Phytochemistry, 1966, Vol. 5, pp. 293 to 300. Pergamon Press Ltd. Printed in England

PHYTOCHEMICAL STUDIES ON THE TOBACCO ALKALOIDS—X.

DEGRADATION OF THE TOBACCO ALKALOIDS AND THEIR OPTICAL ROTATORY CHANGES IN TOBACCO PLANTS

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(*Received* 17 *July* 1965)

Abstract—Myosmine(1,2-dihydronornicotine) was isolated from *Nicotiana glutinosa* at the flowering stage and was proved to be a degradation product of (−)-nornicotine by feeding the latter to tobacco plants grafted onto tomato stock. 1,2-Dehydroanabasine was also found to be a degradation product of anabasine in tobacco leaves. Racemic nornicotine, anabasine and 1-(3-pyridyl)-1-ethylaminoethane which have a similar 3-pyridyliminomethane skeleton were optically activated in tobacco leaves. It can be considered that the first step of the degradation of secondary amine tobacco alkaloids is a stereospecific dehydrogenation. From the findings made here and the previous observation on the optical rotation of secondary amine tobacco alkaloids, it was inferred that such secondary amine tobacco alkaloids as anabasine, anatabine and some nornicotine in the root are biosynthesized in a racemic form in tobacco plants.

INTRODUCTION

THE metabolism of tobacco alkaloids in the plant is still an unsolved aspect of plant biochemistry, whereas that of nicotine in animals and micro-organisms has been studied well. In animals and insects, the pyrrolidine ring of nicotine is first oxidized to pyrrolid-5-one and in micro-organisms this is accompanied by either a conversion of the pyridine ring to pyrid-6-one or dehydrogenation of the pyrrolidine ring to Δ^2-pyrroline. In plants, nicotine may first be demethylated to nornicotine in some types of tobacco and the reaction has been confirmed by isotopic experiment[1] in the cultivated tobacco plant, "Bright Yellow" which produces only nicotine. It can thus be assumed that demethylation of nicotine occurs in all *Nicotiana* plants.

In order to determine the further metabolism of nicotine, nornicotine was first investigated. As the second objective the metabolic products have been examined from other secondary amine tobacco alkaloids, which are always found in any tobacco plant, and especially the preferential degradation of their optical antipodes.

RESULTS

Isolation of Myosmine and its Identification

Extracts from three different harvests of *N. glutinosa*, which is a wild species producing mainly nornicotine, were examined by the procedure described in Experimental, and myosmine (1,2-dehydronornicotine) was isolated only from the second harvest (the flowering stage). Infrared and u.v. spectra, and elemental analyses of the isolated sample were identical

[1] T. KISAKI and E. TAMAKI, *Agr. Biol. Chem.* **28**, 492 (1964).

to those of an authentic specimen. From the other two harvests, nicotine was obtained from the fraction corresponding to that containing myosmine. This appears to suggest that myosmine is not biologically inert.

In order to investigate the origin of myosmine, (−)-nornicotine was introduced to undetached alkaloid-free plants of *N. tabacum* "Cherry Red", prepared by grafting, through cotton inserted into the stalk, and the plant examined after 2 weeks. Myosmine was readily isolated whereas no Koenig positive substance was detected in a control plant. The product had a single peak at a relative retention time of 2·52 (nicotine = 1·0) in gas chromatography,[2] identical with that of myosmine, and its u.v. and i.r. spectra were also identical with the pure substance. Elemental analysis of the picrate of the product was identical with the hydrolytic form[3] of myosmine(1-(3-pyridyl)-4-amino-1-butanone).

Degradation of Myosmine in Tobacco Leaves

As can be seen in Fig. 1, myosmine was further degraded in both nicotine type tobacco leaves (Bright Yellow) and nornicotine type tobacco leaves (Cherry Red). When large

