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Norcotinine (Desmethylcotinine) as a Urinary Metabolite of Nornicotine

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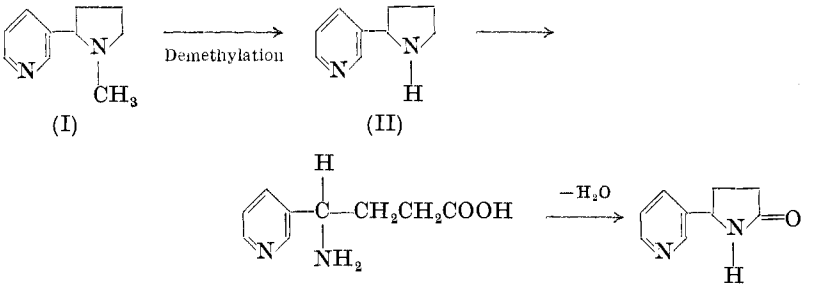
Previous studies[1] from this laboratory have shown that following administration of (−)-nicotine, the urine of dogs contains (+)-γ-(3-pyridyl)-γ-methylaminobutyric acid and its lactam, (−)-cotinine (2-(3-pyridyl)-1-methylpyrrolidone-5) and additional Koenig-positive components. (−)-Cotinine is metabolized further to a variety of Koenig-positive compounds including hydroxycotinine and norcotinine.[2,3] Since the latter arise from both cotinine and nicotine the abbreviated sequence: nicotine ⟶ cotinine ⟶ hydroxycotinine plus norcotinine, represents a metabolic route applicable to the dog. Preliminary evidence suggests that the metabolism of nicotine in man may also involve cotinine as an intermediate.[4]

The suggestion[5] that demethylation of nicotine (I) *in vivo* might provide a route to nornicotine (II) in animals evoked consideration[4] of an additional route to norcotinine (IV):



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Immediate experimental tests of this hypothetical scheme have necessarily involved only consideration of steps II through IV. Metabolic transformation of nicotine to nornicotine (I ⟶ II) has never been unequivocally demonstrated in animals although nicotine can serve[6] as a methyl donor in *Nicotiana tabacum*, and nicotine concentration decreases while nornicotine increases in the harvested leaves of some types of *Nicotiana*.[7]

(−)-Nornicotine was given by slow intravenous administration to anaesthetized dogs under conditions comparable to those employed in studies with nicotine.[8] Hucker and Larson,[9] using similar conditions, reported on the basis of spectrophotometric methods that approximately 33 per cent of the dose of nornicotine was excreted unchanged in the urine of dogs. Excreted nornicotine was isolated in the form of the dipicrate. Prior to the work of these authors, the fate of injected nornicotine and the metabolism of the compound *in vivo* received little or no experimental study.

In our experiments, the pooled urine from sixteen dogs, representing a total intravenous dose of 1·26 g of (−)-nornicotine, was extracted at pH 9 with chloroform. The chloroform solution contained two major Koenig-positive zones, R_f 0·62, R_f 0·76, and a minor Koenig-positive zone, R_f 0·84, upon paper chromatography with an ammonia–ethanol–butanol solvent. The R_f 0·62 and R_f 0·76 values corresponded approximately to those of norcotinine and nornicotine, respectively. The residue from the evaporation of the chloroform was then subjected to a series of chromatographic purifications. This residue was dissolved in water at pH 2 and placed on a column of Dowex 50 (H^+). An ammoniacal eluate of Koenig-positive material was obtained from the column and passed through Dowex 1 (OH^-). The effluent from the column of Dowex 1 (OH^-) after a preliminary treatment with decolorizing carbon was subjected to chromatography on alumina and then on paper. This led to the isolation of 7·1 mg of (−)-norcotinine, m.p. 131–133° after recrystallization from benzene.

