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Nicotine Workshop

arranged by

the Medical Advisory Board of the Swedish Tobacco Company, in Stockholm, in November 1974

A workshop on problems related to the analysis of nicotine and nicotine metabolites in body fluids at levels pertinent to the human situation was held in November 1974 in Stockholm. It was organized by C. Enzell, B. Holmstedt and Å. Pilotti at the request of the Medical Advisory Board of the Swedish Tobacco Company.

The goal of the workshop was to summarize the present state of art in the area outlined by the organizers and to discuss critically the advantages and limitations of the different analytical methods available today.

Eleven experts in the field of metabolism, detection and biosynthesis of nicotine and related compounds were therefore invited to present papers on these topics and to participate in the discussions. All speakers invited were able to attend and the papers were arranged in the following groups:

1. *Metabolism of nicotine*

Speakers: H. Schievelbein
A. K. Armitage
H. McKennis, Jr.
E. Boyland
J. W. Gorrod

2. *Biosynthesis of minor tobacco alkaloids*

Speaker: E. Leete

3. *Detection of nicotine in body fluids*

Speakers: H. van Vunakis
D. M. Turner
L. Neelakantan
C. Feyerabend
E. C. Horning

Each speaker had one hour and a half at his disposal which included the discussion which, due to the informal atmosphere and the small number of participants, was very lively and fruitful.

The papers read at this workshop comprise a very valuable coverage of recent research in the fields of metabolism of nicotine and minor tobacco alkaloids, and of the various methods available for detection of these alkaloids. The abstracts are given below, while full papers, now edited by Å. Pilotti, can be obtained on request from C. Enzell of the Swedish Tobacco Company*.

Absorption of Nicotine under Various Conditions (an introductory review)

by H. Schievelbein

Institut für Klinische Chemie, Deutsches Herzzentrum München, Munich, Germany

Metabolism

After absorption into the organism, nicotine is rapidly metabolized to various compounds. One aspect of nicotine metabolism seems to be of special interest, namely hydroxylation of nicotine by microsomal enzymes and induction of these enzymes. It is known that the concentration of hydroxylating enzymes may be increased by treatment with phenobarbital. This compound and its derivatives are widely used in therapeutics. *Stålhandske* (1) showed that pre-treatment with phenobarbital increased the metabolism of nicotine in the liver of mice and increased significantly the LD_{50} of nicotine in mice.

Earlier investigations by *Yamamoto* and co-workers (2) had shown that pre-treatment with nicotine increased the activity of rat liver 2-acetaminofluorene-hydroxylase and that hydroxylation of benzo(a)pyrene may be stimulated by pre-treatment with benzo(a)pyrene and nicotine.

These findings were confirmed by *Welch* and co-workers (3, 4), who extended their investigations with regard to the application of cigarette smoke and the investigations on humans. Exposure to cigarette smoke increased the hydroxylation of benzo(a)pyrene in the organs of rats, especially in the lungs. The activity of benzo(a)pyrene-hydroxylase in the placenta of women who smoke increases in comparison to the activity of the enzyme in the placenta of women who do not smoke. These results suggest an interaction of tobacco smoke constituents with regard to their toxicity and their possible carcinogenic or co-carcinogenic potency.

* P.O. Box 17007, S — 10462 Stockholm, Sweden.

Estimation

A survey is given on the development of methods for the estimation of nicotine, beginning with early biological methods for the study of nicotine metabolism in *in vitro* assays and leading up to modern methods such as gas-liquid chromatography. The author's own results with regard to the concentration of nicotine in the blood of smokers are presented, which are in the ng/ml range and correspond well with results of other investigators, some of whom work with modern, very sensitive and specific methods, such as mass spectrometry and radioimmunoassay.

Absorption after Oral Application of Nicotine

The absorption of nicotine after oral application in dogs depends on the pH of the solution used. The nicotine concentration in the blood is much higher after application of a nicotine solution of pH 10.2 than after application of a solution of pH 7.4. This pH dependence can be shown also by measuring the rise in blood pressure after application of nicotine solutions with different pH values.