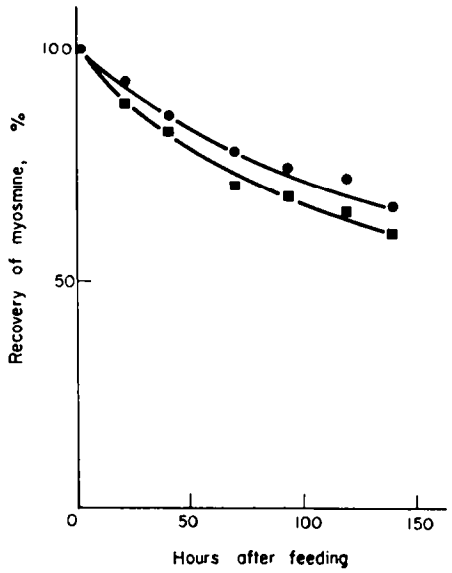

FIG. 1. TIME COURSE OF MYOSMINE DEGRADATION IN EXCISED LEAVES OF *Nicotiana tabacum*, "BRIGHT YELLOW" AND "CHERRY RED".

With each harvest, 5 mg of myosmine was fed to two leaves (fresh weight, approximately 20 g) according to Procedure B. ● = "Bright Yellow", ■ = "Cherry Red".

amounts of myosmine (2 g/kg of fresh weight) were fed to the stem by Procedure A (see Experimental) and the plants examined 2 weeks later, three Koenig positive transformation products were detected by paper chromatography. One of the products was chromatographically identical with nicotinic acid (cf. Griffith *et al.*[4]) and it might be assumed that

[2] Y. KOBASHI, *J. Chem. Soc. Japan* **82**, 1262 (1961).
[3] P. G. HAINES, A. EISNER and C. F. WOODWARD, *J. Am. Chem. Soc.* **67**, 1258 (1945).
[4] G. P. GRIFFITH, T. GRIFFITH and R. U. BYERRUM, *J. Biol. Chem.* **235**, 3536 (1960).

myosmine is also further metabolized to this compound. However, it was observed that its administration caused necrosis on leaves. It is interesting that the nornicotine type tobacco (Cherry Red) grown in the field often shows necrotic symptoms on leaves near the harvesting stage, when conversion of nicotine to nornicotine was taking place vigorously.[5]

Identification of Anabaseine

Anabaseine (1,2-dehydroanabasine) was detected as a degradation product of (±)-anabasine fed to excised leaves of *N. tabacum* "Cherry Red". A small amount of brown oily substance was obtained which gave only one peak at a relative retention time of 4·07 (nicotine = 1·0) by gas chromatography identical with that of authentic anabaseine. Its u.v. spectrum showed absorptions at 229 mμ and 262 mμ in methanol identical with the pure substance. This compound rather readily polymerizes to a resinous brown substance after standing at room temperature for several days, and no i.r. spectrum could be obtained.

Preferential Degradation of the Optical Antipodes of Alkaloids

In a previous report,[6] it has been shown that (±)-nornicotine fed to excised tobacco leaves was not optically activated in a short feeding period. As shown in Table 1, however, (±)-nornicotine which was introduced to intact *N. tabacum* "Cherry Red", through the cotton inserted into the stalk was optically activated to become dextrorotatory after a long feeding period, although only to a slight extent. The result with a feeding experiment of either radioactive (−)- or (+)-nornicotine (Fig. 2) supports the above interpretation. Therefore, such a scheme as Fig. 3 can be postulated as a metabolic pathway of nicotine.

TABLE 1. OPTICAL ACTIVATION OF (±)-NORNICOTINE FED TO *Nicotiana tabacum*, "CHERRY RED"

Culture period (weeks)	Optical rotation ($[\alpha]_D^{24}$)
2	+0·98°
2	+0·73°
4	+2·62°
4	+4·42°

Two g of (±)-nornicotine dissolved in 50 ml (pH = 6·0) was administered to each of four grafted plants with 9–11 leaves, according to Procedure A.

Both (±)-anabasine, which is generally found in a small quantity in all species of *Nicotiana*, and synthetic (±)-1-(3-pyridyl)-1-ethylamino ethane, which has a structure similar to tobacco alkaloids, were also optically activated in tobacco leaves (Table 2). The metabolic fate of the secondary amine alkaloids in plants has been little studied except by Tso and Jeffrey.[7] Optical activation seems to indicate that, in general, these alkaloids are degraded stereospecifically.

