Characterization and Mode of Degradation of the Neurophysiologically Important Transaminase Inhibitors, the α-Aminooxyalkanoic Acids

By EDWARD R. GARRETT†

The α-aminooxyalkanoic acids such as aminooxyacetic acid have shown extraordinary properties of inhibition of the γ-aminobutyric acid-α-ketoglutaric acid enzymatic transamination to glutamate-succinic semialdehyde *in vivo* whereas the β-aminooxyalkanoic acids such as β-aminooxypropionic acid are of low potency. The unexpected lack of significant basicity of the aminooxy group in aminooxyacetic acid and its nondipolar ion nature implies a cyclic form in solution which may be responsible for its ability to cross cell walls and exhibit *in vivo* activity. The aminooxyacetic and β-aminooxypropionic acids are degraded in mild alkali at 60° to form ammonia and nitrite ion in the ratio of 2:1, and a mechanism of hydroxylamine release is postulated to account for this stoichiometry where the rate determining step is the release of hydroxylamine and a carboxylic acid. Hydroxylamine itself is quickly transformed by mild alkali at 60° to ammonia and nitrite ion. This mode of degradation is significant since hydroxylamine itself is a known transaminase inhibitor.

THE IMPORTANCE of the enzymatic transamination of γ-aminobutyric acid, GABA, with α-ketoglutaric acid to form glutamate and succinic semialdehyde in brain tissue and the potential effect on neurological activity has been stressed in the literature (1–3). Thus, inhibition of this transamination is of interest in the possible correlation of GABA levels in the brain with physiological properties such as definite depression of the central nervous system (4). Although suitable doses of hydroxylamine have been shown to increase the GABA levels in the brain of rats (5), a potent inhibitor *in vitro* and *in vivo* of the transaminase involved has been recently shown by Wallach (4) to be aminooxyacetic acid (AOAA), H_2NOCH_2COOH. Recent studies on various analogs have shown that good *in vivo* activity was generally limited to the α-aminooxyalkanoic acids or their easily hydrolyzed derivatives such as esters (6). None of the compounds studied was superior to aminooxyacetic acid (6).

The studies reported in this paper include the titrimetric characterization, stability, and possible modes of degradation of two representative compounds, aminooxyacetic acid and β-aminooxypropionic acid. The purposes were to obtain data for the possible rationalization of the observed difference in *in vivo* activity of these compounds.

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EXPERIMENTAL

Purity by Titration.—Aminooxyacetic acid hemihydrochloride (7) ($H_2NOCH_2COOH.{}^1/_2HCl$, mol. wt. = 109.3) 0.10 mmole, was dissolved in 10.00 ml. solvent and was titrated with 2.000 *N* sodium hydroxide in the Cannon automatic titrator (8) (see Fig. 1). A minor inflection (pH 3.4) was observed at an amount of titer which was $^1/_3$ of the total titer consumed up to a large inflection (pH 6–10). This was equivalent to the $^1/_2$ HCl per molecule. The titer between the minor and major inflections was equivalent to the number of moles.

Fifteen titrations showed that the material was of high purity by titration, 100.0 ± 0.3%.

Similarly, 10.00 ml. of 0.0050 *M* β-aminooxypropionic acid hydrochloride (9) ($H_2NO—CH_2CH_2COOH.HCl$, mol. wt. = 141.6) was titrated (see Fig. 2). The minor inflection was at pH 4.2, and 8 titrations conducted in water and in various aqueous alcohol solvents showed that the material was of high purity by titration, 100.0 ± 1.0%.

The apparent pKa values of these compounds as functions of per cent ethanol by volume are given in Table I. These pKa values are estimated from the pH at half-neutralization of the apparent prosthetic groups.

The compounds studied were provided by E. L. Schumann (6) of these laboratories.

Stability at 60° in Acid and Neutral Regions.—Solutions of 0.01 *M* aminoooxyacetic acid hemihydrochloride as studied up to 554 hours in water (pH 2.75), up to 22 hours at pH 9.5, up to 280 hours in 0.1 *N* hydrochloric acid at pH 1.20 were completely stable as evidenced by no change in the titration curves. Solutions of 0.005 *M* β-aminooxypropionic acid hydrochloride as studied up to 300 hours at pH 8.0 and up to 280 hours in 0.1 *N* hydrochloric acid at pH 1.20 were also completely stable by titration. No differences were observed in the shape of the curves or the apparent equivalent weight for aliquots taken up to these stated times.

