Constituents from *Gymnema sylvestre* Leaves II

Nitrogenous Compounds

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The leaves of *Gymnema sylvestre* were examined for the presence of alkaloids through the establishment of quaternary and nonquaternary nitrogenous fractions. A trace amount of an alkaloidal substance was isolated from a nonphenolic nonquaternary fraction and partially characterized. The plant bases choline, betaine, and adenine were isolated and identified in a combined yield of 0.04 per cent and would appear to be primarily responsible for the positive alkaloid tests previously reported for the leaves. The amino acids leucine, isoleucine, valine, alanine, and γ-amino-*n*-butyric acid were also detected. Of particular interest was the isolation (0.0003 per cent of leaf) and firm identification of trimethylamine oxide which has not been previously reported in plant material.

LEAF EXTRACTS of *Gymnema sylvestre* R. Br. (*Asclepiadaceae*) have an extensive history of pharmacological activity in Indian medicine (1–5) including use as an agent for the treatment of glycosuria and diabetes. It became the objective in these laboratories to examine the chemical nature of plant constituents with potential medicinal importance and to make them available for biological evaluation. The present study describes the isolation and identification of nitrogenous compounds during an investigation of dried *G. sylvestre* leaf for the reported presence (6, 7) of alkaloids.

EXPERIMENTAL

Dried *G. sylvestre* leaf processed during this investigation was commercially supplied by Prachi Gobeson Co., Calcutta, India, and in the later stages by the Himalaya Drug Co., Bombay, India.

Melting points were obtained with the Kofler micro hot stage and are corrected unless noted. An F&M carbon hydrogen nitrogen analyzer model 180 was used to follow nitrogen enrichment in alkaloid-containing fractions and as a deductive lead to structural characterization, but not to confirm a proposed structure. Spectra were determined on Perkin-Elmer models 137B and 337 infrared, Beckman DK-2A ultraviolet, Varian A-60 NMR, and Bendix-Time-of-Flight model 14 mass spectrometers. Neutral alumina, grade 1, Woelm, and silicic acid, 100 mesh (powder) (Mallinckrodt) were used for column chromatography. Silica gel G and alumina G supports and equipment supplied by Warner-Chilcott Laboratories were used for TLC. Continuous extractions of alkaloids were performed with a rotary film liquid-liquid extractor, Rinco VE-4000-x. The adjustments of pH were followed with a Beckman Zero matic pH meter.

Separation Methods

Methanol–Ammonia Extraction—A series of individual extractions was performed with portions (2.4–2.8 Kg.) of finely ground *G. sylvestre* leaf by maceration with methanol saturated with ammonia. The total maceration period was 10 days and included two fresh solvent exchanges. The combined extract was concentrated under reduced pressure to a thick, dark concentrate and diluted with an equal volume of 5% hydrochloric acid. Evaporation to original volume precipitated plant acids which were separated by filtration. The dark filtrate was continuously extracted with chloroform for 48–96 hr. to remove residual plant acids, pigment, and other nonalkaloidal impurities. The acidic aqueous system was made alkaline to pH 11.5 with cold 5 *N* sodium hydroxide and continuously extracted with chloroform for 48 hr. The chloroform extract was washed, dried over sodium sulfate, and evaporated to a brown nitrogenous semisolid, fraction A (0.023% of leaf), as the nonquaternary nonphenolic alkaloid fraction (positive Dragendorff's test). The alkaline solution after chloroform extraction was adjusted to pH 8 with cold 10% hydrochloric acid and continuously extracted with chloroform for 48 hr. The extract was separated, washed, dried over sodium sulfate, and reduced to a semisolid nitrogenous residue, fraction B (0.013% of leaf), as the nonquaternary phenolic alkaloid fraction (positive Dragendorff's test).

