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SYNTHESIS OF HYDROXYL[^{13}N]AMINE AND BINDING OF $^{13}NH_2OH$ TO TWO TRANSAMINASES

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SUMMARY

NH_2OH was labeled with a β^+emitter (^{13}N, $t_{1/2}$ = 9.96 min.). Hydroxyl[^{13}N]amine was synthesized from HSO_3^- and [^{13}N]nitrite (produced via the $^{16}O(p,\alpha)^{13}N$ reaction by proton bombardment of H_2O). The synthesis reactions are:

$$NO_2^- + 2\ HSO_3^- + H^+ \rightarrow HON(SO_3)_2^= + H_2O \tag{1}$$

$$HON(SO_3)_2^= + 2\ H_2O \rightarrow NH_3OH^+ + 2\ SO_4^= + H^+ \tag{2}$$

Hydroxyl[^{13}N]amine was purified by ion-exchange chromatography on Dowex-1 x 8 (AcO^-) to remove unreacted $^{13}NO_2^-$ and $^{13}NO_3^-$. Radiochemical yields of 50-90% were obtained in 25-30 min. The purity and specific activity of $^{13}NH_2OH$ were determined by HPLC analysis and spectrophotometric assay. The binding of $^{13}NH_2OH$ to pig heart glutamate-pyruvate transaminase and to pig heart glutamate-oxaloacetate transaminase was measured. This work demonstrates that quantitation of the binding of hydroxylamine (an active-site directed enzyme inhibitor) labeled with a positron-emitting isotope is technically feasible.

Key Words: Hydroxyl[^{13}N]amine, ^{13}N-labeling, Glutamate-pyruvate Transaminase, Glutamate-oxaloacetate Transaminase

INTRODUCTION

Hydroxylamine (NH_2OH), a potent nucleophile, forms a wide variety of characteristic derivatives such as hydroxamic acids, oximes and amidoximes. Such derivatives have proven useful in understanding the structure and function of many biologically active molecules. Hydroxylamine reacts with Schiff bases,

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aldehydes and "activated" carboxyl groups in proteins. Hydroxylamine is also a versatile synthetic reagent in organic chemistry; it undergoes condensation reactions with many compounds containing reactive carbonyl groups. However, the usefulness of NH_2OH as a biochemical tracer is limited because it contains no atoms that can be labeled with commonly available β^- emitters (^3H would exchange rapidly with protic solvents). We report here the synthesis of $^{13}NH_2OH$ with high radiochemical purity and the use of $^{13}NH_2OH$ as an active-site directed reagent for labeling the pyridoxal 5'-phosphate cofactor of two transaminases.

EXPERIMENTAL

Materials - Hydroxylamine hydrochloride, phenylhydrazine hydrochloride and sodium nitrite were obtained from J.T. Baker. Hydroxylamine hydrochloride was recrystallized from 100% ethanol before use. Sodium bisulfite was purchased from Aldrich. Pyridoxal 5'-phosphate was purchased from Sigma. All other chemicals were ACS reagent grade. Pig heart glutamate-pyruvate transaminase (GPT), 140 U/mg (37° C), and cytosolic pig heart glutamate-oxaloacetate transaminase (GOT), 380 U/mg (37° C), were obtained from Boehringer Mannheim as 3.2 M $(NH_4)_2SO_4$ suspensions. Enzyme solutions were desalted before $^{13}NH_2OH$ binding studies either by dialysis against 0.2 M potassium phosphate buffer, pH 7.8, or by the centrifuge gel filtration (Sephadex G-50-40) column procedure of Penefsky (1).

Assays - GOT and GPT activities were measured as described (2). Hydroxylamine was assayed spectrophotometrically using 8-hydroxyquinoline (3). Protein concentrations were calculated from absorbance at 280 nm (GOT: molecular weight = 93,000 (4), ϵ = 130,000 (5); GPT: molecular weight = 115,000 (6), ϵ = 99,000 (7)). The pyridoxal 5'-phosphate content of the desalted GPT and GOT used in binding studies was measured by spectrophotometric assay of the phenylhydrazone derivative (8), using crystalline pyridoxal 5'-phosphate as a standard.