Although β-aminooxypropionic acid hydrochloride in aqueous solution *ca.* pH 5.1 was stable up to the

TABLE I.—pKa AS FUNCTIONS OF PER CENT ETHANOL BY VOLUME

	Aminooxyacetic Acid			β-Aminooxypropionic Acid		
% Ethanol =	0	50	95	0	50	95
pKa_1	2.75	2.95	2.91	3.65	3.93	4.15
pKa_2	4.50	4.88	6.30	5.22	5.68	7.40

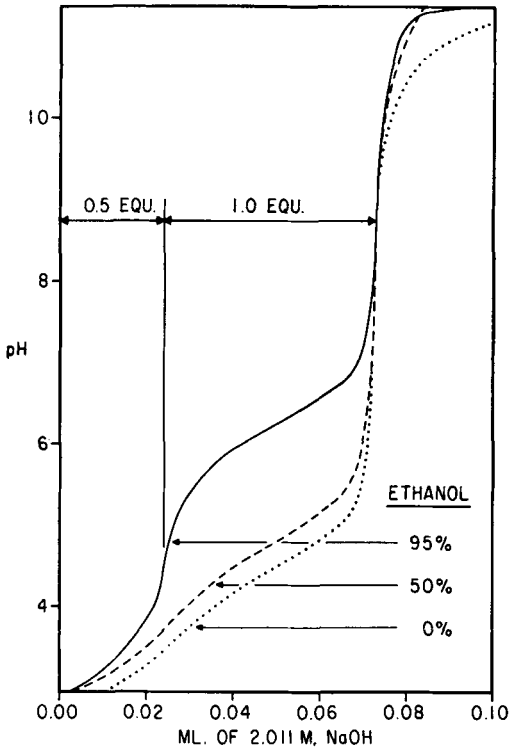


Fig. 1.—Potentiometric titrations of 0.010 *M* aminooxyacetic acid hemihydrochloride in varying per cent ethanol-water solutions.

17 hours studied at room temperature by titration, a change in the titration curve was observed when the solutions were subjected to 60°. This had not been observed with aminooxyacetic acid hemihydrochloride in aqueous solution at 60°. The change in the titration curves resulted in an apparent increase in pKa_2 from 5.22 to 6.0 at 100 hours with a significant decrease in the amount of titer necessary to reach the major inflection at pH 8 by 5% at 18 hours and 10% by 100 hours. This same phenomenon occurred when β-aminooxypropionic acid was subjected to pH 4 at 60°.

Stability in Alkaline Media.—Degradation of the aminooxyalkanoic acids occurred in mild alkali at 60°. Ten-milliliter aliquots of 0.0101 *M* sodium aminooxyacetate in 0.01 *M* sodium hydroxide were acidified with 1 ml. 0.25 *N* hydrochloric acid and potentiometrically titrated with 2.00 *M* sodium hydroxide. In another study, aliquots were directly titrated with 1.904 *M* hydrochloric acid. Typical curves of such titrations as a function of time of subjection to 60° are given in Fig. 3 and pertinent information is tabulated in Table II.

Similarly, typical curves for the titrations with time by 1.904 *M* hydrochloric acid of 10-ml. aliquots of 0.01 *M* sodium β-aminooxypropionate maintained at 60° in 0.01 *M* sodium hydroxide are given in Fig. 4 and pertinent information for such a degradation is given in Table III.

TABLE II.—TITRATION OF 10-ML. ALIQUOTS OF 0.0101 *M* SODIUM AMINOOXYACETATE IN 0.010 *M* SODIUM HYDROXIDE MAINTAINED AT 60°

