on silica gel (E. Merck) with $CHCl_3$. Glyoxylic acid monohydrate was obtained from Sigma Chemical Co. and desferal mesylate was a gift from CIBA Pharmaceutical Co. High-pressure LC analyses were achieved by use of Waters Associates Model 6000A solvent delivery system, Model 440 absorbance detector (254 nm), and Model U6K septumless injector. Melting points were taken on a calibrated Thomas-Hoover Unimelt. UV spectra were obtained on a Beckman Model 35 spectrophotometer, IR spectra were obtained on a Perkin-Elmer Model 180 spectrophotometer, 60-MHz NMR spectra were obtained on a Varian EM 360 A spectrometer, and mass spectra were obtained on a Hewlett-Packard 5980A mass spectrometer. Elemental analyses were performed by Galbraith Laboratories.

Synthesis of *p*-(Dimethylamino)formanilide (3). To a solution of 2.7 g (0.02 mol) of *N,N*-dimethyl-*p*-phenylenediamine in 50 mL of anhydrous Et_2O, which was stirred with a magnetic stirring bar, was added in a single portion 6.2 g (0.02 mol) of dicyclohexylcarbodiimide in 30 mL of anhydrous Et_2O. A solution of 1.5 g (0.03 mol) of 90% formic acid in 10 mL of Et_2O was then added to the reaction over the course of 2 min. Further stirring was continued for 20 min and then the reaction mixture was filtered. The filtrate was dried (Na_2SO_4) and evaporated in vacuo. The residue was purified by chromatography and recrystallized as described below to give 1.5 g (46%) of **3** as white plates, mp 106.5–107.5 °C (lit.[7] mp 104.5–105.5 °C).

Product from Reaction of *N,N*-Dimethyl-*p*-nitrosoaniline (1) with Glyoxylic Acid. To a solution of 18.4 g (0.20 mol) of glyoxylic acid monohydrate in 1.0 L of water, adjusted to pH 6.0 with 10% NaOH, was added 15.0 g (0.10 mol) of **1** dissolved in 100 mL of 95% ethanol. The reaction mixture was stirred at ambient temperature for 4 h and then extracted twice with 500 mL of Et_2O. The combined Et_2O extract was dried (Na_2SO_4) and evaporated in vacuo. The residue was dissolved in 100 mL of $CHCl_3$ and chromatographed on silica gel (60 × 2.6 cm bed size) with 500 mL of $CHCl_3$, 500 mL of 1% MeOH/$CHCl_3$, and finally 1.0 L of 2% MeOH/$CHCl_3$. Those fractions containing the single reaction product were combined and evaporated to give a yellow solid. Repetition of this chromatographic procedure gave 12 g of a pale yellow solid that showed a single spot on TLC (5% MeOH/$CHCl_3$ with silica gel plates). Recrystallization twice from benzene/hexane (5:1) gave 9.6 g (58% based on mol wt 164) of fine white plates, mp 106.5–107.5 °C. The UV, IR, NMR, and mass spectral data were identical with those found for authentic **3**, which was prepared as described above. The C, H, N analysis was consistent with $C_9H_{12}N_2O$.