Aqueous layers after chloroform extraction were combined (representing 23.58 Kg. of leaf) and processed for the recovery of quaternary alkaloids. The system was concentrated to 8.2 L. in a flash evaporator (40°), adjusted to pH 2 with cold 10% hydrochloric acid, and refrigerated for 24 hr. After separation from an insoluble residue, the dark concentrate (9 L.) was treated with a saturated aqueous solution of Reinecke salt to completion of precipitation (3 L.). The reineckates were separated by filtration after chilling 24 hr. and washed with *n*-propanol (3.3 L.) to remove impurities. To the remaining solids on the filter was added acetone until the disappearance of color in

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the filtrate (2.5 L.). The reineckates in acetone solution were diluted with an equal volume of methanol and applied to a column of ion-exchange resin[1] (1075 ml., 3 cm. × 126 cm.), flow rate ≤12 ml./min. Column effluent and washes of acetone–methanol (1:1) were collected until a negative reineckate test was given (6.5 L.) and concentrated under reduced pressure to a solid fraction of quaternary bases.

The crude bases were dissolved in 2 L. of water and passed through a column of resin[2] (250 ml., 2.5 cm. × 211 cm.), flow rate 8 ml./min. Column effluent was concentrated to dryness *in vacuo* (50–60°) to give 3.1 Gm. of a tan residue as fraction C. The column[2] had assumed a dark coloration and was therefore removed and slurried batchwise with water. The dark aqueous supernatant was separated by filtration, evaporated, and dried to yield 1 Gm. of a brown residue as fraction D. The washed resin[2] was returned to the column and eluted with 1 *N* hydrochloric acid until a negative reineckate test was given in the column discharge (2 L.). Eluate was concentrated to dryness *in vacuo* (50–60°) to give tan solids as fraction E.

Aqueous Extraction—A solution resulting from a hot aqueous percolate of 403 Gm. of defatted ground leaf was applied to a column containing 500 ml. of ion-exchange resin.[3] The percolate prior to application to the column was kept under a layer of toluene. The effluent was discarded, and the resin was slurried with water to remove precipitated plant acids. Elution with 2.5 L. of 1 *N* ammonium hydroxide gave six fractions. Fractions 1–3 (1.3 L. of eluate) were reduced to dryness, extracted with hot ethanol, and filtered collectively through a 5-Gm. column of alumina. The yellow filtrate and ethanol washings were combined (200 ml.) and evaporated to a brown residue (1.2 Gm.) giving positive ninhydrin and picrate tests.

A 0.885-Gm. sample was dissolved in 50 ml. of water and freed of impurities by extraction with ether (3 × 25 ml.). To the aqueous layer was added a saturated aqueous solution of picric acid to completion of precipitation. The picrate was recovered and dissolved in 130 ml. of 0.1 *N* hydrochloric acid. Extraction with ether (5 × 65 ml.) removed picric acid and left an aqueous layer which upon repeated concentrations from water *in vacuo* (50°) gave 49.3 mg. of a yellow hydrochloride powder, fraction F.

The supernatant remaining after picrate precipitation was concentrated to 45 ml. and diluted to 50 ml. with 1 *N* hydrochloric acid. Picric acid was extracted with ether (8 × 25 ml.), and the aqueous layer was reduced to a yellow gum, fraction G.

Purification and Characterization

Nonquaternary Alkaloids—A 2.67-Gm. quantity of nonquaternary nonphenolic residue fraction A (representing 13.18 Kg. of leaf) was freed of water-soluble impurities and successively extracted with portions of 2% hydrochloric acid until reineckate spot tests were negative. The extract was treated with a saturated aqueous solution of Reinecke salt until precipitation was completed (60 ml.). After a 16-hr. refrigeration period, the reineckate derivative was filtered and recovered in 50 ml. of acetone. Regeneration of amine hydrochlorides by the classical method (8) gave a residue which was converted to free base form in methanol on a column of ion-exchange resin.[1] Collection of effluent and 80 ml. of methanol wash gave a brown residue, 0.145 Gm., as the purified nonquaternary nonphenolic alkaloid fraction (0.001% of leaf). Alumina column chromatography gave 7.5 mg. of the major component 5[4] from chloroform eluates, as a brown solid, m.p. 60–70°.