Time of Sample, Hr.	pKa_2 (Estd.)	Estd. pKa of New Function[a]	$10^3 \times$ Molarity of New Function[b]	$10^3 \times$ Molarity of NaOH Consumed
Titration with 2.011 *M* NaOH after Acidification with 1 ml. 0.25 *M* HCl				
0.0	4.47	..	0.0	
16.8	4.45	9.3	0.6	
23.8	4.56	9.3	1.2	
40.8	4.57	9.3	1.5	
141	4.52	9.5	2.8	
283	4.60	9.5	4.0	
Titration Directly with 1.904 *M* HCl				
0.0	4.48	...	0.00	0.0
5.0	4.52	...	0.13	0.0
22.0	4.45	9.40	1.03	0.4
47.7	4.50	9.55	1.90	0.8
74.8	4.55	9.62	2.25	0.8
142	4.60	9.65	3.43	1.3
336	4.55	9.58	4.80	2.3

[a] Estimated from point of half-neutralization of between apparent inflection at pH 10.7 and definite inflection at pH 7 (see Fig. 2). [b] Portions of titration curves above pH 10 are superimposed over zero-time curve. The titer difference between the major inflections at pH 7 permits quantification of the amount of pKa 9.4 material appearing.

Hydroxylamine was also subjected to 0.01 *M* sodium hydroxide at 60° and aliquots were titrated at intervals. The pKa = 6.00 of hydroxylamine disappeared within 10–15 minutes and an apparent pKa of 9.3 appeared with a significant loss of free alkali.

The degraded alkaline solutions of both aminooxyalkanoic acids and hydroxylamine showed the presence of large amounts of nitrite ion as tested by the method of Forist (10).

RESULTS AND DISCUSSION

Ionic Nature of Aminooxyalkanoic Acids.—In general, pKa values of carboxylic acids increase with decreasing aqueous content of miscible solvents, whereas the pKa values of ammonium ions show little change or tend to decrease (11). The rationale is that a decrease in dielectric constant should not favor the dissociation of an uncharged species such as a carboxylic group whereas it should show no effect or slightly favor the dissociation of a protonated amine. This latter dissociative equilibrium does not involve a change in the number of ions. The pKa data of Table I indicate very little change in pKa_1 values with increasing alcohol content, whereas the pKa_2 values increase, and the increase is especially large between 50 and 95% (by volume)

TABLE III.—TITRATION OF 10-ML. ALIQUOTS OF 0.010 *M* SODIUM β-AMINOOXYPROPIONATE IN 0.010 *M* SODIUM HYDROXIDE MAINTAINED AT 60°

Time of Sample, hr.	pKa_1	pKa_2	$10^3 \times$ Molarity of New Function	Estd. pKa of New Function	$10^3 \times$ Molarity of NaOH Consumed
0.0	3.70	5.20	0.00	...	0.0
17.8	3.70	5.25	1.33	9.5	0.36
41.9	3.78	5.20	1.90	9.45	0.95
118	3.82	5.25	3.52	9.63	1.15
180	4.10	5.50	4.38	9.76	2.48

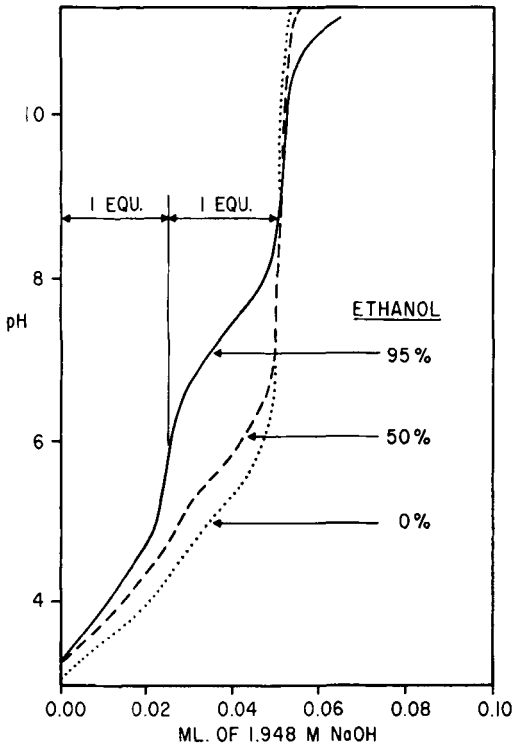


Fig. 2.—Potentiometric titrations of 0.005 *M* β-aminooxypropionic acid hydrochloride in varying per cent ethanol-water solutions.

