Biosynthesis of the *Nicotiana* Alkaloids. XII. The Incorporation of α- and δ-N-Methylornithine into the Pyrrolidine Ring of Nicotine[1]

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Abstract: DL-α-N-Methyl-^{14}C-ornithine-2-^{14}C and DL-δ-N-methyl-^{14}C-ornithine-2-^{14}C were prepared and administered to *Nicotiana tabacum* plants. The δ-N-methylornithine afforded radioactive nicotine (1.25% incorporation) which was labeled solely at C-2′ and on the N-methyl group, the ratio of activity at these two positions indicating that the precursor was incorporated intact without any cleavage of the N-methyl group. The α-N-methylornithine was a much poorer precursor of nicotine (0.10% incorporation). This nicotine was labeled at C-2′, C-5′, and on the N-methyl group, the distribution of activity being consistent with demethylation of the α-N-methylornithine prior to its incorporation into the pyrrolidine ring. The status of δ-N-methylornithine as a normal biosynthetic precursor of nicotine is discussed.

Recently Schröter and Neuman[2] reported that the administration of α-N-methyl-^{14}C-ornithine to *Nicotiana rustica* plants led to the formation of radioactive nicotine (0.2–0.5% specific incorporation),[3] which had 51–57% of its activity located on the N-methyl group of the pyrrolidine ring. On the other hand δ-N-methyl-^{14}C-ornithine was a much poorer precursor of nicotine (0.05% specific incorporation) and only 14% of the activity was found on the N-methyl group. From these results the authors concluded that α-N-methylornithine served as a precursor of the pyrrolidine ring of nicotine, incorporation occurring without prior cleavage of the methyl from the α-amino group. It thus follows that the nitrogen of the pyrrolidine ring is derived from the α-amino group of α-N-methylornithine. However, we[4] found, by feeding α- and δ-^{15}N-labeled ornithine to a sterile culture of *N. tabacum* roots, that the δ-amino but not the α-amino group was utilized in the formation of the pyrrolidine ring of nicotine.

We decided that these conflicting results could be resolved by the administration of α- and δ-N-methylornithines which were labeled with ^{14}C on both their N-methyl groups and at C-2. By determination of the distribution of activity in the resultant nicotine, it could be determined whether the N-methyl group of these amino acids was being incorporated with or without prior cleavage.

α-N-Methylornithine was prepared by the method of Streib.[5] α-N-*p*-Toluenesulfonyl-δ-N-benzoylornithine, dissolved in aqueous sodium hydroxide, was methylated on the α nitrogen with dimethyl sulfate and the product hydrolyzed by heating with hydrochloric acid. The doubly labeled α-N-methylornithine which was fed to the tobacco was actually a mixture of α-N-methyl-^{14}C-ornithine and α-N-methyl-ornithine-2-^{14}C. δ-N-Methylornithine was prepared by an analogous route[6] from α-N-benzoyl-δ-N-*p*-toluenesulfonylornithine. The labeled amino acids were added to the aerated inorganic nutrient solution in which the roots of 5-month-old *N. tabacum* plants were growing hydroponically. There was a dramatic difference in the rate of uptake of the α- and δ-N-methylornithines. After 5 days essentially all the δ-N-methylornithine had been absorbed by the roots and the plants were harvested after 7 days. After 5 days only 50% of the α-N-methylornithine had been absorbed by the tobacco roots. The nicotine from the two experiments was isolated as previously described,[7] final purification being achieved by preparative thin layer chromatography. The nicotine isolated from the plants which had been fed the radioactive δ-N-methylornithine had a much higher activity than the nicotine derived from the α-N-methylornithine. Activity in the N-methyl group of nicotine was determined by demethylation with hydrogen iodide, the resultant methyl iodide being trapped in triethylamine as the quaternary salt.[8] Oxidation of nicotine with nitric acid yielded nicotinic acid and 3-nitro-5-(3′-pyridyl)-pyrazole.[7] This latter compound contains all the carbons of nicotine except C-5′ and the N-methyl group. Activity at C-5′ is thus deduced by difference. Decarboxylation of nicotinic acid yielded pyridine, collected as its oxalate. The activity of nicotine and its degradation products are recorded in Table I.