[5] Private communication from Dr. S. MATSUYAMA, Kagoshima Tobacco Experimental Station, Japan Monopoly Corporation.
[6] T. KISAKI and E. TAMAKI, *Arch. Biochem. Biophys.* **94**, 2321 (1961).
[7] T. C. TSO and R. N. JEFFREY, *Arch. Biochem. Biophys.* **80**, 46 (1959).

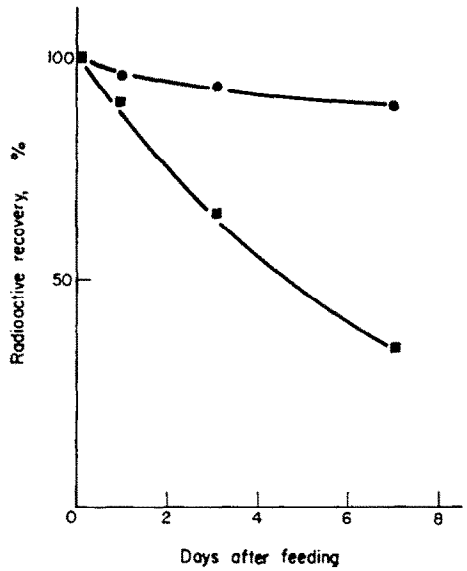

FIG. 2. TIME COURSE OF NORNICOTINE DEGRADATION IN EXCISED LEAVES OF *Nicotiana tabacum*, "CHERRY RED".

With each harvest, either (−)-nornicotine-^{3}H, $3{\cdot}41 \times 10^6$, 4·6 μM or (+)-nornicotine-^{3}H, $1{\cdot}56 \times 10^5$ disintegrations/min, 8·3 μM was fed to two leaves (fresh weight, approximately 20 g) according to Procedure B. ● = (+)-nornicotine, ■ = (−)-nornicotine.

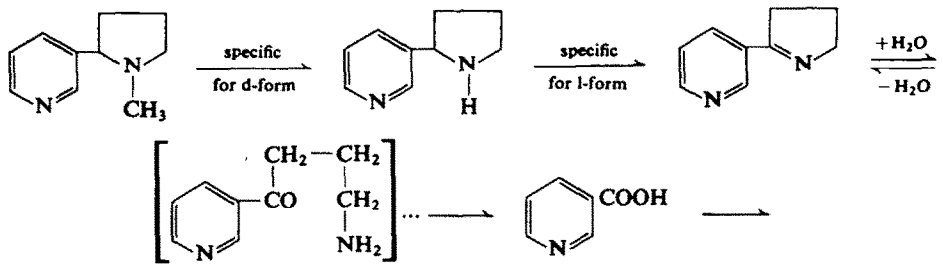

FIG. 3. POSTULATED METABOLIC PATHWAY OF NICOTINE.

TABLE 2. OPTICAL ACTIVATION OF SECONDARY AMINE ALKALOIDS IN EXCISED LEAVES (*Nicotiana tabacum*, "CHERRY RED")

Alkaloid fed (g)	Culture period (hr)	Dry weight (g)	Recovery of alkaloid (%)	$[\alpha]_D^{24}$ of recovered alkaloid
(±)-Anabasine 1·128	116	10·065	91·5	−2·08°
(±)-Anabasine 0·835	164	8·076	89·5	−2·90°
(±)-1-(3′-pyridyl)-1-ethylamino-ethane 0·335	182	8·332	75·9	+4·30°

Six excised leaves were employed for each experiment. Feeding was done according to Procedure B.

Optical Rotatory Changes of Anabasine in Nicotiana glauca

Piriki[8] has reported that anabasine obtained from the various samples of *N. glauca* were racemized over a wide range of $[\alpha]_D = 0 \sim -82°$. He attributed the variance of optical rotation to ecological conditions such as soil and climatic conditions of cultivation. Investigation of the optical rotation of anabasine in *N. glauca* showed that anabasine is dextrorotatory in the leaves but levorotatory in the root, and its optical rotation varied according to maturity. The reason for the direction of the optical rotation of anabasine to be different according to the plant part is unknown.

TABLE 3. OPTICAL ROTATION OF ANABASINE ISOLATED FROM THE VARIOUS PARTS OF *Nicotiana glauca* AT THE VARIOUS STAGES

Plant part	Optical rotation, $[\alpha]_D^{24}$		
	88 day*	115 day*	145 day*
Root	−2·09°	−0·23°	−3·91°
Stalk	−2·13°	+0·28°	−1·12°
Leaf	−1·02°	+1·36°	+2·12°

* Days after germination.

