Nicotine and Its Metabolites. Radioimmunoassays for Nicotine and Cotinine†

John J. Langone, Hilda B. Gjika, and Helen Van Vunakis*,‡

ABSTRACT: Radioimmunoassays for nicotine and one of its major metabolites, cotinine, which permit estimation of these compounds in tissue extracts and physiological fluids at the picomole level, have been developed. The specificities of the antibodies are such that quantitative determination of nicotine and cotinine in the presence of each other and in the presence of other metabolites including cotinine *N*-oxide, desmethylcotinine, γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide, γ-(3-pyridyl)-γ-oxobutyric acid, nicotine *N'*-oxide, and nornicotine can be made. The radioimmunoassays have been used to monitor the oxidation of nicotine to cotinine by NADPH and oxygen-dependent oxidases present in rabbit liver extracts. The results obtained in the radioimmunoassays were confirmed by isolation and quantitation of cotinine and nicotine from the enzymatic reaction mixtures by high-pressure liquid chromatography. The levels of nicotine and cotinine in sera and urine of smokers were also determined.

The major tobacco alkaloid (−)-nicotine is metabolized by man and other mammals to give a number of products (Figure 1), most of which are derived by oxidation of the pyrrolidine ring (McKennis, 1965). (−)-Cotinine (McKennis *et al.*, 1958a) has been shown to be a principal metabolite and the direct precursor of at least three other metabolites: cotinine *N*-oxide (Dagne and Castagnoli, 1972a), *trans*-3'-hydroxycotinine (McKennis *et al.*, 1963; Dagne and Castagnoli, 1972b), and γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide (McKennis *et al.*, 1958b).

Despite the importance of nicotine as a constituent of tobacco smoke, simple assays to estimate the alkaloid and its metabolites in physiological fluids and tissues do not exist. Although gas–liquid chromatography (glc) has been used to estimate nanogram quantities of nicotine in blood (Burrows *et al.*, 1971; Isaac and Rand, 1972), this technique requires that samples be processed extensively prior to analysis. For example, one procedure (Burrows *et al.*, 1971) requires initial steam distillation, extraction, and column chromatography before the sample is concentrated and injected into the gas chromatograph. The nicotine recovery in these experiments was 55–60%. Such a method clearly is not applicable to the analysis of large numbers of samples. In addition, the high temperatures required for glc lead to deoxygenation of cotinine *N*-oxide to give a peak corresponding to cotinine (Dagne and Castagnoli, 1972a). Thus, glc analysis of samples containing cotinine *N*-oxide (and probably nicotine *N'*-oxide) could give erroneous estimates of nicotine metabolites.

On the other hand, the radioimmunoassay technique which is based on the competition between labeled and unlabeled antigen for binding to a limited number of sites on a specific antibody has been used to quantitate biologically important molecules at the picogram and nanogram levels, often in unprocessed serum or urine (Berson and Yalow, 1971). Although initial development of a satisfactory radioimmunoassay may require several months, once specific antibodies are obtained and a sensitive assay developed, the manipulations are simple and can be run on a routine basis.

We report here the development of sensitive and specific radioimmunoassay systems for the quantitative determination of nicotine and cotinine. With these assays, we have been able to follow the enzymatic conversion of nicotine to cotinine by rabbit liver extracts and to determine the levels of nicotine and cotinine in the physiological fluids of smokers.

Materials and Methods

(−)-Nicotine·2HCl was purchased from K & K Laboratories. We are grateful to Drs. H. McKennis, Jr., and E. R. Bowman of the Medical College of Virginia for samples of (−)-cotinine and γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide and to Dr. M. Senkus of the R. J. Reynolds Tobacco Co. for the sample of 6-hydroxynicotine. The chemicals used in the syntheses were from commercial sources, were reagent grade, and were used without purification.