Anal. (CHN analyzer)—C, 68.01; H, 8.41; N, 7.26.

The substance gave a positive Dragendorff's test, was homogeneous by TLC on alumina in 2% methanol in benzene (blue fluorescence), and was soluble in chloroform, ethanol, methanol, benzene and ether and insoluble in water. Neither hydrochloride nor 2,4-dinitrophenylhydrazone derivatives could be prepared. A picrate, exhibiting a crystalline sublimate at 212° and m.p. 260°, indicated a molecular weight of 221 for the conjugate base by a spectrophotometric (Zeiss model PMQ II) method (9). An infrared spectrum (neat) showed $\nu_{max.}$ 3400 cm.$^{-1}$, 2950 cm.$^{-1}$, 1650 cm.$^{-1}$. The ultraviolet spectrum revealed $\lambda_{max.}^{ethanol}$, 231 mμ (*a* 39.68), 250(sh) (24.63), 281 (14.10), 288 (18.35); $\lambda_{max.}^{0.1\ N\ HCl\text{-}ethanol}$ 250 mμ (*a* 27.79), 302–303 (16.48).

A 1-mg. sample of component 5 purified by silica gel chromatography with 5% ethanol in chloroform as eluant gave an NMR spectrum which showed τ 9.10, 9.01, 8.74, 8.16, 3.15. A Varian C-1024 time-averaging computer was used to enhance the signal-to-noise ratio. A mass spectrum revealed the following peaks in order of decreasing intensity: *m/e* 28, 32, 168, 43, 41, 55, 57, 29, 18, 69, 71, 83, 14, 84.

Betaine—Fraction C was reconstituted in 25 ml. of ethanol, filtered from an insoluble residue, and washed with ethanol (3 × 5 ml.). To the solution in an ice bath was added cold concentrated hydrochloric acid, dropwise, until precipitation of base hydrochloride solids was completed (3 ml.). Solids were removed by filtration and washed with portions of ether and dried in vacuum over calcium chloride to give 1.8 Gm. of crude base hydrochloride. TLC on silica gel in methanol–28% ammonium hydroxide (75:25) showed a single zone with a modified Dragendorff's reagent (10) and was similar to a reference sample of betaine hydrochloride prepared from betaine (Nutritional Biochemical). Repeated recrystallization from 95% ethanol deposited a white crystalline solid, m.p. 264–285° dec.

Anal.—Calcd. for $C_5H_{12}ClNO_2$: C, 39.09; H, 7.87; N, 9.12. Found:[5] C, 39.37; H, 8.01; N, 8.73.

An infrared spectrum (KBr) of the substance was identical with that of reference betaine hydrochloride, m.p. 254–265° dec. The plant compound formed a picrate, m.p. 186° [lit. (11) m.p. 180–181°], and chloroaurate, m.p. 207.5–208.5° [lit. (11) m.p. 200–209°], derivatives which were identical with similarly prepared samples of reference betaine