Oral application of suspensions of "total particulate matter" (condensate/TPM) with nicotine at both pH levels show greatly enhanced absorption at higher pH values than after application of nicotine alone. Oral application of nicotine and nicotine + TPM at different pH values and measurement of the increase in arterial blood pressure after administration of the same dose produce a clearly defined absorption pattern. The percentage numbers of responses were at pH 10: 86, pH 8: 67, pH 7.5: 25, and at pH 6.5: nearly 0 (mean values from nicotine and nicotine + TPM). There is a practical implication in the findings of these investigations [*Schievelbein* and co-workers (5, 6)]: As the pH value of the aqueous part of cigarette smoke does not exceed a pH value of 6, merely puffing a cigarette seems to be a subliminal stimulus with regard to pharmacological effects.

Absorption after Inhalation of Cigarette Smoke

In Table 1 the mean rise in blood pressure in dogs after inhalation of smoke from two different brands of cigarettes is registered. It seems that the rise in blood pressure may be in fact dependent upon the concentration of nicotine in the smoke (nicotine content of the two brands registered in Table 1 can be obtained from Table 2).

Table 1. Mean rise in blood pressure of dogs after spontaneous respiration of tobacco smoke (from about 275 mg of tobacco) from different cigarette brands [n = 8].

Type of cigarette	Body weight (kg)	Number of puffs	Rise in blood pressure (mm Hg)
Virginia	7	10	22
Very light German blend	7	13	12

To exclude the influence of spontaneous reactions the experiment was repeated using the "black type" cigarette. Results are registered in Table 2. It seems strange that, even under carefully controlled experimental conditions, there is no indication that the "black type" cigarette increases blood pressure more than the "German blend" cigarette.

Table 2. Mean rise in blood pressure of dogs after artificial respiration of three puffs of tobacco smoke from different cigarette brands [n = 6].

Type of cigarette	Body weight (kg)	Rise in blood pressure (mm Hg)	Time (min)	Nicotine content of smoke (mg)
Virginia	11	20	28	1.9
"Black" type	12	8	4	1.44
German blend	10	5	3	0.52

Table 3. Mean nicotine (ng/ml) content in the blood of dogs after artificial respiration of 1 puff (45 ml) of smoke from different brands of cigarettes [n = 6].

Type of cigarette	Time (s)					Nicotine content of smoke (mg)
	Immediately after puff	15	30	45	60	
Virginia	60	62	39	23	15	1.9
"Black" type	23	24	13	5	5	1.44
German blend	23	26	15	8	0	0.52

Table 3 shows the nicotine content of the animals after inhalation of one puff of the three brands of cigarettes. As can be seen from the table, there is no difference in the nicotine concentration in the "black type" and the "German blend" cigarettes after smoking, in spite of the fact that the "black type" cigarette contains three times as much nicotine as the "German blend" cigarette. This result corresponds with the results described in Table 2. It should be pointed out that these results must be regarded as preliminary, because of the relatively small numbers of animals used in these experiments.

Naturally, the composition of the smoke of these three brands of cigarettes is different. One outstanding difference is the more alkaline pH of the aqueous part of the smoke of the "black type" cigarette. Inhibition of respiration attributable to this property can be excluded as the same amount of smoke entered the lung of the dogs because of controlled artificial respiration.

In spite of the preliminary character of these experiments the results are indicative of influences of cigarette smoke constituents or smoke properties on the pulmonal absorption of nicotine, and certainly more research into these relationships is needed.

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The author's address:

Deutsches Herzzentrum München, Lothstraße 11, D-8000 München 2.

Blood Levels of Nicotine and Cotinine Achieved during Smoking

by A. K. Armitage

Hazleton Laboratories Europe Ltd., Harrogate, England

Standard filter tipped cigarettes and small cigars were uniformly labelled by the addition of 40—60 μCi of [2'-^{14}C]-nicotine, using an apparatus which dispensed a fixed volume from a hypodermic syringe, the solution being deposited along the axis of an intact cigarette as the latter was withdrawn from the syringe at a uniform rate. The smoke transfer characteristics of endogenous and exogenous nicotine were shown to be similar.