The following compounds were prepared according to published directions: *trans*-4'-carboxycotinine, *trans*-4'-carbomethoxycotinine, *trans*-3'-hydroxymethylnicotine (Cushman and Castagnoli, 1972), (−)-cotinine (Bowman and McKennis, 1963), cotinine *N*-oxide (Dagne and Castagnoli, 1972a), γ-(3-pyridyl)-γ-oxobutyric acid, γ-(3-pyridyl)-γ-*N*-methylaminobutyric acid, *dl*-desmethylcotinine (McKennis *et al.*, 1958b), and nicotine *N'*-oxide (Craig and Purushothaman, 1970). In all cases, the melting or boiling points agreed with the reported values, ir and uv spectra were consistent for each compound, and each product was homogeneous by tlc in one or more solvent systems.

trans-3'-Succinylmethylnicotine and *N*-(*p*-hydroxyphenethyl)-*trans*-cotinine carboxamide were synthesized as follows.

trans-3'-Succinylmethylnicotine. A stirred solution of *trans*-3'-hydroxymethylnicotine (300 mg; 1.6 mmol) and succinic anhydride (157 mg; 1.6 mmol) in 30 ml of dry benzene was heated at 60° for 20 hr. Thin-layer chromatography (tlc) of the crude brown reaction mixture (ethyl acetate–methanol–ammonium hydroxide, 65:35:11) showed only one spot with R_F 0.44, while *trans*-3'-hydroxymethylnicotine had an R_F of 0.69. The benzene was concentrated under reduced pres-

† Publication No. 918 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received June 12, 1973.* This work was supported by a contract from the National Cancer Institute (NIH-NCI-E-72-3243).

‡ Public Health Service Research Career awardee (Award No. 5K6-AI-2372 from the National Institute of Allergy and Infectious Diseases).

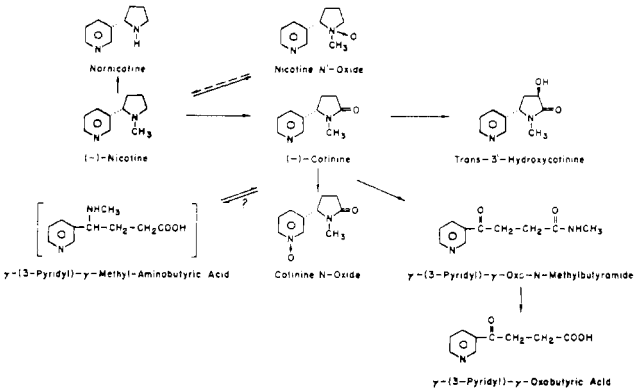

FIGURE 1: Metabolic routes to some nicotine metabolites.

sure to give 440 mg (96%) of semisolid *trans*-3′-succinylmethylnicotine: uv (H_2O) λ_{max} 260 nm (log ϵ 3.53 at pH 1, 3.33 at pH 6 and 13).

Alternatively, the benzene solution of reactants was treated with 1 equiv of butyllithium in hexane. The reaction mixture was stirred at 25° for 3 hr, then concentrated to give 450 mg (98%) of the lithium salt of *trans*-3′-succinylmethylnicotine as a tan solid. The tlc behavior and uv spectrum were identical with those of the above product.

N-(p-Hydroxyphenethyl)-trans-cotinine Carboxamide. Oxalyl chloride (14.9 mg; 0.12 mmol) was added to a stirred solution of *trans*-4′-carboxycotinine (22 mg; 0.11 mmol) in 1.0 ml of dry dimethylformamide in a flask equipped with a drying tube. After 30 min at 25°, triethylamine (14.4 mg; 0.14 mmol) was added followed by 13.7 mg (0.10 mmol) of tyramine. After 16 hr at 25°, the reaction mixture showed three spots by tlc (ethyl acetate–methanol–ammonium hydroxide, 65:35:11) with R_F values 0.31 (*trans*-4′-carboxycotinine), 0.62 (unchanged tyramine), and 0.78 (product). The last spot was β-nitrosonaphthol positive and ninhydrin negative, indicating the presence of a phenol and absence of an amine function, respectively. Preparative tlc of the mixture in the above solvent system showed three bands with R_F values 0.15–0.30 (*trans*-4′-carboxycotinine), 0.41–0.52 (tyramine), and 0.56–0.61 (product). The band with an R_F of 0.56–0.61 was eluted with ethanol. The uv spectrum shown in Figure 2 is consistent for *N*-(*p*-hydroxyphenethyl)-*trans*-cotinine carboxamide. Of special significance is the bathochromic shift of the 262-nm peak to 293 nm at pH 12, indicative of the tyramyl phenolic function, and the increased ϵ value of the 262-nm absorption at pH 2 characteristic of the pyridine ring. Based on this spectrum and that of tyramine, the concentration of the amide was calculated to be 0.38 mg/ml.