General Procedure and Analytical Methods for Reaction of 1 with Glyoxylic Acid. To 40 mL of 0.05 M KH_2PO_4 buffer of the desired pH was added 23 mg (0.25 mmol) of glyoxylic acid monohydrate. The pH of the resulting solution was readjusted to the desired pH with 0.05 M K_2HPO_4 and the solution then brought to 50.0 mL final volume with the desired buffer to yield a 5.0 mM solution of glyoxylic acid. Aliquots of 5.0 mL were placed in test tubes and equilibrated to the desired reaction temperature. The title reaction was initiated by the addition of 50 μL of a solution of either 1.9 mg/mL or 3.8 mg/mL of **1** in 95% ethanol to give a reaction concentration of 0.125 or 0.25 mM, respectively. At the desired time after the start of the reaction, a 10-μL aliquot was injected directly onto a high-pressure LC system consisting of a Waters μBondapak C_{18} column (30 × 3.9 mm i.d.) with 30% MeOH buffered to pH 3.5 with 0.01 M KH_2PO_4 and containing 0.01% desferal mesylate as the elution solvent at a flow rate of 1.5 mL/min. For quantitative determination of **3**, the peak heights at 254 nm were measured and compared to those of pure standard. The retention times in this high-pressure LC system were as follows: **1**, 5.0 min; **2**, 4.57 min; **3**, 2.73 min. The investigation of the reaction by UV spectrophotometry was conducted with **1** at 0.125 mM initial concentration. Simultaneous high-pressure LC analyses were made to correlate high-pressure LC peaks to **1**, **2**, or **3**. Analyses of this reaction by TLC were conducted in a similar manner, except that the concentration of **1** was generally 5 mM with either 5 or 10 mM glyoxylic acid. R_f values on silica gel plates with 5% MeOH/$CHCl_3$ were as follows: **1**, 0.70; **2**, 0.24; **3**, 0.37. The zones were visualized by ultraviolet quenching; in addition the zone corresponding to **2** gave an immediate violet color with 1% $FeCl_3$ that faded to a yellow spot within several minutes.

Registry No. **1**, 138-89-6; **2**, 75767-78-1; **3**, 18606-63-8; *N,N*-dimethyl-*p*-phenylenediamine, 99-98-9; dicyclohexylcarbodiimide, 538-75-0; glyoxylic acid, 298-12-4.

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Specific Deuteration of δ-Aminolevulinic Acid by Pyridoxal-Catalyzed Exchange and Analysis of Products Using an Aqueous Lanthanide Nuclear Magnetic Resonance Shift Reagent

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Samples of δ-aminolevulinic acid (ALA, $H_2NCH_2COCH_2CH_2CO_2H$) with specific isotopic labels have long been of use in studies of heme biosynthesis.[1] While a number of methods are known for specific incorporation of ^{14}C or ^{15}N into ALA,[1,2] none of the published chemical syntheses[1-3] lends itself to substitution of hydrogen isotopes specifically at only one position. This is especially true because most syntheses terminate with an acid-catalyzed hydrolytic removal of an acyl protecting group from the amino nitrogen. This procedure exchanges the protons at both C-3 and C-5 with the aqueous medium,[4] thus altering any deuterium or tritium labeling pattern that might have been introduced at an earlier step. Simple base-catalyzed exchange of ALA with water also fails because the protons at C-3 and C-5 exchange at almost equal rates with a variety of catalysts.[4,5]

We have investigated methods for catalyzing ALA–water hydrogen exchange which lead to regiospecific incorporation of isotope. The use of a water-soluble aldehyde to form a transient Schiff base at the amino group of ALA in situ was anticipated to be effective in accelerating exchange at C-5. Specifically, we turned to pyridoxal, whose biochemical functions include acidifying the proton on the α carbon of α-amino acids.[6]

Results and Discussion

Incubation of ALA with approximately 0.1 molar equiv of pyridoxal in a pyridine buffer at pH 4.4 was found to catalyze the exchange of the hydrogens of the C-5 methylene with those of the aqueous medium much faster than the corresponding exchange at the C-3 methylene. This discrimination results from the transient formation of an

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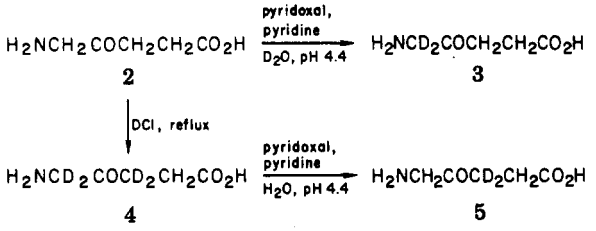
Table I. Chemical Shifts[a] of ALA Protons in the Presence of Europium and Praseodymium Chlorides