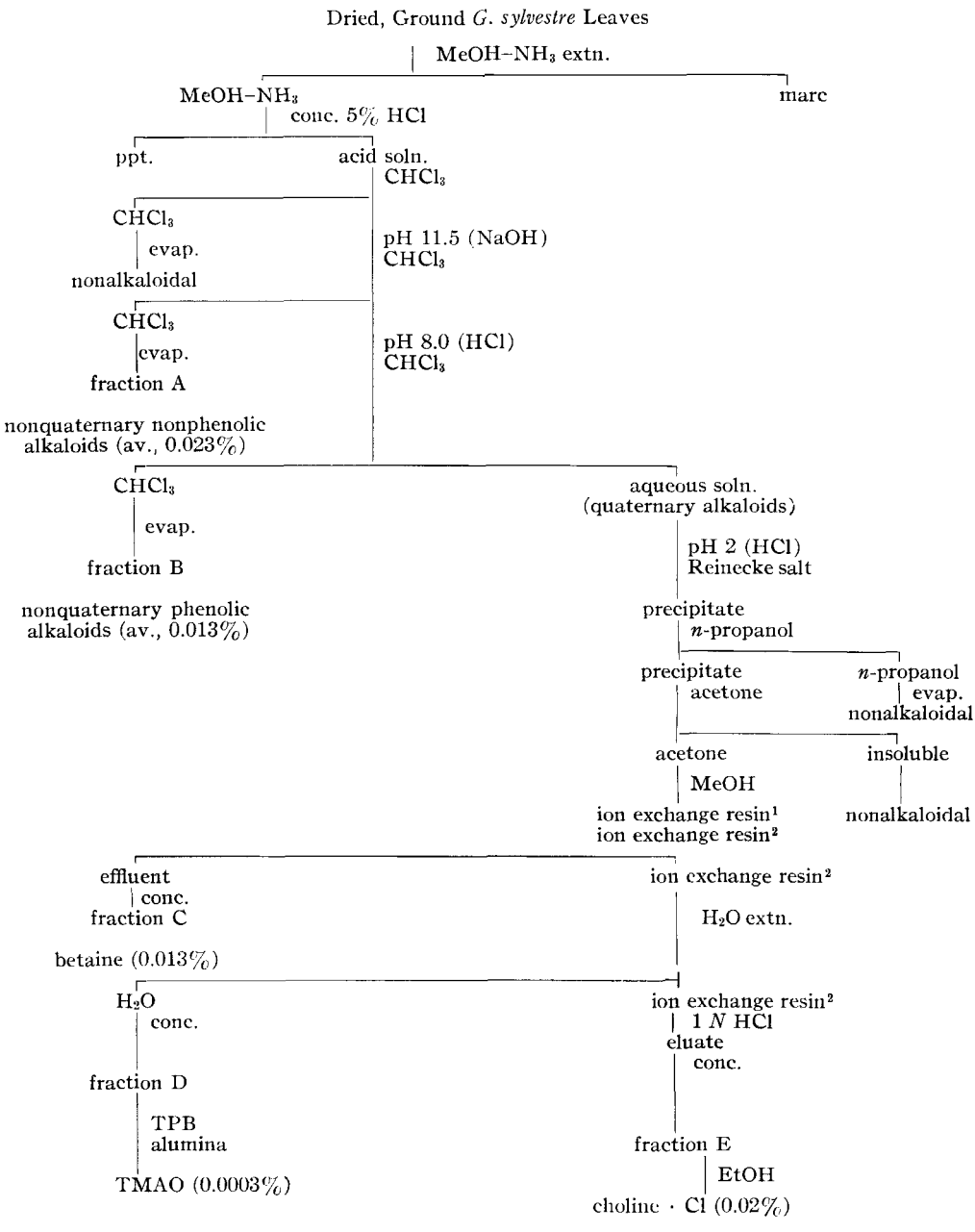
[1] Marketed as Amberlite IRA 401(OH^-) by Rohm and Haas Co., Philadelphia, Pa.

[2] Marketed as Amberlite IRC 50(H^+) by Rohm and Haas Co., Philadelphia, Pa.

[3] Marketed as Amberlite IR 120(H^+) by Rohm and Haas Co., Philadelphia, Pa.

[4] Designated component 5 according to its distribution as the fifth component from the origin on chromatograms.

[5] Performed by Micro-Tech Laboratories, Inc., Skokie, Ill.



Flow Sheet for Methanol–Ammonia Separation of Alkaloids of G. sylvestre

Scheme I

compounds, by melting point, mixed melting point, and infrared (KBr) analyses.

