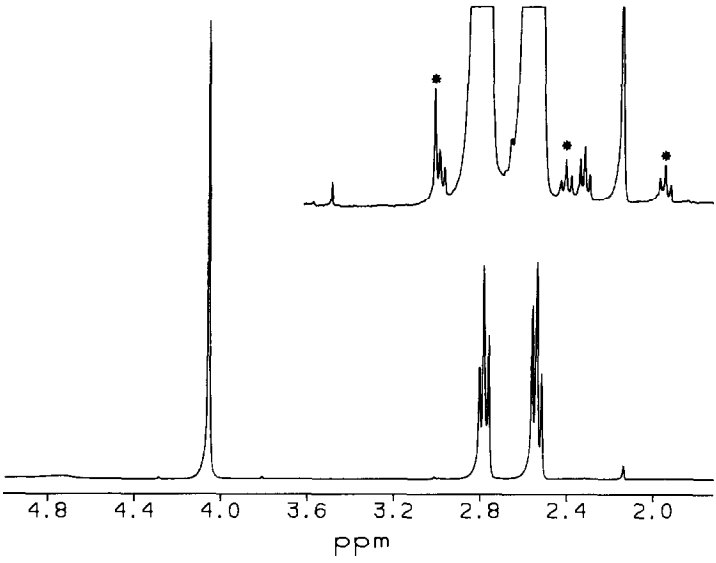


FIG. 3. ^{1}H NMR spectra of 0.9 M ALA. The bottom spectrum illustrates the predominant ^{1}H signals of the ketone. The inset, a 100-fold vertical expansion, illustrates ^{1}H signals (*) at 0.4% which correspond to those predicted for the hydrate of ALA. The remaining two triplets are not spin coupled. The origin of other signals is unknown. Some may arise from spinning side bands.

peaks (Table 1) contains two spin–spin coupled triplets and a singlet. Two triplets of equal intensity (but approximately 4% of ALA triplets) occur at 2.54 and 2.38 ppm. A singlet, 0.01 ppm downfield of the 5-CH_2 signal but only 5% in intensity, is also visible. The chemical shift values of the second set of signals from the ^{1}H NMR ALA at neutral and basic pH (Table 1) are also consistent with a dihydropyrazine structure. At pH 8.5, the signals corresponding to ALA decrease and the second set of signals increase in intensity; after approximately an hour, the second set of peaks are almost of the time area as the peaks from ALA.

^{1}H NMR for Observation of the Hydrate

The methylene protons of the hydrate form of ALA are expected to exhibit a set of ^{1}H NMR signals upfield from the corresponding ketone methylene groups. The α-methylene protons of the hydrate are predicted to be 0.7–0.9 ppm upfield from those of the ketone isoform, and the β-methylene protons are predicted to be 0.14–0.20 ppm upfield from the parent signal (*11*). As illustrated in Fig. 3, a high signal-to-noise spectrum of 0.9 M ALA at pH 4.0 shows a set of resonances of almost equal area which fall within this prediction; there is a singlet 1.03-ppm upfield from the C_4 methylene signal, a triplet 0.83 ppm upfield from the C_3 methylene signal, and a second triplet 0.11 ppm upfield from the C_2 methylene signal (Table 1). The two triplets were shown to be coupled by homonuclear decoupling. There are several other signals in the ^{1}H NMR spectrum of 0.9 M ALA

which confound the spectrum at higher pH. Therefore, on the basis of the spectrum of 0.9 M ALA at pH 4, 0.4% is the upper limit for the mole fraction of the hydrate form of ALA in aqueous solution. ^{1}H NMR of a solution of 40 mM ALA in 0.1 M KP_i, pH 6.8, also shows 0.4% as the hydrate on the basis of the C_5 peak at 3.03 ppm. From the ^{13}C NMR spectrum of [4-^{13}C]ALA, the mole fraction of the hydrate might be as high as 0.6% (δ 93.3, see above). A mole fraction of hydrate at 0.4–0.6% is comparable to an aqueous solution of acetone, where 0.2% exists as the hydrate and 99.8% exists as the ketone (*12*).