Radioisotope Counting - ^{13}N decays by positron emission to ^{13}C, and the positron annihilates with a nearby electron to yield a pair of 0.511 MeV gamma photons. Samples containing $^{13}NH_2OH$ were collected in glass tubes and counted with a Packard Auto-Gamma scintillation spectrometer, Model 5986, as described (9). All ^{13}N counts were corrected for physical decay and natural background.

Synthesis of Hydroxyl[^{13}N]amine - Hydroxyl[^{13}N]amine was synthesized by a rapid, microscale adaptation of the procedure described by Raschig in 1887 (10), and used later by Semon (11). [^{13}N]Nitrite was produced by the $^{16}O(p,\alpha)^{13}N$ reaction by bombardment of H_2O with 14.5 MeV protons from the cyclotron at Memorial Sloan-Kettering Cancer Center as described (12). The target water containing ^{13}N (typically 5-15% $^{13}NO_2^-$, 85-95% $^{13}NO_3^-$ and <0.1% $^{13}NH_4^+$ (12)) was passed through a 1 x 10 cm column of activated basic alumina to remove traces of $^{18}F^-$. The $^{13}NO_2^-$ and $^{13}NO_3^-$ were concentrated by adsorption on a 0.1 x 1 cm column of Dowex-1 x 8 (AcO^-, 200-400 mesh). The column was washed with 5 ml of distilled water, and then eluted with 2 ml of 0.5 M KCl in 0.1 M Na acetate, pH 4.5. Unlabeled $NaNO_2$ was added to give a final concentration of 2.5 mM and 1 M $NaHSO_3$ was added to yield a HSO_3^-/NO_2^- molar ratio of between 2 and 24.

The reaction mixture (vol. 2.2 ml) was incubated at 4° C for 10 min.; the resulting hydroxyl[^{13}N]amine disulfonic acid was hydrolyzed by adding 0.4 ml of 12 M HCl and boiling for 5 min. The mixture was cooled in ice, neutralized with 10 M NaOH, and passed through a 1 x 6 cm column of Dowex-1 x 8 (AcO^-) to remove unreacted $^{13}NO_2^-$ and $^{13}NO_3^-$. An aliquot of the ^{13}N reaction mixture was passed through a 0.5 x 8 cm column of Dowex-50 x 8 ($Tris^+$) and the eluate was counted. No significant radioactivity was bound to the Dowex-50, which indicated that the $^{13}NH_2OH$ reaction mixture was free of $^{13}NH_4^+$. Radiochemical yields (based on conversion of total nitrite to hydroxylamine) of 50-90% were consistently obtained in a production time of 25-30 min. The yields at the end of the syntheses were 0.4-1.0 mCi and specific activities were 1.0-2.5 Ci/mmole (n=9). Since the target water contains considerably less $^{13}NO_2^-$ than $^{13}NO_3^-$, it should be possible to greatly improve the yield of $^{13}NH_2OH$ by converting the $^{13}NO_3^-$ present in the target water to $^{13}NO_2^-$ by reduction on a cadmium column, as described by McElfresh, et al (13).