DISCUSSION

Biological Significance of Myosmine

Fairbarn and Suwal[9] have suggested that γ-coniceine might be interconverted to coiine and participate in oxidation-reduction reactions in *Conium maculatum* L. No reversible conversion from myosmine to the corresponding dihydro-compound, nornicotine, was observed in tobacco leaves, and it can be considered that myosmine is only a degradation product. Neither was anabaseine(1,2-dehydroanabasine) reconverted to anabasine in tobacco leaves.

Kaplan and his group[10] have reported that the nicotinamide moiety of NAD can be replaced by certain pyridylketone derivatives. Fukuzumi *et al.*[11] have shown that nicotinamide moiety of NAD could be enzymically replaced with tobacco alkaloid derivatives and, above all, the modified nucleotide could still work as a hydrogen acceptor of isocitric dehydrogenenase from pig heart, nitrate reductase from soy bean leaves and other enzymes. Since myosmine is hydrolyzable and converts to a 1-(3-pyridyl)-4-amino-1-butanone,[3] the "NAD" which is replaced with this 3-pyridyl ketone also can act as a cofactor for oxidative enzymes. In this sense, it seems probable that myosmine may take part in a reduction-oxidation reaction in tobacco plants.

Optical Rotation of the Alkaloids and their Biogenesis

In a previous paper,[12] it has been described that nornicotine isolated from roots of *N. tabacum* was mainly in the (+) form. The presence of (+)-predominant nornicotine

[8] C. PIRIKI, *Bull. Inform. CORESTA* No. 3, 9 (1958).
[9] J. W. FAIRBARN and P. N. SUWAL, *Phytochem.* 1, 38 (1961).
[10] B. M. ANDERSON, C. J. CIOTTI and N. O. KAPLAN, *J. Biol. Chem.* 234, 1219 (1959); B. M. ANDERSON and N. O. KAPLAN, *J. Biol. Chem.* 234, 1226 (1959).
[11] T. FUKUZUMI, H. TAKAHARA and K. ARAI, *Sci. Papers Central Res. Inst. Japan Monopoly Corp.* No. 106, 63 (1964).
[12] T. KISAKI and E. TAMAKI, *Naturwissenschaften* 47, 540 (1960).

described above can be interpreted as being due to preferential degradation of the (−)-form in the roots. This leads to the conclusion that nornicotine is first synthesized in the root in a racemic form. In a previous paper,[13] it was observed that nornicotine isolated from excised *Nicotiana rustica* root culture was almost optically inactive. This also appears to support the proposed conclusion on the biosynthesis of nornicotine.

Nicotine isolated from the bleeding sap from tobacco roots, which was considered as freshly synthesized, showed the same optical rotation as pure (−)-nicotine ($[\alpha]_D = -168{\cdot}5°$). Therefore, it should be considered that nicotine is stereospecifically biosynthesized in a (−)-form.

Tso and his group[14] have described nornicotine as a direct precursor of nicotine. However, as described above, there appears to be no steric connection between nornicotine and nicotine obtained from the root which is a site of alkaloid biogenesis,[15] and as was proposed earlier[13] these findings seem to indicate that nicotine is not derived from nornicotine.

Anatabine(4,5-dehydroanabasine) isolated from tobacco leaves showed a higher optical rotatory power ($[\alpha]_D = -98{\cdot}15°$) than that from tobacco roots ($[\alpha]_D = -9{\cdot}98°$)[16] which is a site of its biosynthesis ((−)-anatabine has an optical rotation, $[\alpha]_D = -167{\cdot}5°$[17]). This indicated that anatabine may be biosynthesized as a racemate and then optically activated. Therefore, it might be suggested that all the secondary amine tobacco alkaloids are synthesized in a racemic form, excluding any nornicotine secondarily produced from nicotine.