	M = Eu			M = Pr		
$[MCl_3]/[ALA]$[b]	δ(H-2)	δ(H-3)	δ(H-5)	δ(H-2)	δ(H-3)	δ(H-5)
0	2.47	2.77	4.08	2.47	2.77	4.08
0.25	2.26	2.65	4.05	2.93	2.93	4.14
0.50	2.09	2.56	4.03	3.27	3.27	4.23
0.75	1.94	2.49	4.02	3.67	3.43	4.32
1.0	1.79	2.39	3.98	3.96	3.58	4.34
1.5	1.56	2.25	3.93	4.52	3.88	4.35
2.0	1.38	2.14	3.90	4.87	4.14	4.54
average Δδ per equiv of MCl_3[c]	+0.54	+0.32	+0.09	−1.20	−0.68	−0.23

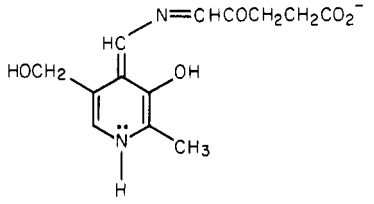
[a] Chemical shift reference was HOD, taken to be δ 4.70. All spectra were at 90 MHz. [b] [ALA] = 0.01 M. [c] Upfield shifts are reported as positive, downfield shifts as negative.

Scheme I



2 3 4 5

imine of ALA and pyridoxal and the concomitant stabilization of the conjugate base at C-5 by the quinonoid resonance structure (1). Side reactions of the amino ke-



1

tone moiety[5] are minimized by the low pH of the reaction mixture.

Carrying out the reaction with unlabeled ALA in D_2O afforded ALA-*5,5-d*$_2$ (3, see Scheme I). Conversely, ALA-*3,3-d*$_2$ (5) was prepared by nonspecific deuteration at C-3 and C-5 in DCl to give ALA-*3,3,5,5-d*$_4$ (4), followed by pyridoxal-catalyzed exchange of the deuterons at C-5 with the protons of H_2O. The specific deuteration reaction has a kinetic isotope effect, k(ALA + D_2O)/k(ALA-*3,3,5,5-d*$_4$ + H_2O) = 1.5.

The deuterium incorporation at various positions was readily monitored by NMR. A problem arises with ALA-*3,3-d*$_2$, however, due to the overlap of the H-2 and H-3 signals at 90 MHz or lower frequency. The addition of europium or praseodymium chlorides to neutral solutions of ALA induces changes in chemical shifts that separate these resonances, simplifying analysis, as shown in Table I for solutions where [ALA] = 0.01 M. The relative magnitudes of the shifts induced on the three resonances are consistent with predominant coordination of only the carboxylate of ALA with the lanthanide ions. The direction of the shifts and the nature of the coordination agree with earlier results on similar compounds in aqueous solutions.[7]

For convenient determinations on the relatively insensitive 60-MHz continuous-wave spectrometer, samples were made up to [ALA] = 0.25 M. The induced shifts at this high concentration are approximately twice as large as those at [ALA] = 0.01 M, presumably due to more complete complexation of ALA to metal ion. Europium was selected over praseodymium to keep the resonances of interest away from the HOD absorption, and the ratio $[EuCl_3]/[ALA]$ was set at 0.5 for routine analyses, giving a separation of 0.92 ppm between the H-2 and H-3 resonances. This is sufficient to render each resonance a first-order triplet instead of the complex AA′BB′ pattern that appears in the absence of additives, especially at 60 MHz.