Subjects smoked a single radiolabelled cigarette or cigar taking one puff of smoke per minute, in as normal a manner as possible. The cigarette was held in a ventilated smoking cartridge designed to collect side-stream smoke, the butt and ash. After each puff, the subject exhaled into a modified face mask through which air was drawn continuously to collect the exhaled main-stream smoke. The brachial artery was cannulated and arterial pressure was recorded continuously. Arterial blood samples were taken at 1 min intervals and analysed for nicotine and cotinine using the methods described by *Turner*. Urine and faeces were collected for 72 h after the experiment.

Four subjects achieved high arterial blood concentrations of nicotine while smoking (30—40 ng/ml) and these were therefore assumed to be "inhalers"; the level of nicotine in the blood fell rapidly when smoking stopped. Two subjects, one of whom was a non-smoker, achieved only low arterial levels of nicotine (8.0 and 2.5 ng/ml) and these were almost certainly "non-inhalers". Arterial blood levels of cotinine were rapidly achieved in the inhalers, and exceeded nicotine levels shortly after smoking ceased.

In another two experiments [^{14}C]-nicotine (15 μCi) was injected intravenously. 10 rapid injections, each of 100 μg, were performed at 1 minute intervals in order to achieve a rate of intake of nicotine comparable with that of the smoking experiment. Peak arterial levels were about half those seen with cigarette smoking in the same subjects and the tachycardia was similarly less. The rate of disappearance of the nicotine from the arterial blood was slower than when the nicotine was given by smoking. This might be explained by differences in the rate of metabolism between the two routes, but most probably reflects the difficulty of achieving rapid complete mixing of the nicotine, since this was given into the antecubital vein and not directly into the right atrium.

The author's address:

Hazleton Laboratories Europe Ltd., Otley Road, Harrogate, HG3 1PY, England.

Experimental Considerations of some Alternate Routes in the Mammalian Metabolism of Nicotine

by Herbert McKennis, Jr.

Department of Pharmacology, Medical College of Virginia, Richmond, Virginia, U.S.A.

Extensive studies on the disposition of nicotine in mammals following ingestion of the alkaloid or the smoking of tobacco have provided evidence that nicotine is excreted partially unchanged, but largely in the form of twenty or more distinctive metabolites that contain an intact pyridine ring.

Progressive metabolic oxidation of the pyrrolidine ring of nicotine leads to the formation of cotinine, 3-pyridylacetic acid and a variety of other compounds. The rapid disappearance of nicotine from blood and many other tissues combined with the ease with which assays for cotinine can be conducted has led to several suggest-

ions that cotinine can play a possible role as an indicator for exposure to nicotine under various conditions.

The concentration of cotinine itself in many body compartments is subject to control by numerous previously described metabolic events including N-demethylation, further degradation of the pyrrolidine ring, pyridino-N-methylation, and pyridino-N-oxide formation.

Studies in the dog and the rabbit indicate that reactions leading to cotinine-N-oxide are reversible, at least in part. Oral administration of cotinine-N-oxide to the dog leads to urinary excretion of this compound, of cotinine, demethylcotinine, and other metabolites that have been previously characterized following administration of nicotine. The possible reversibility of other reactions leading to the formation of metabolites of nicotine and nornicotine is illustrated by urinary elimination of 3-pyridylacetic acid following administration of 3-pyridylacetic acid N-oxide.

As previously described 3-pyridylacetic acid itself enters into conjugation with glycine. The resultant N-3-pyridylacetylglycine when administered to animals is excreted partially in the form of 3-pyridylacetic acid.

Established sources of 3-pyridylacetic acid include nicotine, nornicotine, metanicotine, dihydrometanicotine, cotinine, allohydroxycotinine, and 4-(3-pyridyl)-4-oxobutyric acid. Thus, a number of alternate mammalian routes are available for the production of 3-pyridylacetic acid from nicotine, nornicotine, and associated alkaloids.

The author's address:

Department of Pharmacology,
Medical College of Virginia, Richmond 19,
Virginia, 23298, U.S.A.

Metabolism and Nitrosation of Nicotine

by E. Boyland

London School of Hygiene and Tropical Medicine, Department of Environmental Toxicology, London, England

Nicotine is metabolized to several products but the author deals only with cotinine, the hypothetical 5'-hydroxynicotine and the oxides of nicotine.