It is apparent that the α- and δ-N-methylornithines are utilized in quite different ways for the formation of the pyrrolidine ring of nicotine. The δ-N-methyl-^{14}C-ornithine-2-^{14}C yielded nicotine which was labeled only at C-2′ and on the N-methyl group. Since the ratio of activity in the pyrrolidine ring at C-2′ and on the N-methyl group was the same as the ratio of activity at C-2 and on the N-methyl group of the administered δ-N-methylornithine, we consider that this amino acid is incorporated without cleavage of the N-methyl group. In Scheme I we propose a

(1) This investigation was supported by Research Grant GM-13246 from the U. S. Public Health Service. It is based in part on the Ph.D. Thesis of T. J. Gilbertson, University of Minnesota, Sept 1967.

(2) H.-B. Schröter and D. Neuman, *Tetrahedron Letters*, 1279 (1966).

(3) Specific incorporation is defined as the specific activity of the nicotine (dpm/mmole) divided by the specific activity of the administered precursor.

(4) E. Leete, E. G. Gros, and T. J. Gilbertson, *Tetrahedron Letters*, 587 (1964).

(5) H. Streib, *Z. Physiol. Chem.*, **155**, 279 (1926).

(6) K. Thomas, J. Kapfhammer, and B. Flaschenträger, *ibid.*, **124**, 75 (1922).

(7) E. Leete and K. J. Siegfried, *J. Am. Chem. Soc.*, **79**, 4529 (1957).

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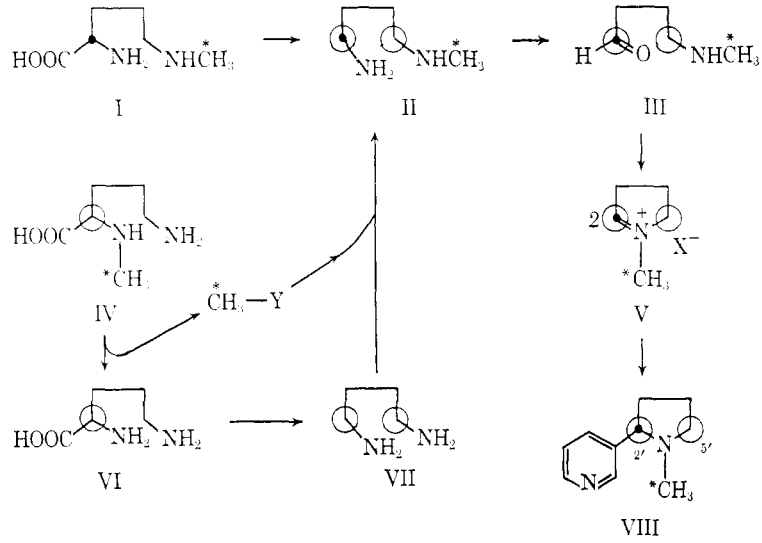
Scheme I. The Incorporation of the Methylornithines into Nicotine



●, ^{14}C arising from C-2 of δ-N-methylornithine; ○, ^{14}C arising from C-2 of α-N-methylornithine; *, ^{14}C arising from the N-methyl group of both amino acids.

biosynthetic sequence which is consistent with the tracer results. The δ-N-methylornithine (I) is decarboxylated yielding N-methylputrescine (II) which is then oxidized at the primary amino group affording 4-methylaminobutanal (III). Cyclization yields an N-methyl-Δ^1-pyrrolinium salt (V), which will be labeled at C-2 and on the N-methyl group. The nicotine (VIII) derived from this salt will be labeled at C-2′ and on the N-methyl group, since we have recently fed N-methyl-Δ^1-pyrrolinium-2-^{14}C chloride to tobacco and obtained nicotine labeled solely at C-2′.[9] Schütte and co-workers[10] have also shown that N-methylputrescine is incorporated without degradation into the N-methylpyrrolidine ring of nicotine.