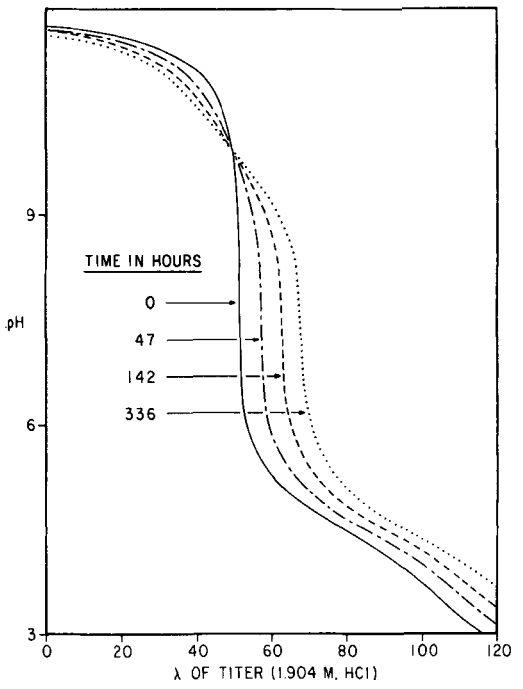
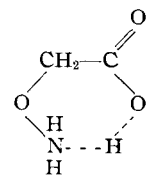


Fig. 3.—Titrations of 0.01 *M* sodium aminooxyacetate in 0.01 *M* sodium hydroxide maintained at 60°. The titer is 1.904 *M* hydrochloric acid.

ethanol content of the solvent. These facts permit the postulation of a nondipolar ion for the aminooxyalkanoic acids in solution, i.e., pKa_1 is assigned to the aminooxy group and pKa_2 is assigned to the carboxyl group. The smaller increase in pKa_2 between 0 and 50% ethanol content of the solvent than between 50 and 95% suggests that unusual molecular interactions may occur in solutions of high dielectric. The presence of a hemihydrochloride is in itself unique and implies that aminooxyacetic acid exists as the dimer in the crystalline form: $Cl^- \cdot NH_3^+—OCH_2COO^- \cdot NH_3^+—OCH_2COOH$.

The apparent pKa_1 values are determined on the premise that pKa_1 = pH at half-neutralization. Actually, the acidity of aminooxyacetic acid hemihydrochloride solutions is no less than what an equivalent molarity of hydrochloric acid would produce and thus strongly indicates that if any aqueous pKa_1 of this compound exists, it is very much less than the 2.75 estimated from half-neutralization and listed in Table I.

These facts indicate that aminooxyacetic acid may exist in cyclic form in solution where the aminooxy group is unavailable for ready protonation, as



If the number of methylene groups is increased between the carbonyl and the aminooxy group, cyclicization is inhibited and the aminooxy group becomes more readily protonated, as in the case of the real pKa_1 of β-aminooxypropionic acid hydrochloride.

Mode of Alkaline Degradation.—When aminooxyacetic acid is subjected to mild alkali and titrations of aliquots are made as a function of time (Fig. 3 and Table II), a new titratable group appears at *ca.* pKa 9.4 and the acidity of the reacting solution increases, indicative of free alkali consumption. The increase in molarity of the new 9.4 pKa func-

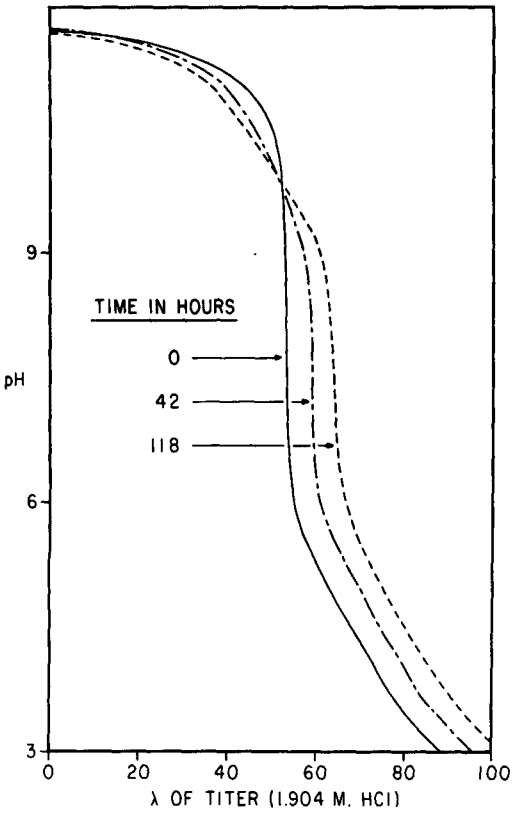


Fig. 4.—Titrations of 0.003 *M* sodium β-aminooxypropionate in 0.01 *M* sodium hydroxide maintained at 60°. The titer is 1.904 *M* hydrochloric acid.