In addition to norcotinine, the extracts of the urine yielded nornicotine which was isolated by chromatography on alumina and then characterized as the dipicrate. This isolation confirmed the results of Hucker and Larson[9] but only in a qualitative way

since our procedures were not designed for the total recovery of compounds with the volatility of nornicotine.

Currently available experimental evidence points to several routes important to the production of the lactam cotinine from nicotine *in vivo*: a direct conversion, without the necessity for the intermediate formation of γ-(3-pyridyl)-γ-methylaminobutyric acid, which can proceed enzymatically as indicated by the experiments of Hucker, Gillette and Brodie[10] or non-enzymatically as foreshadowed by studies *in vitro*,[11] and also by an indirect conversion involving lactamization of γ-(3-pyridyl)-γ-methylaminobutyric acid.[1, 8] It was of interest, therefore, to seek evidence for the presence of γ-(3-pyridyl)-γ-aminobutyric acid in the urine of dogs that had received nornicotine.

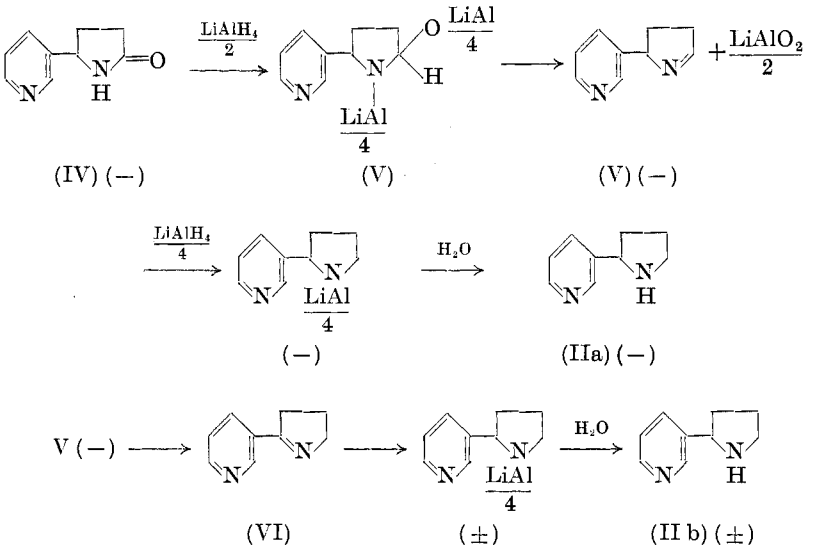
The aqueous phase remaining from the chloroform extraction of the urine of nornicotine-treated dogs was acidified and placed on Dowex 50 (H^+). A Koenig-positive eluate was obtained from this column with ammonia water and placed upon Dowex 1 (OH^-). An early fraction obtained by eluting the column of Dowex 1 (OH^-) with N acetic acid contained two Koenig-positive components. The R_f values of the component giving the most intense colour reaction following paper chromatography corresponded in R_f value to authentic γ-(3-pyridyl)-γ-aminobutyric acid.[12]

After an additional passage through Dowex 50 (H^+), the solids in the fraction were heated at 150° under nitrogen. The residue was treated with dilute aqueous ammonia and then extracted with chloroform. Upon evaporation, the chloroform yielded an oil which showed a single Koenig-positive zone corresponding in R_f value to authentic norcotinine.[3, 12] After chromatography on alumina, the oil afforded crystalline norcotinine which was purified by recrystallization and finally sublimation, and identified by infrared spectra and mixed melting-point determinations. This isolation of norcotinine establishes γ-(3-pyridyl)-γ-aminobutyric acid as a metabolite of nornicotine. In the light of the relationship between cotinine and γ-(3-pyridyl)-γ-methylaminobutyric acid which has already been established, it is reasonable to assume that γ-(3-pyridyl)-γ-aminobutyric acid can serve at least to some extent as a precursor of norcotinine *in vivo*.