Table I

Experiment 1. Administration of α-N-Methyl-[^{14}C]-ornithine-2-^{14}C	Wt, mg	Activity, dpm
DL-α-N-Methyl-^{14}C-ornithine·HCl	24.5	1.26×10^7
DL-α-N-Methylornithine-2-^{14}C·HCl	29.3	4.70×10^7
Activity at C-2/activity at N-methyl = 3.72		

Nicotine and Its Degradation Products	Activity, dpm/mmole	Relative activity
a. Nicotine diperchlorate	1.17×10^5	100
b. Triethylmethylammonium iodide [N–Me]	0.135×10^5	11.5
c. Nicotinic acid	0.531×10^5	45.4
d. Pyridine oxalate	0	0
e. 3-Nitro-5-(2′-pyridyl)-pyrazole	0.536×10^5	45.8
Activity at C-2′ [c − d]		45.4
Activity at C-5′ [a − (b + e)]		42.7
Activity at C-2′ + C-5′/activity at N-methyl = 7.66		

Experiment 2. Administration of δ-N-Methyl-^{14}C-ornithine-2-^{14}C	Wt, mg	activity, dpm
DL-δ-N-Methyl-^{14}C-ornithine·HCl	11.9	2.04×10^7
DL-δ-N-Methylornithine-2-^{14}C·HCl	22.7	4.21×10^7
Activity at C-2/activity at N-methyl = 2.06		

Nicotine and Its Degradation Products	Activity, dpm/mmole	Relative activity
a. Nicotine diperchlorate	2.13×10^6	100
b. Triethylmethylammonium iodide [N–Me]	0.713×10^6	33.5
c. Nicotinic acid	1.44×10^6	67.7
d. Pyridine oxalate	0	0
e. 3-Nitro-5-(3′-pyridyl)-pyrazole	1.44×10^6	67.6
Activity at C-2′ [c − d]		67.6
Activity at C-5′ [a − (b + e)]		0
Activity at C-2′/activity at N-methyl = 2.02		

The nicotine derived from the α-N-methyl-^{14}C-ornithine-2^{14}C had equal labeling at C-2′ and C-5′ (within experimental error). Activity was also present on the N-methyl group; however, the level of activity at this position was about half that required for a direct incorporation of the precursor without cleavage of the N-methyl group. It is suggested that the α-N-methylornithine (IV) is demethylated in the plant, yielding ornithine-2-^{14}C (VI) which is known to afford nicotine labeled equally at C-2′ and C-5′.[7,11–13] Activity on the N-methyl group of nicotine could arise by transmethylation from methyl donors (CH_3–Y) which would have become labeled with ^{14}C by transmethylation from the administered α-N-methyl-^{14}C-ornithine.

Our results do not agree with those reported by Schröter and Neuman.[2] In particular they found that their α-N-methyl-^{14}C-ornithine was a much better precursor of nicotine than the δ isomer. We consider that their reported[14] syntheses may not have yielded authentic α- and δ-N-methylornithines. They claimed to have obtained α-N-methylation by heating α-N-*p*-toluenesulfonylornithine with methyl iodide and aqueous sodium hydroxide in a sealed tube at 80°. Under these conditions primary amines are readily methylated

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(10) H. R. Schütte, W. Maier, and K. Mothes, *Acta Biochem. Polon.*, **13**, 401 (1966).
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(13) A. A. Liebman, B. P. Mundy, and H. Rapoport, *J. Am. Chem. Soc.*, **89**, 664 (1967).
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and it is suggested that the main product from this reaction would be α-N-*p*-toluenesulfonyl-δ-N-methylornithine. Hydrolysis of this compound would yield δ-N-methylornithine. They synthesized the δ-N-methyl isomer by hydrolysis of the product obtained by treating α-N-benzoyl-δ-N-*p*-toluenesulfonylornithine with methyl iodide in the presence of sodium hydroxide at 80°. We have found that the α-N-benzoyl group of this compound is fairly readily cleaved with sodium hydroxide. Therefore they may actually have had considerable δ-N-*p*-toluenesulfonylornithine in their reaction mixture and α-N-methylation on the primary amino group may have occurred. Hydrolysis would then yield α-N-methylornithine. They did not report any physical constants for their methylated intermediates. If the above interpretation of their synthetic reactions is correct, their results agree with ours; *i.e.*, the δ-N-methylornithine is a much better precursor of the pyrrolidine ring than the α isomer.

Our present experiments have shown that the *N. tabacum* plant is able to utilize δ-N-methylornithine for the synthesis of the N-methylpyrrolidine ring of nicotine. However, this compound cannot be a metabolic intermediate between ornithine and the pyrrolidine ring. It is recalled that ornithine-2-^{14}C yields nicotine labeled equally at C-2′ and C-5′. If it were methylated to yield δ-N-methylornithine-2-^{14}C, the resultant nicotine would be labeled only at C-2′. It is therefore suggested that the tobacco plant contains nonselective enzymes which are capable of catalyzing the decarboxylation of δ-N-methylornithine, affording N-methylputrescine which is then utilized as previously suggested for the formation of the pyrrolidine ring of nicotine.