Oxygen Exchange at C_4 of ALA

Supporting evidence for formation and breakdown of the hydrate of ALA was demonstrated by monitoring oxygen exchange at C_4. Oxygen exchange was followed by ^{18}O incorporation from $H_2{}^{18}O$ into [4-^{13}C]ALA by ^{13}C NMR. The ^{13}C chemical shift change expected when ^{18}O replaces ^{16}O in an aldehyde or ketone is 0.045–0.050 ppm upfield (*13*). When [4-^{13}C]ALA is dissolved in 0.1 M KP_i, pH 6.8, 50% $H_2{}^{18}O$, the incorporation of ^{18}O into C_4 is complete in less than 20 min at room temperature demonstrating the facile formation and breakdown of the C_4 hydrate. The observed isotope shift is −0.046 ppm (3.5 Hz) at 37°C.

Deuterium Isotope Exchange at C_3 and C_5 of ALA as Evidence for the Enolization of ALA

The protons on C_3 and C_5 of ALA are labile to deuterium exchange through enolization. The evolution from CH_2 to CD_2 can be monitored by ^{1}H NMR as total proton loss by signal integration with respect to the C_2 protons and by analysis of peak multiplicity. At C_5, CH_2 is a singlet at 4.050 ppm; CHD is a 1 : 1 : 1 triplet at 4.033 ppm, J_{HD} = 2.5 Hz. These signals are reasonably well resolved and can be integrated separately[4] (Fig. 4A). For C_3, which is coupled to the methylene protons at C_2, the protons from the CH_2 species exhibit a 1 : 2 : 1 triplet line shape at 2.746 ppm, J_{HH} = 6.7 Hz; CHD is a 1 : 2 : 1 triplet of 1 : 1 : 1 triplets which is difficult to decipher (Fig. 4B). Isotope exchange at C_3 can also be monitored by the structure of the signals from the C_2 protons. When adjacent to CH_2, the C_2 proton signal is a 1 : 2 : 1 triplet at 2.445 ppm; when adjacent to CHD, the C_2 proton signal is a doublet at 2.440 ppm (J_{HH} = 6.6 Hz), and the signal is slightly broadened by unresolved coupling to the deuterium atoms; when adjacent to CD_2, the C_2 proton signal is a singlet at 2.435 ppm, substantially broadened by unresolved coupling to the deuterium atoms (Fig. 4B).

An estimate of the rates of proton loss from C_5 or C_3 was made by integrating over the entire chemical shift range of the protonated and partially deuterated species and fitting the data to a pseudo-first-order reaction rate (Fig. 1 and Table 2). This method ignores secondary deuterium kinetic isotope effects, which are expected to be small, and is the only feasible method for analysis of the C_3 protons.

[4] From these data one can derive the secondary deuterium isotope effect on enolization at C_5, which is approximately 1.45.