Norcotinine obtained from the chloroform extraction of urine

or from the thermal lactamization of γ-(3-pyridyl)-γ-aminobutyric acid on the basis of melting-point data is the *laevo*-rotatory isomer. The relationships which have been established in the metabolism of nicotine suggested a conversion of (−)-nornicotine to (+)-γ-(3-pyridyl)-γ-aminobutyric acid and to (−)-norcotinine with retention of the absolute optical configuration of the administered (−)-nornicotine. Experimental confirmation of this relationship between nornicotine and its metabolites was afforded by reducing (−)-norcotinine with lithium aluminium hydride[12-14] to nornicotine. The nornicotine obtained from this reduction was optically active and *laevo*-rotatory in methyl alcohol. The full optical rotation of (−)-nornicotine was not obtained, however. The low rotation of the reduction product persisted in nornicotine which was regenerated from an analytically pure sample of nornicotine dipicrate. In contrast, the reduction of (−)-cotinine with lithium aluminium hydride readily afforded[3] (−)-nicotine with no evidence of racemization. Nornicotine is generally considered more susceptible to racemization than nicotine. Since, however, after the treatment with lithium aluminium hydride, (−)-nornicotine was recovered with unchanged optical rotation, other explanations must be sought for the low optical activity of nornicotine arising from the reduction of (−)-norcotinine.

N-Methylpyrrolidone-2 and *N*-methylpiperidone-2 yield, on reduction with 0·25 mole of lithium aluminium hydride, the corresponding ω-methylaminoaldehydes according to Galinovsky and coworkers,[15] and these aldehydes are considered to be in equilibrium with the corresponding cyclic alkylolamines. In analogous reductions of (−)-cotinine, i.e. 2-(3-pyridyl)-1-methylpyrrolidone-5, the intermediate 2-(3-pyridyl)-2-hydroxy-1-methylpyrrolidine would, in accordance with the mechanisms proposed by Gaylord and others[16] for related compounds, react further with a total consumption of 0·5 mole of lithium aluminium hydride to give (−)-nicotine with complete retention of optical activity. The reduction of (−)-norcotinine by the foregoing route would provide (−)-nornicotine in a similar fashion. Racemic nornicotine may, on the other hand, arise via the reactions:



These equations provide for the synthesis of both (−)-nornicotine (IIa) via the optically active 5-pyrroline (V), and of optically inactive nornicotine (IIb) via myosmine, i.e. optically inactive 2-pyrroline (VI). Substantiation of this scheme would involve isolation of some of the hypothetical intermediates and a consideration of the more sophisticated mechanisms for the reduction of lactams, imines, and amides which have been discussed by Gaylord.[16]

Experimental

Administration of (−)-nornicotine to mongrel dogs. (−)-Nornicotine (5 mg/kg) was administered over an 8-h period into the femoral vein of mongrel dogs (mixed sexes) under pentobarbital anaesthesia. Bladder urine was collected during administration and a subsequent 10-h period by an indwelling catheter.

Examination of urine for metabolites. The pooled urine of sixteen dogs, representing 1·26 g of (−)-nornicotine, was adjusted to pH 9·0 with ammonium hydroxide and then extracted continuously with chloroform for approximately 20 h. The chloroform extract was concentrated under diminished pressure to a dark brown oil (4·95 g). Paper chromatograms developed in

'base'* indicated the presence of three Koenig-positive components,† R_f 0·62, 0·76 and 0·84. The oil was shaken with 80 ml of water. After an adjustment to pH 2 with 4N HCl, the solution was filtered. The Koenig reaction of the filtrate was positive and that of the precipitate negative. The filtrate was placed on Dowex 50 (H^+). After a water wash, Koenig-positive material was eluted with N ammonium hydroxide. The eluate was placed on Dowex 1 (OH^-). The effluent and water washes were combined and concentrated under diminished pressure. The residue was dissolved in a small amount of methanol and then treated with decolorizing carbon. After removal of the solvent, the residue (0·813 g) was dissolved in chloroform and placed on an alumina column. The column was eluted with successive solutions of methanol–ether (5, 10, 20 and 60 per cent) and finally with 100 per cent methanol. The early fractions giving Koenig-positive zones at R_f 0·62 and 0·83 ('base') were evaporated to a dark brown oil (19 mg) which requires further study. The subsequent fractions, giving Koenig-positive material, R_f 0·27 ('acid'), 0·62 ('base') were combined. The last fractions contained Koenig-positive material, R_f 0·23 ('acid'), 0·76 ('base').