Trimethylamine Oxide—A 1% aqueous solution of fraction D was acidified to pH 2.5 with 5 *N* acetic acid, filtered, and treated with 70 ml. of 0.1 *M* sodium tetraphenylboron (aqueous). The precipitate was separated and converted to its chloride salts by passage through a column of ion-exchange resin.[6] Reduction of the effluent to dryness gave a residue which was fractionated by alumina column chromatography. Elution with 2% ethanol in chloroform (1.2 L.) gave 70.8 mg. of a hygroscopic white solid (0.0003% of leaf) which could be crystallized from ethanol–ether as needles, m.p. 80–81°. The addition of a 5% solution of oxalic acid dihydrate (0.6 ml.) to a 5.5% solution of the isolated compound in ethanol (0.5 ml.) gave crystals upon chilling. Recrystallization from ethanol gave needles (8.8 mg.), m.p. 119°, which showed a single peak in an NMR (D_2O) spectrum, τ 8.12.

Anal. (CHN analyzer)—C, 36.40; H, 7.05; N, 7.99.

Reference trimethylamine oxide (TMAO) di-

[6] Marketed as Amberlite IRA 401(Cl⁻) by Rohm and Haas Co., Philadelphia, Pa.

hydrate (K & K Laboratories) showed an infrared spectrum (KBr) that was superimposable with the isolated substance and gave an oxalate, which was identical with the unknown oxalate by mixed melting point, infrared (KBr), and NMR analyses.

Anal. (reference TMAO oxalate)—Calcd. for $C_3H_9NO \cdot C_2H_2O_4$: C, 36.36; H, 6.67; N, 8.48. Found:[7] C, 36.55; H, 6.80; N, 8.31.

A picrate derivative, m.p. 208.5–209.5° dec., indicated a molecular weight of 72 for the conjugate base of a monopicrate, by a spectrophotometric (Zeiss model PMQ II) method (9). This picrate derivative was subsequently shown to be identical in melting point, mixed melting point, and infrared (KBr) to reference TMAO picrate.

Residues from chromatographic fractions 4 and 5 of the aqueous extraction procedure (see under *Aqueous Extraction*) representing 1.3–2.0 L. of 1 *N* ammonium hydroxide eluate, were extracted with hot ethanol, combined, and filtered over 2 Gm. of alumina. The colorless filtrate and ethanol washings were collected (50 ml.) and evaporated to a white scale. Thin-layer chromatography on alumina G in methanol–carbon tetrachloride–acetic acid (10:30:1) revealed a zone (iodine vapors) that corresponded to that of trimethylamine oxide when developed side by side. Response to modified Dragendorff's reagent (delayed orange coloration) was the same with each sample.

Choline—Fraction E was separated from considerable inorganic chloride content by reconstituting the fraction in 200 ml. of ethanol and concentrating to a small volume. The dark supernatant was decolorized[8] and concentrated to dryness *in vacuo* (60°) which left a yellow semisolid (0.02% of leaf). A 0.500-Gm. quantity of crude base chloride was chromatographed over alumina to yield 0.211 Gm. which was recrystallized from ethanol–ether to give 0.177 Gm. of needles, m.p. gradual dec. above 250°. Paper chromatography in three solvent systems (10, 12) gave R_f values and characteristic color responses with modified Dragendorff's and Levine Chargaff reagents consistent with reference choline chloride (Eastman recrystallized). The infrared spectra (KBr) of plant base chloride and authentic choline chloride were similar. Chloroaurate derivatives of the plant compound and reference choline were identical with m.p. 230.5–232.5°[9] and by mixed melting point and infrared (KBr) analyses. The melting point of tetraphenylborate derivatives of the plant isolate, reference choline chloride, and a mixture of the two were identical at 227–229°. [Lit. (14) m.p. 219–221° uncorrected.] The infrared spectra (KBr) of these derivatives as well as a reineckate from the isolated plant base were identical with the spectra of the corresponding derivatives from reference choline chloride.