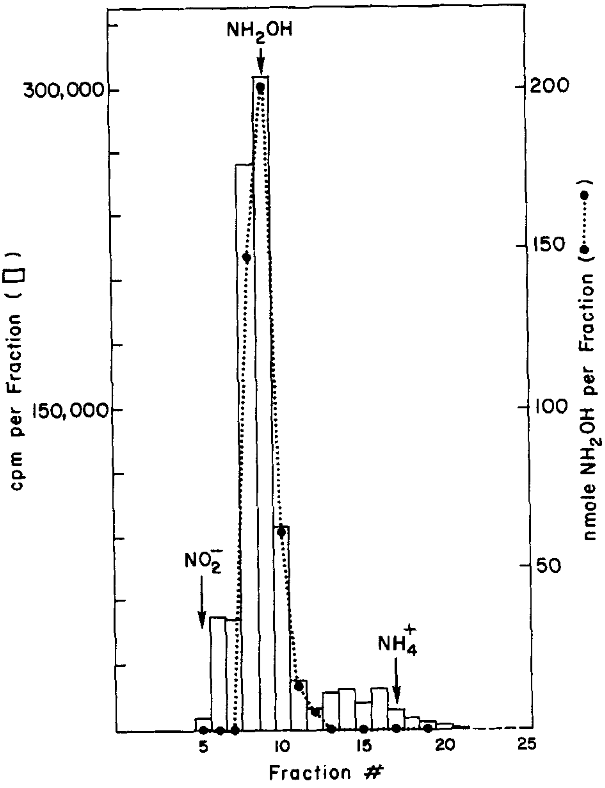

Figure 1. HPLC elution profile of Hydroxyl[^{13}N]amine. To 1.0 ml of final product containing 0.32 mCi of $^{13}NH_2OH$ was added 1.0 ml of a solution containing 250 μmoles of unlabeled hydroxylamine. A portion (20 μl) of this solution was injected into the HPLC system 14 min. after the end of the synthesis and was chromatographed on a 25 cm Whatman Partisil PXS-10/25 SCX cation exchange column (mobile phase: 10 mM potassium phosphate, pH 6.5, flow rate 1.1 ml/min). The HPLC system consisted of a minipump and pulse dampener (Laboratory Data Control, model 711-31) and Rheodyne injection valve. Pressure was maintained at 750 p.s.i. Fractions (30 sec., 0.55 ml) were collected and counted, then assayed for NH_2OH. Retention times for authentic NH_2OH, NO_2^-, and NH_4^+ are indicated by arrows. Nitrite was determined by the method of Csaky et al (14), ammonia was detected with Nessler's reagent. Counts are decay-corrected to the start of the HPLC run, 44 min. after the end of cyclotron bombardment.

In preliminary studies with unlabeled NH_2OH, the overall yield of NH_2OH was found to be approximately constant when the HSO_3^-/NO_2^- molar ratio was between 2 and 24, but the yield decreased substantially if the HSO_3^-/NO_2^- molar ratio was less than 2 or more than 24. It was necessary to add unlabeled $NaNO_2$ to the reaction because at NO_2^- concentrations lower than 2.5 mM the rate of formation of hydroxylamine disulfonic acid was too slow to achieve maximal $^{13}NH_2OH$ yield in the 10 min. time interval chosen for step 1 of the synthesis.

The purity of the synthesized $^{13}NH_2OH$ was confirmed by high performance liquid chromatography (HPLC), as shown in Figure 1. HPLC analyses of the $^{13}NH_2OH$ product from several syntheses showed that in each case about 90% of the total radioactivity eluted in a single peak coincident with authentic NH_2OH; in the HPLC analysis shown in Figure 1, the specific activity (1500-1800 cpm/nmole $^{13}NH_2OH$, decay corrected to the start of the HPLC run) remained essentially constant over the entire width of the peak. The total recovery of ^{13}N radioactivity (decay-corrected) from the HPLC column was 97-98% of the amount injected.

Binding Studies with Hydroxyl[^{13}N]amine and Purified Transaminases-

Glutamate-pyruvate transaminase (1.5 mg, 13.0 nmole dimeric holoenzyme) or glutamate-oxaloacetate transaminase (1.5 mg, 16.1 nmole dimeric holoenzyme) were incubated in 1.4 ml of 0.2 M potassium phosphate buffer, pH 7.8, containing $^{13}NH_2OH$ synthesized as described above. After incubation at 23° C for 10 min., the mixture was applied to a 1 x 18 cm Sephadex G-50-40 column and was eluted with 0.2 M potassium phosphate buffer, pH 7.8.Fractions (0.7 ml) were collected, counted, and assayed for NH_2OH and protein. The gel filtration profiles obtained from these experiments are shown in Figs. 2 and 3.