Cotinine is 50 times less toxic than nicotine on injection in mice. In tests with adult mice using subcutaneous injection or skin painting, cotinine did not produce any tumours. However, when implanted into the bladders of these animals or when injected into newborn mice it induced some tumours. Moreover, after administration in the drinking water, some tumours were also encountered in rats. The evidence that cotinine is carcinogenic is not conclusive, but as the substance is a major metabolite of nicotine it should be investigated further in long-term tests, notably as cotinine is formed by the lung tissue of rats and also by adult and foetal human lung.

When rats are pre-treated with nicotine they become resistant to the lethal action of this compound. This could be due to a nicotine metabolite protecting the animal from the effects of nicotine itself. Cotinine does not have this protective effect, but experiments indicate that the hypothetical intermediary metabolite, 5'-hydroxynicotine, obtained on reduction of cotinine could be the compound that reduces the acute toxic action of nicotine.

The two stereoisomers of nicotine-1'-N-oxide are both formed by the liver and lung and are excreted in urine. The relative amounts of the R- and S-isomers produced vary with different species. By analogy with the 7-N-oxides of xanthine and guanine, which are carcinogenic on injection in Wistar rats, the N-oxides of nicotine could be carcinogenic.

Recent research has shown that many tertiary amines react with nitrous acid to yield nitrosamines. Nicotine reacts slowly with nitrous acid to give nitrosonornicotine which is carcinogenic when injected into mice. The reaction probably proceeds via demethylation and subsequent nitrosation.

The author's address:

London School of Hygiene and Tropical Medicine,
Dept. of Environmental Toxicology, Keppel Street
(Gower Street), London, WC1E 7HT, England.

The Excretion and Metabolism of Nicotine and some Minor Tobacco Alkaloids in Man

by J. W. Gorrod

Department of Pharmacy, Chelsea College, University of London, London, England

Nicotine and some minor tobacco alkaloids can be measured in urine by a sensitive specific gas-liquid chromatographic technique. Application of this technique to the study of nicotine excretion and metabolism in man has shown that

a) Nicotine excretion was dependent upon urinary pH

whereas the excretion of a metabolite, cotinine, was but slightly pH dependent and the excretion of an alternative metabolite, nicotine-1'-N-oxide was independent of urinary pH.

b) Female smokers excreted less nicotine but more cotinine than female non-smokers after an intravenous dose of nicotine. Male smokers could be divided into two groups: those which gave a high total recovery of nicotine and cotinine after an intravenous dose of nicotine and another group which gave a lower total recovery. The first group excreted much more cotinine than their non-smoking counterparts, whereas the latter group excreted rather less.

c) The urinary excretion of unchanged nornicotine, methylanabasine or anabasine after an oral dose of the alkaloid was dependent upon the urinary pH, whereas no unchanged β-nicotyrine, β-nornicotyrine or myosmine were detected in the urine after an oral dose.

d) Less of the tertiary bases, nicotine and methylanabasine, were excreted under controlled acidic urinary pH conditions than the corresponding secondary bases.

e) Good correlation between buccal absorption characteristics and excretion data was obtained.

f) Nicotine-1'-N-oxide is well absorbed after an oral dose, poorly absorbed after rectal administration and quantitatively excreted unchanged after an intravenous dose. Nicotine-1'-N-oxide is reduced to nicotine in the gastrointestinal tract.

g) The ratio of cotinine to nicotine-1'-N-oxide excreted by smokers with cancer of the urinary bladder was higher than in the case of smokers without this disease.

The author's address:

Department of Pharmacy, Chelsea College, University of London, Manresa Road, London, SW3 6LX, England.

Biosynthesis of the Minor Alkaloids of Tobacco

by E. Leete

Department of Chemistry, University of Minnesota, Minneapolis, Minnesota, U.S.A.

Most of the biosynthetic work which has been carried out on the alkaloids of tobacco has been concerned with nicotine or anabasine. A fairly detailed biosynthetic scheme can now be outlined for these alkaloids, and the enzymes which catalyze some of the biosynthetic steps leading to nicotine have been isolated from tobacco roots. Much less is known about the origin of other alkaloids in *Nicotiana* species.