Samples of the purified solid hydrochlorides of the specifically deuterated ALA's were subjected to analysis by NMR in the presence of 0.5 equiv of $EuCl_3$ as described in the Experimental Section. ALA-*5,5-d*$_2$ showed no detectable NMR absorption at the position of the H-5 resonance of ordinary ALA under the same conditions. From this we estimate a minimum of 95% deuterium incorporation at C-5. The H-2 and H-3 resonances (in the presence of $EuCl_3$) were 1:2:1 triplets with equal integrals, and we estimate a maximum of 5% deuterium incorporation at these two positions. Moreover, since no exchange at C-2 was expected under the reaction conditions, the equal integrations of H-2 and H-3 strongly suggest that the true deuterium content at C-3 is more nearly zero. Correspondingly, ALA-*3,3-d*$_2$ showed no detectable absorbance at the position of the H-3 resonance, and the H-2 and H-5 signals were both singlets of equal integral, with the H-2 resonance being slightly broadened due to coupling to the deuterium nuclei at C-3.

We thus conclude that the workup procedure in acidic H_2O produces no alteration of the labeling pattern and that therefore either isomer can be efficiently synthesized in a single pyridoxal/pyridine-catalyzed exchange. Samples that are specifically tritiated rather than deuterated can easily be prepared by the same reactions.

Experimental Section

NMR spectra were obtained at 60 MHz on a Perkin-Elmer R-12B spectrometer or at 90 MHz on a Perkin-Elmer R-32 spectrometer at Bryn Mawr College. Melting points were measured in open capillaries on a Thomas-Hoover apparatus and were corrected. The pH measurments on D_2O solutions were uncorrected meter readings with electrodes standardized in H_2O buffers. D_2O was obtained from Sigma or Norell and contained a minimum of 99.8 atom % deuterium. Europium and praseodymium chlorides were purchased from Alfa.

ALA-*5,5-d*$_2$. Sigma ALA hydrochloride (110 mg, 660 μmol) was dissolved in 1 mL of D_2O and then mixed with a solution of 14 mg (69 μmol) of pyridoxal hydrochloride (Sigma) in 0.5 mL

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of D_2O. The reaction was started by adding 53 μL (660 μmol) of neat pyridine, which made the pH 4.4.

The loss of protons at C-5 was monitored by NMR and had a half-life of 25 min at 36 °C, the temperature of the NMR probe. After 130 min, the reaction was stopped by adding 60 μL of 38% DCl (Norell), which dropped the pH below 2.

The mixture was applied to a 15.5 × 1 cm diameter column of Dowex 50-X8 which had been equilibrated with purified 0.4 M HCl, made by diluting redistilled concentrated HCl. (The azeotropic distillate was titrated and found to be 6.97 M.) Elution was carried out at 2.4 mL/min with the same purified 0.4 M HCl while 2-min fractions were collected. The effluent was monitored continuously at 280 nm with an ISCO UA-5 monitor. The three major components of the mixture were well separated and eluted in the order ALA, pyridoxal, and pyridine, after the early elution of a small peak of absorbance which gave a negative test for amino groups with fluorescamine.[8]

The ALA fractions were pooled and evaporated under reduced pressure at room temperature, yielding 73.6 mg of ALA-*5,5-d*$_2$ hydrochloride (67%), mp 153–156.5 °C dec.[9] The analysis of this material by NMR is described under Results and Discussion.

ALA-*3,3,5,5-d*$_4$. In our hands, the base-catalyzed exchange procedure of Lester and Klein[5] produced colored impurities which were difficult to remove. We found that acid-catalyzed exchange eliminated this difficulty.

ALA hydrochloride (2.46 g, 14.7 mmol) was refluxed in 40 mL of 15% DCl for 2.5 h. The solution was evaporated under reduced pressure at room temperature and the residual solid was dissolved in a fresh 30-mL portion of 20% DCl and then refluxed a further 2.5 h. A second evaporation gave a white crystalline solid, which was recrystallized by dissolving in 250 mL of boiling absolute ethanol and precipitating by chilling on ice and adding 300 mL of anhydrous ether. The yield of recrystallized ALA hydrochloride was low (1.43 g, 58%) due to solubility difficulties and esterification under these conditions.