The desalted enzyme solutions were assayed for pyridoxal 5'-phosphate to determine the amount of holoenzyme available for reaction with $^{13}NH_2OH$. The desalted GOT used in the $^{13}NH_2OH$ binding studies contained 1.16 moles pyridoxal 5'-phosphate/93,000 g (dimer) and the GPT contained 1.26 moles pyridoxal 5'-phosphate/115,000 g (dimer). Although these values are lower than the

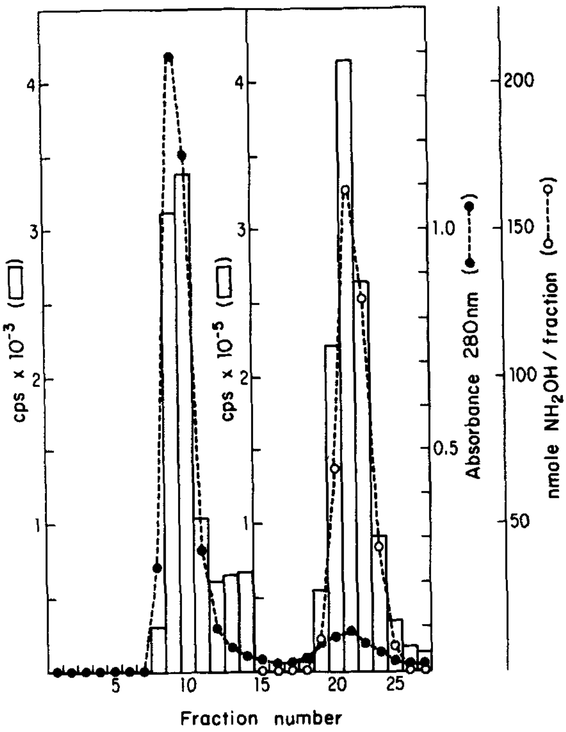

Figure 2. Binding of $^{13}NH_2OH$ to glutamate-oxaloacetate transaminase. Counts are decay-corrected to the start of the gel filtration.

expected maximum pyridoxal 5'-phosphate content of 2 moles per dimer, they are comparable to the pyridoxal 5'-phosphate content of GPT and GOT reported in previous studies. Purified GPT was reported to contain 1.53 moles pyridoxal 5'-phosphate/115,000 g enzyme when assayed with phenylhydrazine (6), and the pyridoxal 5'-phosphate content (measured by spectrophotometric assay of the 2,4-dinitrophenylhydrazone) of GOT (estimated to be 81 ± 3% pure) was 1.27 moles/93,000 g protein (15); the latter value is about 9% higher than the pyridoxal 5'-phosphate content of the GOT used in the $^{13}NH_2OH$ binding studies reported here.

The amount of $^{13}NH_2OH$ bound to protein in the experiments shown in Figs. 2 and 3 was 0.17 nmole $^{13}NH_2OH$/nmole GOT pyridoxal 5'-phosphate and 0.32 nmole