Immunization Procedures. The following general procedure was used to conjugate *trans*-4′-carboxycotinine and *trans*-3′-succinylmethylnicotine to human serum albumin, hemocyanin (from giant keyhole limpets), and poly-L-lysine.

A solution of the functionalized hapten (10 mg) and the macromolecule (10 mg) in 1.0 ml of water was adjusted to pH 6.8–7.0, then 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (10 mg) (Goodfriend *et al.*, 1966) was added. After being stirred at 25° overnight, the reaction mixture was chromatographed on a column (1.9 × 35 cm) of Sephadex G-50 and eluted with saline–phosphate buffer at pH 7.0; Fractions (1.5–2.5 ml) were examined for absorption at 260 nm to determine the presence of hapten–protein conjugates. Based on the uv spectra, the degree of coupling ranged between 6 and 18 mol of hapten per mol of macromolecule.

New Zealand albino rabbits were immunized with complete Freund's adjuvant according to the following schedule. Approximately 1 mg of each conjugate in 1 ml of buffer was emulsified with an equal volume of adjuvant. Animals were given the material by injection into the toe pads and leg muscles. Three weeks later, the animals were bled. About every 6 weeks, the rabbits were given booster injections and bled 1 week later.

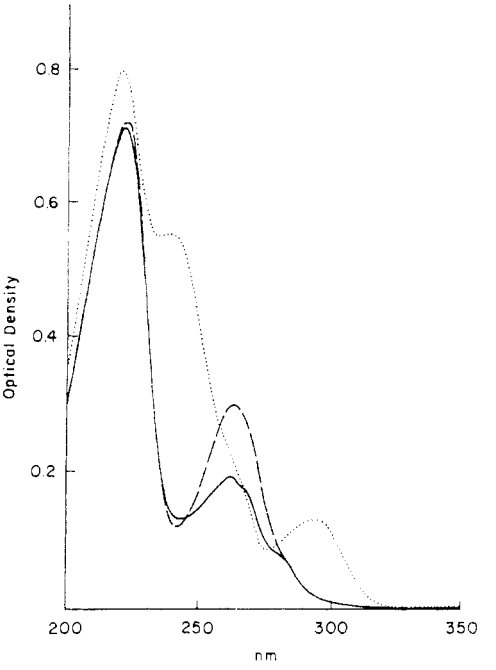

FIGURE 2: Absorption spectra of *N*-(*p*-hydroxyphenethyl)-*trans*-cotinine carboxamide at pH 1.0 (---), pH 5.5 (——), and pH 13.0 (. . . . .).

Labeled Haptens. [^{3}H]Nicotine was prepared at New England Nuclear Corp. by random catalytic tritium exchange on the pyridine ring to a specific activity of 15 Ci/g. *N*-(*p*-Hydroxyphenethyl)-*trans*-cotinine carboxamide was iodinated with Na^{125}I according to the procedure of Greenwood *et al.* (1963) to a specific activity of 100 Ci/g of derivative.