Isolation of norcotinine from urine. The fractions (R_f 0·27, 'acid') were concentrated to dryness and treated with a small volume of benzene. After filtration, the benzene solution was applied to Whatman No. 1 paper and chromatographed in 'acid'.

* Descending paper chromatography was conducted on Whatman No. 1 paper. For simplicity the solvent system 0·5N ammonium hydroxide (1 vol)–95 per cent ethanol (1 vol)–*n*-butanol (1 vol) is designated 'base', and the system *sec*-butyl alcohol (45 vol)–formic acid (8·4 vol)–water (6·6 vol) [Hausman, W., *J. Amer. chem. Soc.*, **74**, 3181 (1952)] is designated 'acid'.

† Koenig-positive components were located by a standard procedure. The dried chromatographic papers (Whatman No. 1) were sprayed with an alcoholic solution of *p*-aminobenzoic acid. The papers were then redried and exposed for several minutes in an atmosphere of cyanogen bromide. The formation of yellow, red or orange spots which takes place during this exposure is designated a positive Koenig reaction. A positive reaction under these conditions is generally considered evidence for the presence of pyridine compounds with an unquaternized nitrogen in the pyridine ring. Y. Okuda (*J. Biochem.*, **46**, 73, 1959) has reported that dried paper chromatograms of *N*-methylated pyridine, following a spray with *p*-aminobenzoic acid in a dilute alcoholic solution of HCl, produce a pink-red colour on exposure to cyanogen bromide vapours for 1 h. Samples of *N*-methylpyridinium iodide and *N*-ethylpyridium bromide, chromatographed on paper by our procedure, gave very weak colours after several minutes in the presence of *p*-aminobenzoic acid and exposure to cyanogen bromide.

The zone at R_f 0·27–0·29, located as a quenching area in ultraviolet light, was cut from the chromatograms and then extracted with methanol. The combined methanolic extracts yielded upon evaporation 7·1 mg of norcotinine as an oil which solidified on standing. The compound was recrystallized from benzene, m.p. 131–133°. The mixed melting point with authentic (−)-norcotinine[3] was 132–135°. The melting point was depressed to 110–114° by admixture with (+)-norcotinine. The R_f values of the compound, 0·27 ('acid') and 0·64 ('base'), corresponded to authentic (−)-norcotinine chromatographed under the same conditions. The infrared spectra[3] of isolated and authentic (−)-compound in chloroform showed no differences.

Isolation of nornicotine from urine. The fourth fraction from the alumina column (above) was concentrated to a brown oil. The oil was dissolved in chloroform and treated with decolorizing carbon. The oily residue remaining after evaporation of the solvent was dissolved in benzene and filtered. The filtrate gave upon concentration under diminished pressure 85·4 mg of oil with R_f values 0·23 ('acid') and 0·76 ('base') corresponding to authentic nornicotine. Upon treatment with a saturated aqueous solution of picric acid, the oil afforded crystalline nornicotine dipicrate which was recrystallized three times from water, m.p. 190–192° (83 mg). Admixture with authentic (−)-nornicotine dipicrate did not depress the melting point.