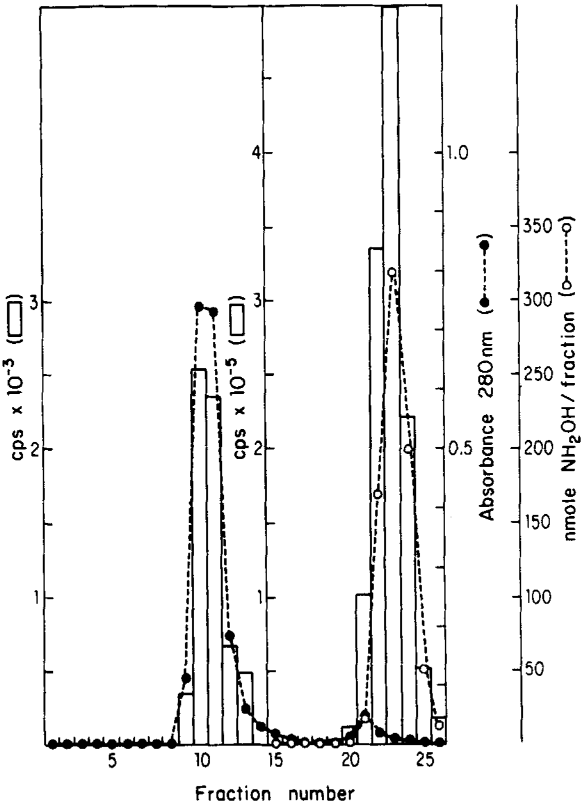

Figure 3. Binding of $^{13}NH_2OH$ to glutamate-pyruvate transaminase. Counts are decay-corrected to the start of the gel filtration.

$^{13}NH_2OH$/nmole GPT pyridoxal 5'-phosphate. Hydroxylamine reacts reversibly only with the bound pyridoxal 5'-phosphate of GOT and is a competitive inhibitor of the amino acid substrates (16). To demonstrate this competition, a reaction mixture containing 2.5 mg GOT (26.8 nmoles dimeric holoenzyme), 1.1 μmoles of $^{13}NH_2OH$, and 10 μmoles of L-glutamate in 1.1 ml of 0.2 M potassium phosphate buffer, pH 7.7, was incubated at 23° C for 10 min., then chromatographed on a Sephadex G-50-40 column as described above. A control mixture contained no L-glutamate. Quantitation of the binding of $^{13}NH_2OH$ to GOT following gel filtration showed a 22% decrease in NH_2OH binding in the mixture containing

L-glutamate as compared to the control mixture. The relatively small decrease in $^{13}NH_2OH$ binding, even in the presence of a large excess of the amino acid substrate, reflects a several hundred-fold difference in the dissociation constants of the enzyme-inhibitor complex and the enzyme-substrate complex. For GOT, the K_m for L-glutamate is about 4 mM, whereas the K_i for NH_2OH is approximately 0.01 mM (16). The binding of $^{13}NH_2OH$ was substantially lower than the theoretical maximum; this is in accord with the fact that oxime formation is reversible (16), and therefore, dissociation of $^{13}NH_2OH$ from the pyridoxal 5'-phosphate cofactor during gel filtration may occur. The pyridoxamine 5'-phosphate form, as well as other non-covalently bound derivatives (e.g. the oxime) are also more easily resolved than the pyridoxal 5'-phosphate aldimine form (17). Thus, some of the ^{13}N-labeled pyridoxal 5'-phosphate oxime may be lost during gel filtration. In view of these factors, it is probable that the initial binding of $^{13}NH_2OH$ to GOT and GPT prior to gel filtration is higher than that reported above.

DISCUSSION

Spectrophotometric studies of the reaction of NH_2OH with glutamate-oxaloacetate transaminase showed that inhibition is accompanied by formation of an active-site pyridoxal 5'-phosphate oxime (16). The results reported here show that this protein-bound oxime, despite its lability, can be readily quantitated by the use of $^{13}NH_2OH$, and that the protocol employed in these experiments is adequate to detect the binding of $^{13}NH_2OH$ to proteins at the nanomolar level.

During the past few years, ^{13}N has been used as a metabolic tracer in many studies (reviewed by Cooper, et al (18)) of plants and microorganisms. These include investigations of: 1) assimilation (incorporation of nitrate, nitrite, or ammonia into amino acids), 2) dissimilation (loss of nitrate or nitrite as non-utilizable ammonia), 3) nitrogen fixation (conversion of N_2 to utilizable ammonia), 4) denitrification (conversion of inorganic or organic nitrogen compounds from an oxidized to a more reduced state with subsequent loss of nitrogen as N_2 (or possibly N_2O)), and 5) nitrification (conversion of inorganic or organic nitrogen from a reduced to a more oxidized state).