tion was estimated by superimposition of the titration curve above pH 10 and estimating the differences in titer between the major inflection of a titration curve at any time with inflection of the titration curve taken at zero time (12). The loss of free alkali was estimated from differences in standard acid titer necessary to achieve the initial pH (12) and is given in Table II from the data on standard acid titration of aliquots. Standard alkaline titration of acidified aliquots gave variable results and indicated that the strong acid anion produced by basic treatment was unstable in acidic solutions.

These facts were consistent with the assignment of ammonia to the observed pKa 9.4 (NH_3, pKa = 9.25) and nitrite ion (pKa 3.6), produced in the ratio of 2:1 (see Tables II and III). The possible explanation of this degradation in this ratio is given in the following equations

$$H_2NO{-}CH_2COOH \xrightarrow[HOH]{OH^-} H_2NOH + HOCH_2COOH \quad \text{(Eq. 1)}$$

$$3NH_2OH + OH^- \rightarrow NO_2^- + 2NH_3 + 2H_2O \quad \text{(Eq. 2)}$$

Equation 2 predicts the formation of NH_3 and NO_2^- in the ratio of 2:1. The formation of the latter would lower the apparent acidity and consume free alkali. Chemical tests have unambiguously shown the presence of nitrite. Hydroxylamine (pKa = 6.00) was not observed by titration. This indicates that the possible reaction shown in Eq. 2 is the fast one and that reaction shown in Eq. 1 is rate determining. This fact was substantiated by subjecting hydroxylamine to 0.01 *M* sodium hydroxide at 60°. Within several minutes the pKa 6.0 disappeared, a pKa of 9.3 appeared with an alkali consumption in

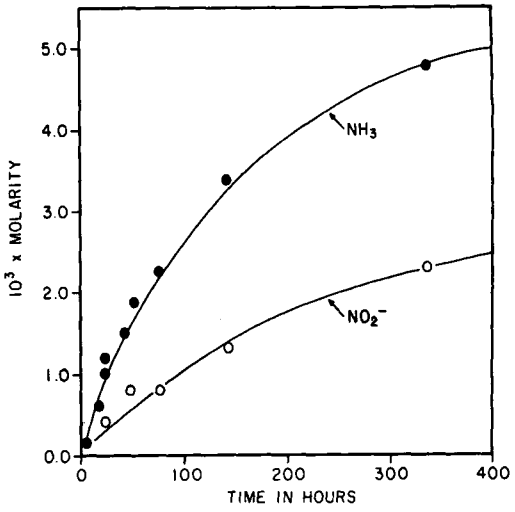


Fig. 5.—Appearance of NH_3 and NO_2^- as a function of time from the degradation of 0.010 *M* sodium aminooxyacetate in 0.010 *M* sodium hydroxide.

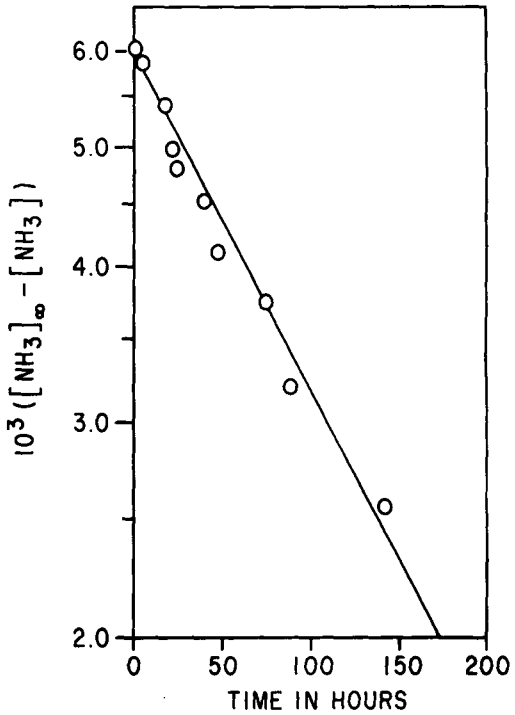


Fig. 6.—First-order plot for the appearance of $[NH_3]$ from the degradation of 0.010 *M* sodium aminooxyacetate in 0.010 *M* sodium hydroxide. The $[NH_3]_\infty$ is taken as 6.0×10^{-3}.

the ratio 1:2 with the pKa 9.3, and nitrite ion was conclusively identified as present.