Nornicotine is formed by the demethylation of nicotine; however, the mechanism by which this occurs in the tobacco plant is still obscure. Nicotine-N'-oxide does not seem to be an intermediate in this transformation. Myosmine is formed by the dehydrogenation of nornicotine, a reaction which is not reversible in tobacco. Metabolism of myosmine ultimately affords nicotinic acid. Cotinine, a well established metabolite of nicotine in animals, is not formed to any appreciable extent in the healthy plant. Anatabine (4',5'-dehydroanabasine) is apparently unrelated biosynthetically to anabasine. Feeding experiments with [2-^{14}C]-lysine, [carboxyl-^{14}C]- and [6-^{14}C]-nicotinic acid have enabled us to deduce a novel biosynthetic scheme for anatabine.
Nicotelline and anatalline are probably trimers of dihydropyridines derived from nicotinic acid. These alkaloids are thought to be formed by non-enzymatic reactions.

The author's address:

Dept. of Chemistry, University of Minnesota, 139 Smith Hall, Minneapolis, Minnesota, 55455, U.S.A.

Radioimmunoassays for Nicotine and its Metabolite: Applications and Results

by H. van Vunakis

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts, U.S.A.

Radioimmunoassays for nicotine and two of its metabolites, cotinine and 4-(3-pyridyl)-4-oxo-N-methylbutyramide, have been developed which permit estimation of these compounds at the picomole level in physiological fluids and tissue extracts [*Langone, Gjika, van Vunakis:* Biochemistry 12 (1973) 5025 / *Langone, Francke, van Vunakis:* Arch. Biochem. Biophys. 164 (1974) 536]. The specificities of the antibodies are such that quantitative determination of these compounds in the presence of each other and in the presence of other metabolites

including cotinine-N-oxide, desmethylcotinine, 4-(3-pyridyl)-4-oxo-N-methylbutyramide, 4-(3-pyridyl)-4-oxobutyric acid, nicotine-N'-oxide, and nornicotine can be made.

The levels of cotinine and/or nicotine in the sera, urine, spinal and amniotic fluids and in cord arterial, cord venous and maternal blood of smokers were determined. Cotinine was also found to be present in the urine of tobacco croppers who were non-smokers, indicating that nicotine was absorbed through the skin during harvesting of the plants.

The radioimmunoassays have been used to monitor the oxidation of nicotine to cotinine and cotinine to 4-(3-pyridyl)-4-oxo-N-methylbutyramide by enzyme systems present in rabbit liver extracts. The results obtained in the radioimmunoassays were confirmed by isolation and quantitation of the compounds from the enzymatic reaction mixtures by high pressure liquid chromatography. Recently, the nicotine analogues of TPN and DPN formed in incubation mixtures containing nicotine, TPN or DPN and a source of DPNase have been isolated and characterized [*Shen* and *van Vunakis:* Biochemistry 13 (1974) 5362].

The author's address:

Graduate Dept. of Biochemistry, Brandeis University, Waltham, Massachusetts, 02154, U.S.A.

The Use of a Radiochemical Method to Study the Metabolism of Nicotine in Experimental Animals

by D. M. Turner

Hazleton Laboratories Europe Ltd., Harrogate, England

A radiochemical method for the analysis of [^{14}C]-nicotine in biological fluids was developed some years ago in these laboratories. It is based on solvent extraction and thin-layer chromatographic separation. Nicotine and cotinine are recovered quantitatively and using [^{14}C]-nicotine at a specific activity of 20 mCi/mmole, nanogram amounts of both compounds can be measured. The method has been used to study the fate of [2'-^{14}C]-nicotine in the cat after intravenous injection of a single dose or multiple doses. The excretion into urine and bile and the disposition of the drug in tissues has also been investigated. The brain accumulates [^{14}C]-nicotine rapidly and there is evidence of regional concentration of the drug in the thalamus and hypothalamus. Administration of [^{14}C]-nicotine as a series of small intravenous injections (4 µg/kg every 60 s for 20 min) yields a peak blood nicotine level in the cat, of approximately 100 ng/ml. The blood levels are in fact higher than those achieved after intravenous infusion of the same dose, using the isolated perfused dog lung. We have shown that the increase in blood radioactivity, during serial injections of [^{14}C]-nicotine or [^{14}C]-labelled smoke inhalation, is not smooth but fluctuates.