Certain species of the nitrifying bacteria Arthrobacter (19,20) convert inorganic ammonium salts in a $2e^-$ transfer reaction to NH_2OH (-1 oxidation state) via N-oxygenation (21). Most of the NH_2OH formed does not immediately undergo further oxidation by the organism, but is excreted into the medium. It has been found that hydroxylamine formation occurs most rapidly during the exponential growth phase (19), and, indeed, studies carried out in this laboratory on two species of Arthrobacter showed that well-aerated log-phase cultures (10^4-10^5 cells/ml), produced NH_2OH concentrations as high as 2 mM in the medium.

Prior to the development of the chemical synthesis of $^{13}NH_2OH$ presented in this work, we attempted several syntheses using cell suspensions of Arthrobacter sp. and $^{13}NH_4^+$ (prepared by reduction of $^{13}NO_2^-$ and $^{13}NO_3^-$ with Devarda's alloy (22)). However, this microbiological approach has thus far proven unsuccesful for several reasons: 1) Although we used log-phase cells (harvested by centrifugation), the cells were resuspended in the $^{13}NH_4^+$-containing reaction mixture at 10^7-10^8 cells/ml, a cell density that is inconsistent with exponential growth-phase conditions. Under these conditions, we observed an overall conversion of $^{13}NH_4^+$ to $^{13}NH_2OH$ of less than 1% in 10 min. in the cell suspension, as compared to 50-90% conversion of $^{13}NO_2^-$ to $^{13}NH_2OH$ in the chemical synthesis. 2) Although NH_2OH is known to be the major nitrogen-containing metabolite of Arthrobacter that is released into the culture medium, other products, such as acetohydroxamic acid and 1-nitrosoethanol, are also formed (19). Formation of these compounds during incubation of cell suspensions with $^{13}NH_4^+$ complicated the purification of $^{13}NH_2OH$. 3) The cell suspension released large amounts of soluble protein into the reaction mixture, which, along with protein from lysed cells, resulted in contamination of the purified $^{13}NH_2OH$ solution.

Cell-free extracts of Arthrobacter do not catalyze the nitrification of ammonia (23), and the enzymology of NH_2OH formation remains obscure. Thus, despite the apparent unsuitability of Arthrobacter for use in the synthesis of $^{13}NH_2OH$, this unusual bacterium remains an intriguing candidate for further studies of ammonia oxidation using $^{13}NH_4^+$ as a tracer.

Many microorganisms (e.g. Nitrosomonas europaea) nitrify by direct conversion of NH_4^+ (-3 oxidation state) to NO_2^- (+3 oxidation state) by a six electron transfer (24). This transfer is thought to proceed via three $2e^-$ steps (25). In the presence of hydrazine, complete oxidation of NH_4^+ to NO_2^- does not occur, but a $2e^-$ transfer results in accumulation of NH_2OH (26,27). These findings provide evidence that NH_2OH is an intermediate in the oxidation of NH_4^+ to NO_2^-. The reverse reaction, i.e. a $2e^-$ reduction of NH_2OH to NH_4^+, has also been reported to occur in several bacteria (e.g., Pseudomonas aeruginosa) and fungi (e.g., Neurospora crassa) that contain hydroxylamine reductase (28).

The availability of $^{13}NH_2OH$ may be expected to facilitate further exploration of these diverse oxidation-reduction reactions, and also to contribute to the study of NH_2OH-protein interactions. Although ^{13}N is available only at institutions possessing a cyclotron or a Van de Graaff generator, The present synthesis of $^{13}NH_2OH$ may have a wider applicability; $^{15}NO_2^-$ is available commercially, and the method should be useful for the synthesis of $^{15}NH_2OH$.

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