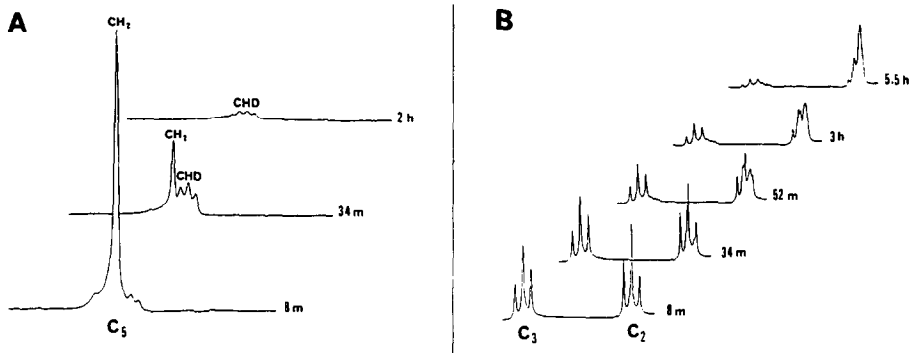


FIG. 4. ^{1}H NMR spectra of ALA during deuterium exchange through enolization. (A) Successive deuteration of C_5. The spectrum at 8 min is predominantly CH_2; the spectrum at 34 min is a nearly equimolar mixture of CH_2 and CHD; the spectrum at 2 h shows a trace of CHD remaining. (B) Successive deuteration of C_3, illustrating the effect on the chemical shifts and multiplicities at both C_2 and C_3. The spectrum at 8 min shows no evidence of deuteration. The C_2 signal of the spectrum at 34 min shows the beginnings of the isotope-shifted doublet for those C_2 atoms adjacent to CHD at C_3. By 52 min the C_2 signal is an overlay of the triplet and doublet signals for those atoms adjacent to CH_2 and CHD, respectively. The signal from CHD at C_3, which should be a 1 : 2 : 1 triplet of 1 : 1 : 1 triplets, deuterium isotope shifted upfield, appears as a broad signal, mostly beneath the triplet. By 5.5 h, the C_3 signal is nearly gone and the predominant structure at C_2 is a broad singlet for those atoms adjacent to CD_2.

Table 2 lists the exchange rates of the protons at C_3 and C_5 under a variety of conditions. The most rapid exchange is observed at pH 6.8 in 0.1 M KP_i; the half-times for proton exchange at C_3 and C_5 are about 2 and 0.5 h, respectively. When the pH is lowered to 6.3, 50 mM KP_i, the half-times for proton exchange at both C_3 and C_5 increase to 6.9 and 0.9 h, respectively.

Although phosphate is invisible to ^{1}H NMR and is thus an attractive buffer for

TABLE 2

Proton Exchange Rates at C_3 and C_5 of 5-Aminolevulinate (ALA), Levulinate (LA), and 5-Chlorolevulinate (5-CLA)

Sample (10 μmol ALA, 5-CLA, or LA in 0.5 ml)	C_3 proton exchange				C_5 proton exchange			
	Percentage exchange[a]	Rate (h^{-1})	Correlation coeff.	Number of points	Percentage exchange	Rate (h^{-1})	Correlation coeff.	Number of points
ALA in 0.1 M KP_i pH 6.8	0–35	0.36[b]	0.995	20	0–50	1.4		
LA in 0.1 M KP_i pH 6.8	0–75	0.0028	0.995	7	0–54	0.0010	0.990	11
5-CLA in 0.1 M KP_i pH 6.8	0–75	0.123	0.998	15	0–86	1.3	0.985	11
ALA in 50 mM KP_i pH 6.3	0–35	0.10	0.965	20	0–48	0.77	0.90	
ALA in 0.1 M TES–KOH, pH 6.8	0–51	0.028	0.991	31	0–92	0.12	0.978	31

[a] Percentage exchange refers to the region of the exchange reaction for which the rates are calculated.
[b] All rates are calculated on the bais of total proton intensities (loss of 2 protons, or 3 protons for C_5 of LA).

proton exchange studies, alternative buffers were examined to test if the rapid proton exchange observed in KP_i was buffer catalyzed. In all the zwitterionic buffers tested at 0.1 M, pH 6.8, (BES, HEPES, PIPES, MOPS, and TES), proton exchange on ALA was substantially slower than in KP_i. TES was selected for additional proton exchange studies because the proton signals from TES are particularly well resolved from those of ALA. The half-times for exchange of the C_3 and C_5 protons of ALA in 0.1 M TES, pH 6.8, are 25 and 6 h, respectively, both 12-fold less than in 0.1 M KP_i (Table 2).

Deuterium Exchange at C_3 and C_5 of 5-CLA and LA

5-CLA and LA also undergo deuterium exchange at C_3 and C_5 which can be monitored by ^1H NMR. For 5-CLA the ^1H NMR chemical shifts for protons at C_2, C_3, and C_5 are 2.64, 2.99, and 4.65 ppm respectively. Deuterium incorporation at C_3 or C_5 causes similar changes in lineshape and deuterium isotope shifts as for ALA (Fig. 4B). For LA the ^1H NMR chemical shifts for protons at C_2, C_3, and C_5 are 2.28, 2.65, and 2.10 ppm, respectively. Deuterium incorporation at C_3 causes the same change in lineshape for C_3 and C_2 as for the C_3 and C_2 positions of ALA (Fig. 4B). Deuteration at C_5 results in the transformation of CH_3 (a singlet) to CH_2D (a deuterium-shifted, deuterium-coupled 1 : 1 : 1 triplet) to CHD_2 (further deuterium-shifted, deuterium-coupled 1 : 2 : 3 : 2 : 1 quintet); at 300 MHz the triplet and quintet signals overlap (spectra not shown). The deuterium exchange rates at C_3 and C_5 of 5-CLA and LA were determined by integrating over all protonated species at C_3 and C_5, normalizing to the proton intensity at C_2. The half-time for deuteration at C_5 of 5-CLA is almost identical to that for ALA while exchange of the C_3 protons is about three times slower (in 0.1 M KP_i, pH 6.8, 37°C). The half-times for deuteration of LA are at least two orders of magnitude slower than those for ALA, about 12 and 29 days for C_3 and C_5, respectively (in 0.1 M KP_i, pH 6.8, 37°C).