The effect of route of administration on the metabolism of [^{14}C]-labelled nicotine has been investigated in four different animal species: cat, rabbit, rat and squirrel monkey. In all species, intravenous administration of small multiple doses of [^{14}C]-nicotine (4 µg/kg every 60 s for 1 h) results in peak blood levels of approximately 100 ng/ml. The proportions of [^{14}C]-cotinine differ widely between species however. Subcutaneous injection of 0.4 mg/kg [^{14}C]-nicotine also yields peak blood nicotine levels of the same order but the time course, for each species, is different to that observed after intravenous administration.

Intragastric instillation of 1 mg/kg [^{14}C]-nicotine produced relatively low blood levels of [^{14}C]-nicotine and higher levels of [^{14}C]-cotinine in both cat and rabbit. In the rat however peak blood nicotine levels of approximately 140 ng/ml occur. The results have important implications for all *in vivo* animal model experiments where the relevance of data thus derived are to be interpreted in relation to smoking and health in man.

The author's address:

Hazleton Laboratories Europe Ltd., Otley Road, Harrogate, HG3 1PY, England.

GC Estimation of Nicotine in Picomole Concentrations (some problems encountered)

by L. Neelakantan

College of Pharmacy, University of Kentucky, Lexington, Kentucky, U.S.A.

The perfluoropropionyl derivative of octahydronicotine constitutes an electron-capture sensitive compound by which nicotine can be quantitated in subpicomole amounts. Preliminary studies indicate that nicotine at levels achieved by smoking one cigarette can be extracted from a sample, hydrogenated, derivatized and quantitated by this procedure. However, in working with submicrogram amounts of nicotine, it is necessary to use $Pd/BaSO_4$ rather than Pd/C as a hydrogenation catalyst to prevent adsorption losses. The reliability and efficiency of this procedure has been monitored by using [^{14}C]-nicotine.

The author's address:

College of Pharmacy, University of Kentucky, Lexington, Kentucky, 40506, U.S.A.

Nicotine Estimation in Biological Fluids

by C. Feyerabend
Poisons Unit, New Cross Hospital, London, England

A method for nicotine measurement is described which uses a GC equipped with either FID or nitrogen detector. The extraction procedure involves two diethylether extractions from plasma, followed by an acid back extraction, and reextraction into the solvent for injection on the GC.
The method is relatively quick (24–32 samples analysed per day) and sensitive, measuring down to 0.1 ng/ml nicotine. It is also very simple to perform and does not require sophisticated or expensive equipment and yet gives both precise and accurate results.
Nicotine contamination in the atmosphere and the levels in non-smokers — attained by passive inhalation — and in smokers have been investigated using this method. The results show that it might be erroneous to designate nicotine levels measured in non-smokers as "blank values" unless they have deliberately refrained from contact with smokers or a smoking environment for at least twelve hours.

The author's address:

Poisons Unit, New Cross Hospital,
Avonley Road, London, SE14 5ER, England.

Detection of Nicotine by Atmospheric Pressure Ionization (API) Mass Spectrometry

by E. C. Horning
Institute for Lipid Research, Baylor College of Medicine, Texas Medical Center, Houston, Texas, U.S.A.

Atmospheric pressure ionization (API) mass spectrometry is a novel form of mass spectrometry in which ionization occurs in a small reaction chamber external to the low pressure region of a quadrupole mass spectrometer. The primary source of electrons is a ^{63}Ni foil or a corona discharge. Ions are admitted to the mass analyzer through a small aperture (25 μ) in a disk separating the ion chamber (which is at atmospheric pressure) from the low pressure region. Subpicogram sensitivity of detection has been established for this instrument.

Analyses of nicotine in urine, for both smokers and non-smokers, were carried out by API techniques. Extracts of urine were injected directly into the vaporizer-source assembly. The urine of non-smokers was found to contain nicotine. The route of transfer was by room air.

The author's address:

Institute for Lipid Research, Baylor College of Medicine,
Texas Medical Center, Houston, Texas, 77025, U.S.A.