Radioimmunoassay Procedures. For radioimmunoassay, 0.1 ml of the labeled antigen (approximately 20,000 cpm), 0.1 ml of buffer (0.1% gelatin–0.15 M sodium chloride–0.01 M Tris, pH 7.4), and 0.1 ml of antiserum at the appropriate dilution were incubated at 37° for 1 hr at which time 0.1 ml of goat anti-rabbit γ-globulin (previously calibrated to be in antibody excess with respect to the rabbit γ-globulin) was added. To control for nonspecific binding, normal rabbit serum was used in place of immune serum. The reaction mixtures were mixed and incubated at 2–4° overnight. The precipitate was collected by centrifugation at 1000*g* for 30 min at 4°, and the supernatant fluid was decanted. Residual traces of fluid were removed by wiping the inside of the tube and the precipitate was counted in a Packard Auto-Gamma spectrometer. For the inhibition experiments, a 0.1 ml of solution containing different amounts of inhibitor was added in place of the buffer. All subsequent procedures were the same.

Binding was determined by the level of radioactivity in the immune precipitate (antibody-bound hapten). The per cent inhibition was calculated by determining the percentage of counts displaced from the complex by known quantities of the nonradioactive inhibitor. The amounts of hapten in physiological fluids were calculated by comparing its inhibition with the standard inhibition curve. Other details of the assay are given in the figure and table legends.

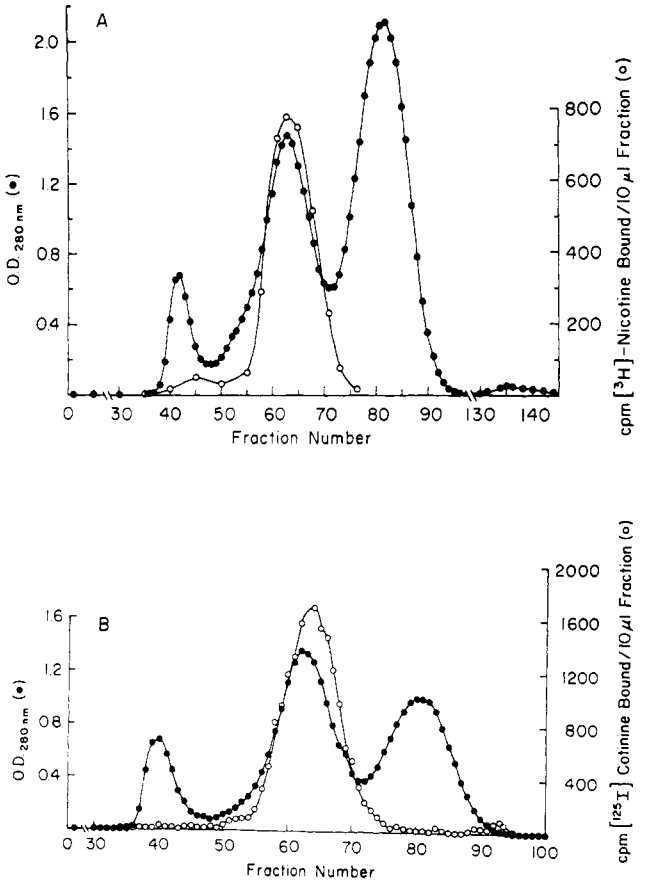

FIGURE 3: Gel filtration on Sephadex 200 of (A) rabbit anti-nicotine and (B) rabbit anti-cotinine.