NMR analysis of this material showed no signal above noise level at the positions of the H-3 and H-5 resonances.

ALA-*3,3-d*$_2$ was prepared by the procedure described for ALA-*5,5-d*$_2$ above, using ALA-*3,3,5,5-d*$_4$ as starting material and H_2O instead of D_2O as solvent. The H-5 resonance in the NMR is far enough from HOD, even at 60 MHz, that its growth can be monitored in the H_2O solution. The reaction had a half-life of 38 min at 36 °C; this retardation relative to the isotopically inverse reaction is the product of a deuterium kinetic isotope effect at the C-5 methylene and a solvent isotope effect.

NMR Analysis with $EuCl_3$. For measurement with a high signal-to-noise ratio at 60 MHz, 17 mg (100 μmol) of a sample of ALA hydrochloride was dissolved in 340 μL of D_2O and neutralized with 1 equiv of NaOD (typically 40 μL of 2.5 M). The pH after such neutralization was 6.5. $EuCl_3$ (0.5 equiv) in D_2O was then added (typically 33 μL of 1.5 M) and the spectrum recorded. If the europium is added before the base, precipitation occurs.

Analogous procedures were used for the 90-MHz measurements at lower concentrations with $EuCl_3$ and $PrCl_3$.

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Registry No. 2·HCl, 5451-09-2; **3**·HCl, 75700-34-4; **4**·HCl, 75700-35-5; **5**·HCl, 75700-36-6.

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A Simple Procedure for the Analysis of Multisite Hydrogen–Deuterium Exchange Rates Obtained by Mass Spectrometry

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The measurement of hydrogen–deuterium exchange rates usually relies upon NMR or mass spectrometry. While the former technique allows the monitoring of specific hydrogen atoms undergoing exchange, it can be imprecise, especially when the substrate exhibits a complex spectrum. On the other hand, mass spectrometry yields very precise data on the isotopic composition of the substrate undergoing exchange, but it is frequently difficult to relate the results to the degree of reaction at any particular site. For example, consider the two-site exchange process in Scheme I where 1 bears diastereotopic protons H_x and H_y. If only d_0 material is present initially, then

$$[d_0] = [d_0]_0\, e^{-k_1 t}$$

and

$$[d_1] = \frac{k_1[d_0]_0}{k_2 - k_1}\,(e^{-k_1 t} + e^{-k_2 t})$$

Present interpretive methods[1-3] which rely on equations such as those shown above suffer from the drawback that the basic exchange rate constants k_x and k_y are not easily available from k_1 and k_2, except when $k_x = k_y$ and the exchange is statistically controlled.

An alternative and simple analysis lies in considering the progress of the reaction in terms of exchange at the specific sites themselves. Accordingly, if secondary isotope effects are neglected in Scheme I, then

$$[H_x] = [1] + [2] = [H_x]_0 e^{-k_x t}$$

and

$$[H_y] = [1] = [3] = [H_y]_0 e^{-k_y t}$$

Hence, if only d_0 material is present initially,

$$e^{-k_x t} + e^{-k_y t} = 2[1] + [2] + [3] = 2d_0 + d_1$$

where d_0 and d_1 are fractional isotopic species. Generalizing for n-site exchange, eq 1 is derived. For symmetrical

$$\sum_i^n e^{-k_i t} = \sum_i^n (n + 1 - i)d_{i-1} \qquad (1)$$

substrates with p nonequivalent sites, the above equation reduces to eq 2.

$$\sum_i^p e^{-k_i t} = \sum_i^n (n + 1 - i)d_{i-1} \qquad (2)$$

Equations 1 and 2 describe most exchange situations and may readily be incorporated into a simple and general computer program for routine use. We have written an iterative program[4] based on Kim's procedure,[5] and we have

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