As observed with *Nicotiana*, it has been reported that nicotine and anabasine isolated from *Duboisia myopoloide* were a pure (−)-form and a racemic form ($[\alpha]_D = -0{\cdot}44°$), respectively.[18] It has been known that many kinds of optically impure alkaloids having various optical rotatory activities occur in alkaloid-bearing plants. Such optical rotatory variance of alkaloids might be speculatively interpreted by a preferential diminution of an optical antipode of the alkaloids which was biosynthesized as a racemic form.

EXPERIMENTAL

Plant Materials

Both *Nicotiana glutinosa* and *Nicotiana glauca* were cultivated in a usual manner at the field (Utsunomiya Tobacco Experimental Station) from early May to late July in 1960. Both *Nicotiana tabacum* "Cherry Red" and "Bright Yellow" free of alkaloids were prepared by grafting onto tomato stock as described in a previous paper.[19] Their sixth and seventh leaves from the grafted position which were grown for about three months after grafting, were employed for Experiments in Procedure B.

Chemicals

(−)-Nornicotine and (±)-nornicotine,[6, 19] and (±)-anabasine and (±)-1-(3-pyridyl)-1-ethylaminoethane were prepared previously.[20] Myosmine was prepared by the same method as described by Woodward *et al.*[21] Anabaseine was kindly supplied by Dr. Yamamoto, Laboratory of Pesticide Chemistry of Tokyo Agricultural University.

[13] S. MIZUSAKI, T. KISAKI and E. TAMAKI, *Agr. Biol. Chem.*, **29**, 714, (1965).
[14] B. LADESIC and T. C. TSO, *Phytochem.* **3**, 541 (1964).
[15] R. F. DAWSON, *Advan. Enzymol.* **8**, 203 (1948).
[16] E. WADA, T. KISAKI and M. IHIDA, *Arch. Biochem. Biophys.* **80**, 258 (1959).
[17] E. SPÄTH and F. KESZTLER, *Ber. Deut. Chem. Ges.* **70**, 239 (1937).
[18] P. I. MORTIMER and S. WILKINSON, *J. Chem. Soc.* 3967 (1957).
[19] T. KISAKI and E. TAMAKI, *Arch. Biochem. Biophys.* **92**, 351 (1961).
[20] T. KISAKI and E. TAMAKI, *Agr. Biol. Chem.* **28**, 492 (1964).
[21] C. F. WOODWARD, A. EISNER and P. G. HAINES, *J. Am. Chem. Soc.* **66**, 911 (1944).

Tracers

(+)- and (−)-[U-^{3}H] Nornicotine were prepared in the following way. A mixture of 100 mg of cold (±)-nicotine and 2·5 mc of-[U-^{3}H] nicotine (purchased from Radiochemical Centre, Amersham, England) was given to excised alkaloid-free leaves of Cherry Red according to the Procedure B. After one week, the leaves were harvested and were subjected to steam distillation to separate the alkaloid. The steam distillate was divided into two halves. After 70 mg of (−)- and (+)-nornicotine* were added to the two distillates as carriers, respectively, the transformed radioactive nornicotine was extracted with chloroform. Following two chromatographic purifications on alumina columns, the nornicotine thus purified was converted to its perchlorate and repeatedly recrystallized to a constant melting point (185°) from methanol–ether. Radiochemical purity was checked with a liquid scintillation counter by counting a paper chromatogram cut centimeter by centimeter. The activity of (−)-nornicotine was $9{\cdot}84 \times 10^7$ disintegration/min, 19·6 mg and that of (+)-nornicotine was $2{\cdot}38 \times 10^6$ disintegration/min, 18·8 mg.

Experimental Procedure

(*a*) *Procedure A.* To feed chemicals to the undetached plants, the stem of an alkaloid-free plant was bored 5 mm in diameter, 5 cm above from the grated position, and wet absorbent cotton was inserted. Then 50 ml (adjusted with acetic acid to pH = 6·0) containing 1–2 g of alkaloids with a small amount of dihydrostreptomycin were introduced into plants (approximately 3 months old *N. tabacum* "Cherry Red"), through the wick. Administration of the feeding solution was accomplished within 12 hr. After the culture period, the whole part of the plant was harvested, and the juice was immediately obtained by maceration. About 400 ml (per plant) of the plant juice thus obtained was heated for 10 min in a boiling water bath, cooled, filtered, the filtrate basified, and the alkaloids extracted with chloroform.