Identification of γ-(3-pyridyl)-γ-aminobutyric acid. One-half of the aqueous solution remaining after removal of the chloroform-soluble components (above) was acidified to pH 2 and placed on Dowex 50 (H^+). After a water wash, Koenig-positive eluates were removed by elution with N ammonium hydroxide. The ammoniacal solution was placed on Dowex 1 (OH^-). After a water wash the column was eluted with N acetic acid. The first fraction contained components giving a faint Koenig-positive zone at R_f 0·28 upon paper chromatography with 'acid' and a strong Koenig-positive zone at R_f 0·14 ('acid') and R_f 0·11 ('base') corresponding approximately to values obtained with authentic γ-(3-pyridyl)-γ-aminobutyric acid. The second fraction contained, in addition to these two components, three other Koenig-positive zones, R_f 0·08, 0·13, and 0·20 ('base'). This fraction was not investigated further.

The first fraction was placed on a column of Dowex 50 (H^+). After a water wash the column was eluted with N ammonium hydroxide. The residue obtained by removing the solvent from the eluate was treated with 5 ml of methanol. The methanolic solution was filtered and evaporated to a gummy residue (100 mg). The latter was heated to 150° in an atmosphere of nitrogen for 10 min. The resultant brown oil was dissolved in water. After alkalinization with ammonium hydroxide, the solution was extracted several times with chloroform. The combined chloroform extracts were dried over anhydrous sodium sulphate and filtered. The solution upon evaporation afforded 35 mg of orange yellow oil, R_f 0·23 ('acid') and R_f 0·62 ('base'), corresponding in value to authentic (−)-norcotinine chromatographed on Whatman No. 1 paper under the same conditions. The oil was dissolved in chloroform and placed on an alumina column. The column was eluted with ether containing successively 5, 10 and 20 per cent methanol. Fractions containing the higher concentrations of methanol which gave a positive Koenig reaction were concentrated to an oil (12 mg) which solidified upon standing. The crude (−)-norcotinine was recrystallized twice from benzene and finally purified by sublimation at 0·5 mm. The colourless crystals (7·2 mg) melted at 131–133°. Admixture with authentic (−)-norcotinine did not depress the melting point. The melting point was depressed to 112–114° by admixture with (+)-norcotinine. The isolated (−)-norcotinine in chloroform solution gave an infrared absorption spectrum which did not differ from that obtained with an authentic sample.

Reduction of (−)-norcotinine to nornicotine. A solution of synthetic (−)-norcotinine (4·5 g) in dry tetrahydrofuran (200 ml) was refluxed with lithium aluminium hydride (1·6 g) for 40 h. After decomposition of excess lithium aluminium hydride with acetone, the mixture was added to 5N sodium hydroxide (100 ml) and extracted with three portions of ether. The combined ether extracts were dried (Na_2SO_4 anhyd.) and evaporated to a brown oil. The latter was dissolved in ether and placed on a column of acid-washed alumina (100 g). The column was eluted with ether and 10 per cent methanolic ether. The fraction obtained with 10 per cent methanolic ether was concentrated to a brown oil; yield, 1·93 g. Upon treatment with a saturated aqueous solution

of picric acid, the latter yielded nornicotine dipicrate which was recrystallized from water and methanol, m.p. 189–189·5° (yield, 1·5 g). The mixture melting point with authentic (−)-nornicotine dipicrate (m.p. 190–191°) was 189–190·5°.

Anal. Calcd. for $C_{21}H_{18}N_8O_{14}$: C, 41·59; H, 2·99; N, 18·48. Found: C, 41·94; H, 3·03; N, 18·4.

The synthetic nornicotine dipicrate was decomposed with a small volume of N HCl, and the mixture extracted with benzene. The aqueous layer was made alkaline with ammonium hydroxide and extracted with ether. The concentrated ether extracts were dried over anhydrous sodium sulphate, and the filtrate was concentrated to dryness. The resultant oil, after drying under diminished pressure to remove residual water, weighed 0·19 g. A solution in chloroform was placed on a column of acid-washed alumina. The column was developed with ether containing 5 per cent methanol and subsequently 10 per cent methanol to give Koenig-positive fractions which were combined and concentrated. The residual nornicotine was a yellow oil; yield, 0·16 g. Upon re-chromatography on alumina, the nornicotine weighed 0·13 g, $[\alpha]_{5461}^{24·5} - 20·6°$ (*c* 2·8, in methanol). A pure sample of authentic (−)-nornicotine, $[\alpha]_{5461}^{24·5} - 48·0°$ (*c* 2·75, in methanol), co-chromatographed on paper with the isolated sample in both the acidic and basic solvent systems.