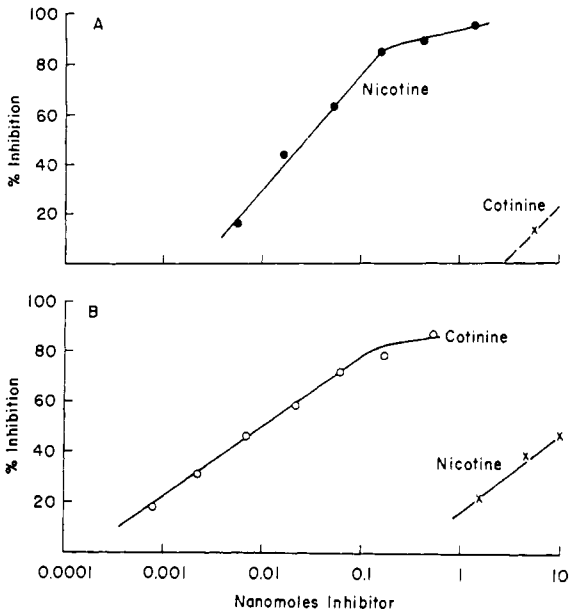

FIGURE 4: (A) Inhibition of the [³H]nicotine–anti-nicotine binding by nicotine and cotinine. Radioimmunoassay conditions as described in Methods. Each reaction mixture contains [³H]nicotine (20,000 cpm) and 2 μg of antisera (added as 0.1 ml of a $^1/_{50}$ dilution). In the absence of inhibitor, approximately 2000 cpm is precipitated. When 2 μl of normal rabbit sera was added instead of immune sera to determine nonspecific binding, approximately 140 cpm was precipitated. (B) Inhibition of the [¹²⁵I]cotinine derivative–anti-cotinine binding by cotinine and nicotine. Each reaction mixture contains *N*-[¹²⁵I]-(*p*-hydroxyphenethyl)-*trans*-cotinine carboxamide (20,000 cpm) and 2 μl of antisera (added as 0.1 ml of a $^1/_{50}$ dilution). With the immune sera, approximately 3900 cpm was precipitated. With the normal rabbit sera, 320 cpm was precipitated.

Results

Low molecular weight molecules generally must be linked to macromolecules in order to render them antigenic (Landsteiner, 1945). Since nicotine and cotinine do not possess suitable functional groups for this purpose, carboxyl derivatives of these compounds (*i.e.*, *trans*-4′-carboxycotinine and *trans*-3′-succinylmethylnicotine) were synthesized and covalently linked to proteins or polyamino acids to prepare the immunizing conjugates. Antibody production in the immunized rabbits was followed by monitoring the binding of [³H]nicotine or an ¹²⁵I-labeled cotinine derivative to the homologous antibody. Immune precipitation with goat anti-rabbit γ-globulin separated the labeled hapten bound to the antibody from the free labeled hapten. As shown in Figure 3, most of the binding activity was found in the 7S γ-globulin fraction of the rabbit antisera after gel filtration in Sephadex G-200. The per cent inhibition of binding of [³H]nicotine by anti-nicotine in the presence of different amounts of nicotine under standard conditions is shown in Figure 4A. For 50% inhibition, 3.2 ng of nicotine is required, while the lower limit of detection is about 350 pg. Cotinine is 2500 times less effective an inhibitor than nicotine of the [³H]nicotine–anti-nicotine reaction.

In Figure 4B is shown the inhibition of the [¹²⁵I]cotinine derivative–anti-cotinine reaction by cotinine. Only 1.2 ng of cotinine is required for 50% inhibition in the cotinine anti-cotinine system, while the lower limit of detection is about 200 pg. Nicotine is about 2000 times less effective than cotinine in inhibiting this reaction.

Table I shows data for a comprehensive list of nicotine metabolites and other compounds structurally related to nicotine and cotinine which were examined for inhibitory effectiveness in the anti-nicotine and anti-cotinine systems. The data are interpolated from curves like those shown in Figure 4 which were obtained for each compound.

Since nicotine is such a poor inhibitor of the cotinine anti-cotinine reaction, it was possible to use the radioimmunoassay procedure to follow the enzymatic production of cotinine from nicotine by rabbit liver extracts in the presence of NADPH and O_2 (expt 1, Table II), or in the presence of a NADPH-regenerating system and O_2 (expt 2, Table II). Since [³H]-nicotine had been added to the mixture, it was also possible to quantitate cotinine production by an independent method, *i.e.*, by determining the radioactivity which emerged from the liquid chromatograph and had the same retention volume as a standard sample of cotinine (Table II). Both analytical methods are in good agreement.

Sera and urine samples from two smokers were analyzed for cotinine and nicotine by the radioimmunoassay procedure. The results obtained for these two individuals before and after they stopped smoking are shown in Table III. Nicotine is rapidly metabolized having a plasma half-life in smokers of less than 30 min (Isaac and Rand, 1972). In our experiments, nicotine levels in the sera are not given, since the time that elapsed between smoking the last cigarette and withdrawing the blood for analyses was not strictly controlled.