The same phenomena were observed for the alkaline degradation of β-aminooxypropionic acid (Table III).

It can be concluded that carbonate or hydroxylamine is not an end product of the mild alkaline degradation of aminooxyalkanoic acid (no pKa's 6.0–6.5 are observable) and that the most probable sequence is the hydrolysis to hydroxylamine and some carboxylic acid, with a subsequent fast degradation of hydroxylamine to ammonia and nitrite ion.

Figure 5 plots the ammonia and nitrite produced as a function of time in 0.01 *M* sodium hydroxide at 60° as based on the above discussion. Apparent first-order plots for the production of ammonia and nitrite are given in Fig. 6 for aminooxyacetic acid with an apparent half-life of 110 hours under these conditions.

It is interesting to note that although β-aminooxypropionic acid is quite stable at 60° below pH 2 and between pH 7–10, degradation other than alkaline also occurs with an apparent maximum at pH values of *ca.* 3–4. This implies a significant acid catalyzed hydrolysis of the neutral molecule where

$$H_2NO{-}CH_2CH_2COOH + H_3O^+ \rightarrow H_3\overset{+}{N}OH + HOCH_2CH_2COOH \quad \text{(Eq. 3)}$$

the hydroxylamine was identified by the appearance of a pKa 6.0 since hydroxylamine does not readily degrade at this pH. It may be inferred that at a pH less than 2 the protonated compound, $^+H_3NO{-}CH_2CH_2COOH$, is the major species and resists hydrogen ion attack. At a pH greater than 7, the hydrogen ion and hydroxyl ion concentrations are insufficient for catalytic action.

Biological Activity and the Chemistry of Aminooxyalkanoic Acids.—The increased biological activity of aminooxyacetic acid over all other compounds studied (4, 7) can be assigned to its unusual chemical characteristics which include the absence of any apparent basicity of the aminooxy group in aqueous solution, perhaps due to a tendency to exist in the form of a five-membered ring. This in turn may be responsible for its ability to cross cell walls.

The ability of these α-aminooxyalkanoic acids to release hydroxylamine *in vitro* is of interest in light of the known action of hydroxylamine as an inhibitor of transaminase. This suggests that these α-aminooxyalkanoic acids may uniquely carry and release hydroxylamine at the physiological site, wheras hydroxylamine itself is too readily metabolized or not readily transportable. Of course, the possibility that the intact acids are the primary agent is not refuted.

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Sulfide Derivatives of Cysteine II

Some Sulfonamide Derivatives of Cysteine and Methionine

By MATTHEW VERDERAME

Seven new sulfide derivatives of cysteine have been prepared. In addition sulfonamides of cysteine, L-3-mercapto-N-sulfanilylalanine, and methionine, DL-N-sulfanilylmethionine, among others, have also been prepared. Except for L-3-[(3′-hydroxy-4′-carboxyphenyl)-carbamylmethylthio]-alanine (compound 2), which was slightly effective against a *Klebsiella pneumoniae* infection in Swiss mice, all other compounds tested for various activities showed a lack of physiological effectiveness.

A PREVIOUS paper (1) was concerned with the synthesis and physiological testing of several new amide derivatives of L-3-carboxymethylthioalanine as possible antiviral agents. This paper is, in part, involved with the synthesis and testing of seven additional sulfide derivatives of cysteine. The second half of this publication essentially deals with the preparation and biological study of L-3-mercapto-N-sulfanilylalanine, DL-N-sulfanilylmethionine, and other related compounds.

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DISCUSSION

The sulfide derivatives of cysteine, L-3-(substituted)-methylthioalanines (Table I), were made by