Solution Chemistry of ALA under Physiologic Conditions

The alternative forms of ALA (Scheme 1) have been probed by ^{13}C and ^1H NMR. All of the NMR studies demonstrate that the major form of ALA under physiologic conditions is the ketone **(I).** ^{13}C NMR of [4-^{13}C]ALA and ^1H NMR of unlabeled ALA provide evidence that the hydrate may account for 0.4–0.6% of the species in solution **(II).** Observation of ^{18}O exchange at C_4 provides additional support for the existence of the ALA hydrate. We conclude that *none* of the signals observed in the spectra of [3-^{13}C], [4-^{13}C), and [5-^{13}C]ALA derive from the enol or enamine structures **(III, IV, VIII,** or **IX).** Based on the ^{13}C NMR spectrum of [4-^{13}C]ALA the enolic and enamine forms must each be less than 0.3% of the keto form of ALA. In the absence of direct observation of the enols and/or enamines, indirect evidence for their existence comes from the proton exchange reactions which we have observed at both C_3 and C_5. At neutral pH, 4–7% ALA exists as a dihydropyrazine **(VII)** and the mole fraction of **VII** is dependent on the concentration of ALA. It is interesting to note that a pyrazine, probably a deriva-

tive of ALA, has been reported in the urine of patients suffering from acute intermittent porphyria (*14*).

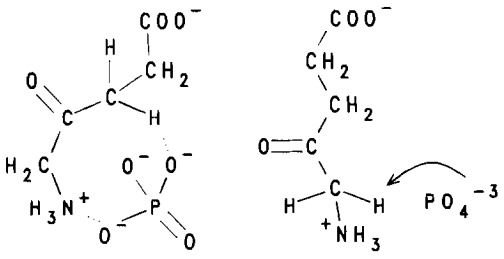
Mechanisms for Isotope Exchange on ALA, 5-CLA, and LA

The mechanisms for C_4 oxygen exchange on ALA can be either through hydration and dehydration or through reversible formation of the condensation products illustrated in Scheme 1 **(I–VI).** We have presented evidence for the existence of both the hydrate and the condensation products; thus both mechanisms must contribute to the observed isotope exchange.

Deuterium exchange at C_3 and C_5 can occur either through enolization or through transient bimolecular enamine formation. Isotopic exchange via imine formation generally proceeds at a much higher rate than through enolization. For example, catalysis of enolization of acetone via ketimine formation is several orders of magnitude higher than in the absence of an amine catalyst (*15*). However, the enolization rate at C_5 of ALA is only slightly higher than that of 5-CLA (which contains a chloro substituent in place of the amino group and thus cannot form an enamine), which suggests that deuterium exchange principally results from enolization in both cases. It is possible that the dihydropyrazine is formed by dehydration of a cyclic condensation product of ALA (resulting from intramolecular condensation of the hemiaminal of ALA) instead of from the mono-Schiff base **(VI);** this could account for no significant accumulation of either the imine or enamine form of ALA. Moreover, electron-withdrawing substituents β to the amino groups tend to decrease the equilibrium constants for the formation of imines such as **VI** (*16, 17*).