Experimental Section

Melting points are corrected. Radioactivity measurements were carried out in a Nuclear Chicago liquid scintillation system, Model 724, using as solvents either toluene or dioxane–water with the usual scintillators.[15] Microanalyses were carried out at the University of Minnesota by J. Canterbury and his assistants. Nmr spectra were determined on a Varian A-60 nmr spectrometer.

DL-α-N-Methylornithine-2-^{14}C Monohydrochloride. DL-Ornithine-2-^{14}C[16] (500 mg, 0.39 mcurie) was dissolved in water (8 ml) and heated to boiling. Basic copper carbonate ($CuCO_3 \cdot Cu(OH)_2$) (663 mg) was added in small portions and the mixture refluxed for 25 min after the addition was complete. The filtered solution was then cooled, sodium carbonate (1.5 g) added, and the solution stirred with benzoyl chloride (1 ml) and ether (5 ml). The copper salt of δ-N-benzoylornithine separated out and was filtered off. This salt was dissolved in water (10 ml) with the aid of a little concentrated hydrochloric acid and hydrogen sulfide passed in. The mixture was filtered and the filtrate made neutral by the addition of ammonia. On concentration of the solution, DL-δ-N-benzoylornithine-2-^{14}C separated out. Recrystallization from water afforded colorless plates (334 mg, 40%), mp 241–246° dec (lit.[17] mp 243–248°). This N-benzoyl derivative (334 mg) was dissolved in water (5 ml) containing sodium hydroxide (112 mg) and the solution stirred at 20° with *p*-toluenesulfonyl chloride (266 mg) dissolved in ether (1 ml). After 1 hr the reaction mixture usually failed to give a purple color with ninhydrin. If a positive reaction was observed, additional small quantities of *p*-toluenesulfonyl chloride and sodium hydroxide were added and the stirring was continued until no color was obtained with ninhydrin. The solution was then filtered and cooled to 0°. Acidification with concentrated hydrochloric acid caused the separation of DL-δ-N-benzoyl-α-N-*p*-toluenesulfonylornithine (443 mg, 80%), mp 184–185° (lit.[5] mp 183°). This compound (443 mg, 1.13 mmoles) was dissolved in water (4 ml) containing sodium hydroxide (96 mg) and the solution stirred at 0° with dimethyl sulfate (0.42 ml, 4.42 mmoles) until the solution became cloudy (about 40 min). Then 10% sodium hydroxide (2 ml) was added and the mixture stirred for an additional 15 min. Concentrated hydrochloric acid was added to the filtered solution when an oil separated. On scratching, this oil solidified. Recrystallization from a mixture of acetone and ethyl acetate afforded colorless needles of DL-α-N-methyl-α-N-*p*-toluenesulfonyl-δ-N-benzoylornithine (446 mg, 90%), mp 184–186° (lit.[5] mp 185°). A mixture melting point with the unmethylated compound showed considerable depression. The nmr spectrum in deuterated dimethyl sulfoxide had a singlet at τ 7.2 assignable to the N-methyl group.

Anal. Calcd for $C_{20}H_{24}N_2O_5S$: C, 59.39; H, 5.98; N, 6.93. Found: C, 59.42; H, 6.18; N, 6.91.

The methylated compound (446 mg) was heated with concentrated hydrochloric acid (9 ml) in a sealed tube at 130° for 24 hr. After cooling, the reaction mixture was filtered and the filtrate evaporated to dryness. The residue was dissolved in water and chromatographed on a column (30 × 1.5 cm) of Dowex 50-8X resin (H^+ form). The column was washed successively with water (50 ml), 1 *N* HCl (50 ml), water (50 ml), and 1 *N* ammonium hydroxide (100 ml). The α-N-methylornithine (detected with ninhydrin) was present in the fractions eluted with ammonia. These fractions were evaporated to dryness and the residue was dissolved in hydrochloric acid. The residue obtained on evaporation of this solution was dissolved in ethanol (10 ml) and a little pyridine added when DL-α-N-methylornithine-2-^{14}C hydrochloride separated (166 mg, 83%). Recrystallization from aqueous ethanol afforded colorless plates, mp 229–233° (lit.[18] mp 229–230°). The nmr spectrum in D_2O had a singlet at τ 7.3 assignable to the N-methyl group.