Authentic (−)-nornicotine in tetrahydrofuran was treated with lithium aluminium hydride under the same conditions employed in the reduction of (−)-norcotinine. After decomposition of the excess lithium aluminium hydride, (−)-nornicotine was isolated after chromatography on alumina with unchanged optical rotation, $[\alpha]_{5461}^{24·5} - 48·0°$ (*c* 2·75, in methanol).

Summary. Following intravenous administration of (−)-nornicotine, the dog excretes in the urine a variety of Koenig-positive compounds. A chloroform extract of the alkalinized urine contained norcotinine, nornicotine and other components. The aqueous phase remaining from the chloroform extract contained γ-(3-pyridyl)-γ-aminobutyric acid, which was lactamized to (−)-norcotinine [2-(3-pyridyl)-pyrrolidone-5]. Reduction of optically active norcotinine with lithium aluminium hydride resulted in the formation of a mixture of optically active and optically inactive nornicotine. Possible routes to the inactive product are discussed.

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References

[1] McKennis, H., Jr., Turnbull, L. B. and Bowman, E. R. *J. Amer. chem. Soc.*, **80**, 6597 (1958)

[2] McKennis, H., Jr., Turnbull, L. B. and Bowman, E. R. *Abstracts of Communications*, IV International Congress of Biochemistry, Vienna, Austria, September 1–6, 1958, p. 184

[3] McKennis, H., Jr., Turnbull, L. B., Bowman, E. R. and Wada, E. *J. Amer. chem. Soc.*, **81**, 3951 (1959)

[4] Bowman, E. R., Turnbull, L. B. and McKennis, H., Jr. *J. Pharmacol.*, **127**, 92 (1959)

[5] Werle, E., Schievelbein, H. and Spieth, D. *Arzneimittel-Forsch.*, **6**, 322 (1956)

[6] Leete, E. and Beil, V. M. *J. Amer. chem. Soc.*, **81**, 4358 (1959)

[7] Griffith, R. B., Valleau, W. D. and Stokes, G. W. *Science*, **121**, 343 (1955); Jeffrey, P. N. *Tobacco Science*, **3**, 89 (1959); Wada, E. *Arch. Biochem. Biophys.*, **62**, 471 (1956)

[8] McKennis, H., Jr., Turnbull, L. B. and Bowman, E. R. *J. Amer. chem. Soc.*, **79**, 6342 (1957)

[9] Hucker, H. B. and Larson, P. S. *J. Pharmacol.*, **123**, 259 (1958)

[10] Hucker, H. B., Gillette, J. R. and Brodie, B. B. *Nature, Lond.*, **183**, 47 (1959)

[11] Frankenburg, W. G. and Vaitekunus, A. A. *J. Amer. chem. Soc.*, **79**, 149 (1957)

[12] McKennis, H., Jr., Turnbull, L. B., Wingfield, H. N., Jr. and Dewey, L. J. *J. Amer. chem. Soc.*, **80**, 1634 (1958)

[13] Sugasawa, S., Tatsuno, T. and Kamiya, T. *Pharm. Bull. (Tokyo)*, **2**, [1], 39 (1954)

[14] Zymalkowski, F. and Trenktrog, B. *Arch. Pharm., Berl.*, **292**, 9 (1959)

[15] Galinovsky, F., Wagner, A. and Weiser, R. *Mh. Chem.*, **82**, 551 (1951)

[16] Gaylord, N. G. *Reduction with Complex Metal Hydrides*. 1956. New York; Interscience Publishers