Based on the proton exchange data, enolization of ALA occurs to form both **III** and **IV.** Enolization at C_5 **(IV)** is the more rapid of the two reactions, by about a factor of 4. The enolization rate at C_5 of 5-CLA is almost identical to that of ALA, while enolization at C_3 is about threefold slower for 5-CLA. On the other hand, the enolization rates at C_3 and C_5 of LA, which does not contain a C_5 substituent, are two to three orders of magnitude slower than for either ALA or 5-CLA. Clearly both the amino and chloro substituents at C_5 significantly enhance the enolization rates of both C_3 and C_5. The question arises as to why this reaction should be 12-fold slower for ALA in TES than in KP_i. These observations indicate that the phosphate buffer may act as a general base through electrostatic catalysis (Fig. 5, **X**) and enhance the rate of enolization at C_3 of ALA. In the case of 5-CLA, the absence of such electrostatic catalysis at C_3 may account for the slower enolization rate at C_3 relative to ALA. At C_5, the phosphate may still act as the general base abstracting the proton, as shown in Fig. 5, **XI,** but the rate of enolization at C_5 is almost the same for ALA and 5-CLA probably because of high kinetic acidities of the protons at C_5.

The complexes shown in Fig. 5 may also account for the difference in rates of deuterium exchange in ALA between TES and phosphate. In TES, the sulfonate group is too weak a base to abstract protons and the amino group is the only other general base. It has been previously observed that for amino ketones the proton exchange rates are enhanced by approximately 10-fold by anionic bases such as



X XI

FIG. 5. Potential mechanism for buffer catalysis of proton exchange.

carboxylate anion when compared to neutral bases such as pyridine (*18*). This effect has been attributed to electrostatic catalysis by the oppositely charged buffer acting as the general base.

In the absence of the C_5 substituents, as for LA, the rates of enolization at C_3 and C_5 are more nearly equivalent, with C_3 exchange being about 50% faster (on a per proton basis). Unfortunately, the steady-state concentrations of the enols of LA, 5-CLA, and ALA (e.g., **III** and **IV**) are too low to detect by NMR and kinetic isotope exchange studies cannot quantify the equilibrium concentrations of the various isomers. Simple ketones exhibit isomers analogous to **I, II, III,** and **IV** at a ratio of about $10^5 : 200 : 1 : 1$, respectively (*12*). For ALA, the mole fraction of the hydrate **(II)** is comparable to the value expected. The mole fractions of the enols of ALA are below 0.3% and may be as low as 0.001% as in the case of simple ketones.

Biosynthetic Significance of the Isomeric Chemistry of ALA

ALA is the common precursor of all biologic pyrroles. It is formed by three enzymes: ALA synthase (*1*), glutamate-1-semialdehyde transaminase (*19*), and L-alanine : 4,5-dioxovalerate aminotransferase (*20*). Investigation of the chemical reaction mechanisms of these enzymes must consider the rapid equilibration between the ALA forms illustrated in Scheme 1. The alternative forms **II, III,** and **IV,** if produced at the active sites of these enzymes, would collapse to the ketone when released from the enzyme.

The sole pathway for ALA utilization is the asymmetric condensation of two ALA molecules to form porphobilinogen (PBG) (*1*). In the PBGS-catalyzed reaction, both ALA enols (**III** and **IV**) are potentially significant because they each lack one of the four protons which are lost to water in PBG formation. ^{13}C NMR has demonstrated that the ALA which becomes the propionyl side of PBG contains both C_5 protons when bound to the enzyme as the Schiff base intermediate (*21*). Therefore enol **IV** is excluded as an active site species. Proton exchange rates described herein establish that formation of enol **III** is too slow for it to serve as the form of ALA which binds to PBGS and becomes the acetyl side of PBG (A-side ALA). To test the suggestion that C_3–C_4 enol formation is catalyzed by PBGS,

one would like to demonstrate PBGS catalysis of C_3 proton exchange of A-side ALA. Unfortunately, the spontaneous proton exchange rates have thwarted all efforts to observe PBGS catalysis of proton exchange at either C_3 or C_5 of ALA even at enzyme : substrate ratios of 1 : 20.[5] Consequently, observation of PBGS catalysis of C_3 proton exchange must rely on a form of PBGS which could bind A-side ALA without catalyzing PBG formation at a rate greater than 10^{-5} the normal turnover rate. To date, chemical modification efforts have failed to produce such a species of PBGS. It has, however, been possible to observe PBGS catalysis of the C_3 protons of LA,[5] but the rates are orders of magnitude slower than turnover for the reaction of ALA to PBG.

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[5] Unpublished results.