Anal. Calcd for $C_6H_{15}N_2O_2Cl$: C, 39.45; H, 8.28; N, 15.34. Found: C, 38.87; H, 8.21; N, 15.53.

The product was identical (nmr, infrared, mixture melting point) with a sample of α-N-methylornithine obtained by Fischer's method[19] (acid hydrolysis of the reaction product of 5-phthalimido-2-bromovaleric acid and methylamine).

DL-α-N-Methyl-^{14}C-ornithine Monohydrochloride. Dimethyl sulfate-^{14}C[20] (0.05 ml, 0.52 mmole, 0.5 mcurie) was added to a stirred solution of DL-α-N-*p*-toluenesulfonyl-δ-N-benzoylornithine (250 mg, 0.64 mmole) dissolved in water (3 ml) containing sodium hydroxide (96 mg). After 45 min, inactive dimethyl sulfate (1 ml) was added and the stirring continued for an additional 45 min. The α-N-methyl-^{14}C-α-N-*p*-toluenesulfonyl-δ-N-benzoylornithine (255 mg, 98%), mp 177–180°, was isolated as previously described. Acid hydrolysis of this compound afforded DL-α-N-methyl-^{14}C-ornithine hydrochloride (47 mg, 41%), mp 225–229°.

DL-δ-N-Methylornithine-2-^{14}C Monohydrochloride. Basic copper carbonate (663 mg) was added to a boiling solution of DL-ornithine-2-^{14}C hydrochloride (500 mg, 0.49 mcurie) in water (8 ml). After 25 min the solution was filtered and sodium bicarbonate (2.5 g) added to the cooled filtrate. *p*-Toluenesulfonyl chloride (570 mg) in acetone (8 ml) was added and the mixture stirred overnight. The resultant precipitate of the copper salt was removed and decomposed with hydrogen sulfide, as described for the preparation of the corresponding δ-N-benzoyl derivative, affording DL-δ-N-*p*-toluenesulfonylornithine-2-^{14}C (492 mg, 60%), mp 226–230° (lit.[21] mp 229–232°). This compound was benzoylated on the α-amino group with benzoyl chloride in the presence of sodium hydroxide affording DL-α-N-benzoyl-δ-N-*p*-toluenesulfonylornithine (649 mg, 90%), mp 164–165° (lit.[6] mp 160–164°). Methylation was carried out with dimethyl sulfate as previously described for the preparation of the α-N-methyl isomer, affording DL-α-N-benzoyl-δ-N-methyl-δ-N-*p*-toluenesulfonylornithine-2-^{14}C (641 mg, 91%), mp 190–192° (lit.[6] mp 188°). The nmr spectrum in deuterated dimethyl sulfoxide had a singlet at τ 7.4 due to the N-methyl group.

Anal. Calcd for $C_{20}H_{24}N_2O_5S$: C, 59.39; H, 5.98; N, 6.93. Found: C, 58.81; H, 6.05; N, 6.95.

Hydrolysis of the above compound as described for the α-N-methyl isomer afforded DL-δ-N-methylornithine-2-^{14}C hydrochloride (223 mg, 77%), mp 236–237° (lit.[6] mp 215–225°). The

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nmr spectrum in D_2O had a singlet at τ 7.3 assigned to the N-methyl group.

Anal. Calcd for $C_6H_{15}N_2O_2Cl$: C, 39.45; H, 8.28; N, 15.34. Found: C, 39.58; H, 8.43; N, 15.38.

DL-δ-N-Methyl-^{14}C-ornithine monohydrochloride was obtained by methylation of DL-α-N-benzoyl-δ-N-*p*-toluenesulfonylornithine with dimethyl sulfate-^{14}C as described for the α isomer.

Chromatography of the Amino Acids. Thin layer chromatography of the amino acids on silica gel (Eastman chromatogram sheet 6061) with methanol–triethylamine (9:1) resulted in the following R_f values: α-N-methyornithine 0.28, δ-N-methylornithine 0.18, ornithine 0.24.