Adenine—Ether addition to a concentrated methanolic solution of fraction F afforded a flocculent precipitate which dried to 33 mg. of an off-white powder (0.008% of leaf), m.p. 280–292° dec., uncorrected. It gave a weakly positive Dragendorff's test for alkaloids, did not form a reineckate, and yielded a picrate, m.p. 284–288° dec., uncorrected, and picrolonate, m.p. 256–259°. An infrared spectrum (KBr) of the powder was similar to that of adenine·HCl·$3H_2O$. A picrate derivative, m.p. 284–288° dec., uncorrected [lit. m.p. 279–281° (15), 292° (16)], was prepared from reagent adenine sulfate (Nutritional Biochemical) and converted to adenine hydrochloride, m.p. 280–292° dec., uncorrected, in the same manner as was the amine picrate of plant origin. A reference sample of adenine picrolonate, m.p. 256–259° [lit. (15) m.p. 265°], was prepared from the hydrochloride. Mixed melting points of reference picrate and picrolonate derivatives of adenine were not depressed with the corresponding derivatives of plant origin; the infrared spectra (KBr) of reference adenine hydrochloride, picrate, and picrolonate were superimposable with the respective samples derived from the plant compound.

Amino Acids—Fraction G (positive ninhydrin and negative picrate tests) as a 0.5% aqueous solution was applied successively to two ion-exchange resin columns.[2, 10] No basic or acidic amino acids could be recovered from these columns. However, the final effluent passing through both columns was reduced to a yellow semicrystalline residue, 0.545 Gm. (0.23% of leaf), as the purified neutral amino acid fraction. The residue was examined by paper (17) and silica gel thin-layer (18) chromatography with a series of naturally occurring reference L-amino acids. Agreement of R_f values in three paper chromatographic systems and a two-dimensional thin-layer pattern with reference mixtures run in parallel and in admixture revealed the presence of leucine, isoleucine, valine, alanine, and γ-amino-*n*-butyric acid as the major amino acid constituents of the plant fraction.

RESULTS AND DISCUSSION

The methanol–ammonia extraction for alkaloids from *G. sylvestre* (Scheme I) gave residues of nonquaternary nitrogenous substances. After nitrogen enrichment by acid exchange resin adsorption and binary phase extraction, TLC indicated that both phenolic and nonphenolic fractions contained only small amounts of a complex mixture of alkaloid positive constituents (0.001% of leaf) and considerable nonalkaloidal impurities. Thus, interest was limited to the isolation of the major alkaloid of the nonquaternary nonphenolic fraction A. Several milligrams of the base (5×10^{-5}% of leaf) were made available by alumina column chromatography as a brown solid, m.p. 60–70°. The fluorescent substance formed a picrate from which a molecular weight of 221 was estimated for the base by a spectrophotometric method (9). A mass spectrum did not substantiate this value and showed a peak of highest mass at *m/e* 168, suggesting the presence of a stabilized fragment ion from the intact molecule. The base had ultraviolet absorption characteristics of an aniline derivative, particularly the indoline chromophore (19), but exhibited a bathochromic shift in acid and lacked the features of aromatic structure from infrared and mass spectrometric data. NMR and mass spectra

[7] Performed by Spang Microanalytical Laboratories, Ann Arbor, Mich.

[8] The decolorizing agent was Norit, American Norit Co., Jacksonville, Fla.

[9] This melting point was increased to 245–250° when rate of heating was increased to 30°/min. [Lit. (13) m.p. 250–255° with rapid heating.]

[10] Marketed as Amberlite IR-4B(OAc⁻) by Rohm and Haas Co., Philadelphia, Pa.

indicated significant aliphatic nature. Efforts to isolate sufficient quantities of the alkaloid substance for further characterization are in progress.