(*b*) *Procedure B.* The feeding procedure to excised tobacco leaves was similar to the method described in the previous paper.[19] The harvested samples were freeze-dried and used for analysis.

Isolation Procedure of Myosmine and Anabaseine

(*a*) *Myosmine from* Nicotiana glutinosa. Leaves from 120 plants of *Nicotiana glutinosa* were harvested at three periods (88, 115 and 145 days after germination) and each harvest was extracted with 70% methanol at room temperature. The methanol extract was concentrated *in vacuo*. The concentrate was subjected to chloroform extraction after alkalinization. The alkaloids obtained were transferred to aq. HCl, from which they were re-extracted with chloroform after basification. The alkaloid obtained was chromatographed on an alumina column (5 × 25 cm). The ether eluate (300 ml) was concentrated to dryness to yield 0·8 g of orange crystals, which on distillation *in vacuo* (b.p. 135°/10 mm) gave 0·45 g of white crystals. (Found: C, 73·90; H, 6·96; N, 19·20) Calc. for $C_9H_{10}N_2$: C, 73·94; H, 6·90; N, 19·16%.

(*b*) *Myosmine from* N. tabacum *fed* (−)-*nornicotine.* The alkaloid was extracted with chloroform from the plant juice of four plants which were grown for four weeks after administration of 1 g of (−)-nornicotine by Procedure A. The alkaloids were extracted with aqueous HCl and such alternative extractions were repeated twice more to remove impurities. Finally, the acidic aqueous solution was adjusted to pH = 5·0 and shaken with ether several times.

* (+)-Nornicotine was prepared as described previously.[20]

The combined ether extracts were dried (Na_2SO_4) and gave 30 mg of oil. After vacuum distillation of the oil, the distillate was converted to its picrate, m.p. 185°. (Found: C, 40·58; H, 3·51; N, 17·81) Calc. for $C_{21}H_{20}N_8O_{15}$: C, 40·49; H, 3·23; N, 17·95%.

(*c*) *Anabaseine from Leaves of* N. tabacum *fed* (±)-*anabasine. N. tabacum*, "Cherry Red" leaves were fed with (±)-anabasine by Procedure B and were cultured under the experimental condition as described in Table 2. According to the isolation Procedure (b) of myosmine, a very small amount of brown oily substance was obtained.

Analytical Procedure

Quantitative determination of alkaloids was made by the u.v. spectrophotometric method.[22,23] Absorptivities used for calculation were A = 38·7 at λ_{max} = 259 mμ for nornicotine, A = 32·6 at λ_{max} = 259 mμ for anabasine, A = 32·5 at λ_{max} = 259 mμ for 1-(3-pyridyl)-1-ethylaminoethane and A = 34·5 at λ_{max} = 260 mμ for myosmine in acidic aqueous solution.

Isolation and purification of alkaloids for optical rotatory measurement were described previously.[19,24] The samples fed with tracers were steam-distilled from alkaline solution, an aliquot of the distillate was dried in a glass vial, and assay samples were prepared by adding 0·5 ml of Hyamine-10 and 10 ml of toluene scintillator solution.[25] Assay of radioactivity was carried out with Packard Tri-Carb 3002 liquid scintillation spectrometer. Gas chromatography was carried out with a Perkin Elmer Model 188, triple-stage vapor fractometer according to the method of Kobashi.[26] Paper chromatography was carried out in the same manner described previously.[27] Ultraviolet spectra were obtained with a Hitachi EPS 2U spectrophotometer. Elemental analyses were run at the Laboratory of Microanalysis, Faculty of Agricultural Chemistry, Tokyo University. Polarimetry of alkaloids was described previously.[19] Infrared spectra were obtained with a Nihon Bunko IRS IR spectrophotometer.

Acknowledgement—Authors wish to acknowledge preparation of plant materials employed in this work to the staff of Utsunomiya Tobacco Experimental Station and carrying out gas chromatography of tobacco alkaloids to Dr. Y. Kobashi.

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[24] T. KISAKI and E. TAMAKI, *Arch. Biochem. Biophys.* **94**, 252 (1961).
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[27] T. KISAKI and E. TAMAKI, *Bull. Agr. Chem. Soc. Japan* **38**, 549 (1964).