Administration of the Tracers to the Plants and Isolation of the Nicotine. The methylornithines (see Table I for amounts) were added to the nutrient solution[22] in which the roots of 5-month-old *N. tabacum* plants were growing. After 7 days the plants which had been fed the δ-N-methylornithine (residual activity in nutrient solution, 1.3%) were harvested (wet weight of plant, 856 g) and the crude alkaloids isolated as previously described.[7] After distillation the nicotine was purified by preparative thin layer chromatography on silica gel PF, eluting with a mixture of chloroform and methanol (9:1). Nicotine, having an R_f of 0.5, was extracted from the silica gel with ethanol. Evaporation of the ethanol in the presence of 70% perchloric acid (0.1 ml) yielded nicotine diperchlorate (133 mg), having an activity of 5.87×10^3 dpm/mg (1.25% absolute incorporation, 0.65% specific incorporation). After 13 days the plants which had been fed the α-methylornithine (residual activity in the nutrient solution, 11.1%) were harvested (wet weight of plants, 880 g) and afforded nicotine diperchlorate (192 mg), having an activity of 322 dpm/mg (0.10% absolute incorporation, 0.053% specific incorporation).

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Mechanism and Catalysis of the Hydrolysis of a Formamidinium Compound[1]

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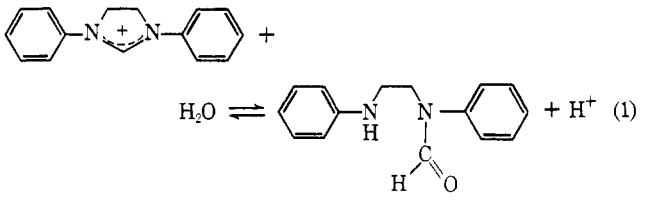
Abstract: The hydrolysis of the amidine, 1,3-diphenyl-2-imidazolinium chloride (DPIC), to N-(2-anilinoethyl)-formanilide (AEF) in aqueous solutions at 25°, ionic strength 1.0 *M*, follows the rate law $v = k_2[\text{DPIC}][\text{B}] + k_3[\text{DPIC}][\text{B}]a_{\text{OH}^-}$, where B is a general base or hydroxide ion. Catalysis was observed by all of the 26 different bases examined, many of which have no dissociable proton, and the k_3 terms account for most of the observed reaction above pH 9. Nucleophilic attack of the bases on DPIC is ruled out. The presence of the term in the rate law which is second order in base is evidence for a two-step mechanism. The mechanism proposed involves addition of solvent to DPIC to form a tetrahedral addition intermediate followed by rate-determining general base catalyzed breakdown of the intermediate to form AEF. Mechanistically, the observed general base catalysis in the k_3 reaction is shown to involve the general acid catalyzed breakdown of an anionic tetrahedral intermediate. Structure–reactivity correlations and comparison with the mechanism of imido ester aminolysis suggest that the mechanism of the k_2 reaction involves general base catalyzed breakdown of the conjugate acid of the neutral tetrahedral intermediate. The increased reactivity of bicarbonate ion and the greater reactivity of phosphate dianion than methyl phosphate dianion in the k_3 reaction suggest that bifunctional acid–base catalysis by these compounds is slightly more efficient than monofunctional catalysis.

The hydrolysis of 1,3-diphenyl-2-imidazolinium chloride (DPIC) to N-(2-anilinoethyl)formanilide (AEF) (eq 1) has been considered as a model for the reaction of



(1)

formyl derivatives of tetrahydrofolic acid, but the mechanism of the reaction has not been previously studied in detail.[2] The hydrolysis of the related N,N′-diarylformamidines has been found by DeWolfe to occur by pH-independent and hydroxide ion catalyzed pathways.[3] It was concluded from the effects of substituents that the pH-independent hydrolysis in alkaline solutions represents the reaction of hydroxide ion with the protonated amidine or its hydrate and it was pointed out that the nonlinear Hammett plot for these reactions could indicate a change in rate-determining step. These reactions were found to be subject to buffer catalysis but the mechanism of the buffer effects was not established.[3a]

The work reported here and in the accompanying paper was carried out in order to obtain further information on the mechanism and catalysis of the hydrolysis of amidines in general and on some reactions of formyltetrahydrofolates in particular.

Experimental Section

N-(2-Anilinoethyl)formanilide (AEF) was prepared by the method of Zienty,[4] and was recrystallized once from methanol and

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(3) (a) R. H. DeWolfe, *J. Am. Chem. Soc.*, **82**, 1585 (1960); (b) R. H. DeWolfe, *ibid.*, **86**, 864 (1964).