The major alkaloid-positive constituents of *G. sylvestre* were detected in quaternary base fractions and were indicated as the biological amines choline and betaine (combined yield, 0.03%) by chromatography and infrared data. The compounds were isolated by reineckate precipitation and separated by an exchange resin technique. Firm identification was established by analysis of choline as reineckate, tetraphenylborate, and chloroaurate derivatives, and betaine as chloroaurate and picrate derivatives.

During the separation process of choline and betaine, minor amounts of a third alkaloid-positive component were isolated (0.0003% of leaf). The substance was identified as trimethylamine oxide (TMAO) by NMR, infrared, molecular weight, and elemental analyses, and by agreement of oxalate and picrate derivatives with reference samples. The oxalate of TMAO has not been described previously in the literature. However, the ease with which a crystalline, nonhygroscopic compound with sharp melting point was obtained makes trimethylamine oxide acid oxalate an excellent derivative for characterization of TMAO.

Aqueous leaf extractions removed an alkaloid-positive substance which was separated by adsorption on a strong acid exchanger and isolated as an insoluble picrate derivative. Conversion to the amine hydrochloride (fraction F) and precipitation gave a compound (0.008% of leaf) which was similar to adenine·HCl·$3H_2O$ by infrared analysis. A picrate of reference adenine sulfate was processed in the same manner to give a hydrochloride with similar chromatographic and melting point characteristics as the plant product. Their infrared spectra were superimposable. Picrate and picrolonate derivatives of both reference and plant samples of adenine were identical by melting point and infrared analysis. The occurrence of compounds such as adenine in plants and their relationship to nucleic acids is discussed by Markham (20).

The supernatant remaining after precipitation of adenine from aqueous extracts contained a series of neutral amino acids (0.23% of leaf). The possibility that an uncommon amino acid of potential pharmacological significance (*e.g.*, as a hypoglycemic agent) might reside in this fraction prompted the characterization of major constituents by paper and thin-layer chromatographic methods. By agreement of R_f values with reference mixtures in a series of solvent systems, the amino acids leucine, isoleucine, valine, alanine, and γ-amino-*n*-butyric acid were detected. A two-dimensional thin-layer chromatogram confirmed these results.

Identification of the protoalkaloids (21), choline and betaine, and the pseudoalkaloid (22), adenine, offers an explanation for Webb's report of positive alkaloid tests from 1% hydrochloric acid extracts of *G. sylvestre* leaves (7). Phosphomolybdic acid, the only reagent in Webb's survey that showed appreciable alkaloid content, affords heavy precipitates with betaine, choline, and adenine. Minor amounts of complex alkaloid mixtures in nonquaternary plant fractions exclude *G. sylvestre* as a true alkaloid-bearing plant in taxonomic terms, which require an accumulation of 0.01% alkaloid from dried tissue (23). Hooper's original alkaloid-positive extract (6) most likely contained this group of trace nonquaternary alkaloidal substances. The level of occurrence of all nitrogenous compounds found in *G. sylvestre* and the chemical identity of major constituents show essentially no correlation with pharmacological activity of the plant.

The occurrence of TMAO in dried leaves was reaffirmed when direct aqueous extractions, which minimized artifact production by chemical and bacterial influences, gave chromatographic evidence for the presence of the oxide. The natural occurrence of TMAO in plant tissue has not been previously described in the literature. However, the biogenesis of TMAO in biological systems has been discussed by Guggenheim (24). Also, evidence has recently been presented for the ability of alfalfa (25) and *Chenopodium vulvaria* (26) seedlings to convert radioactive trimethylamine to TMAO *in vivo*, while inherent oxidative mechanisms are proposed for the biogenesis of amine oxides in the seeds of *Piptadenia* species (27). It would be of particular interest to examine fresh *G. sylvestre* leaves for the natural occurrence of TMAO, and to explore the conversion of trimethylamine to TMAO in this plant.

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