Discussion

The radioimmunoassays for nicotine and cotinine permit estimation of these compounds at the pmole level in tissue extracts and physiological fluids. In addition to being sensi-

TABLE I: Inhibition of the Nicotine and Cotinine Antigen–Antibody Reactions.

Compound	nmol Required for 50% Inhibn: [^{3}H]Nicotine–Anti-nicotine	nmol Required for 50% Inhibn: [^{125}I]Cotinine Derivative–Anti-cotinine
(−)-Nicotine	*0.02*	12.7
(−)-Cotinine	>50.0[a]	*0.0067*
(−)-Cotinine *N*-oxide	>50.0[a]	6.2
dl-Desmethylcotinine	>50.0[b]	2.2
γ-(3-Pyridyl)-γ-oxo-*N*-methylbutyramide	>25.0[a]	>20.0[a]
γ-(3-Pyridyl)-γ-oxobutyric acid	200.0	>25.0[b]
Nicotine *N'*-oxide	3.7	44.9
(−)-Nornicotine	2.2	67.5
(−)-Anabasine	6.1	>50.0[a]
dl-2-Aminonicotine	0.83	42.0
dl-6-Aminonicotine	3.3	>25.0[b]
6-Hydroxynicotine	100.0	>25.0[b]
Pyridine	>50.0[b]	>50.0[b]
Nicotinic acid	>50.0[b]	>50.0[b]
Nicotinamide	>50.0[b]	>50.0[b]
2-Aminopyridine	>50.0[b]	>50.0[b]
2-Acetamidopyridine	>50.0[b]	>50.0[b]
Ethyl nicotinate	>50.0[b]	>50.0[b]
L-Proline	>50.0[b]	>50.0[b]
N-Methylpyrrolidine	>50.0[b]	>50.0[b]
N-Methyl-2-pyrrolidone	>50.0[b]	>50.0[b]

[a] 20% inhibition at this level. [b] Less than 10% inhibition at this level. 3-Hydroxycotinine, a minor metabolite in which the two rings of the parent molecule are intact, was not available for assay. Based on the data in this table, other metabolites which retain only one of the rings would not be expected to inhibit either antigen–antibody reaction significantly.

TABLE II: Enzymatic Oxidation of (−)-Nicotine to (−)-Cotinine by Rabbit Liver Extracts.

Expt	Time (min)	% Cotinine Determined by: Radioimmunoassay	% Cotinine Determined by: Liquid Chromatography[b]
1[a]	0	0.13	0.19
	20	5.90	6.90
	40	9.97	11.8
	60	15.2	18.0
	80	17.2	19.2
2[a]	0	0.1	
	30	7.3	7.9
	60	14.0	
	120	30.5	
	600	36.7	38.2

[a] The supernatant fraction from a rabbit liver homogenate that had been centrifuged at 17,000*g* for 15 min was used (Papadopoulos and Kintzios, 1963). Expt 1: Each milliliter of reaction mixture contains 25 mg of protein in phosphate–KCl buffer (pH 7.5), 500 μg of nicotine·2HCl, [^{3}H]nicotine (7×10^3 cpm), and 1.3 mg of NADPH. Expt 2: Each milliliter of reaction mixture contains 20 mg of protein in buffer, 500 μg of nicotine·2HCl, [^{3}H]nicotine (3×10^6 cpm), and a NADPH-regenerating system consisting of 0.167 mg of NADP, 2.53 mg of glucose 6-phosphate, 1.39 μg of glucose-6-phosphate dehydrogenase, and 2.4 mg of nicotinamide. After incubation in an O_2 atmosphere at 37°, aliquots from each reaction mixture were withdrawn, heated in a boiling-water bath for 5 min to terminate the reaction, and then centrifuged. The supernatant fraction was diluted in the gel–Tris buffer for radioimmunoassay or used without dilution for liquid chromatography. [b] Liquid chromatography (in cpm in cotinine peak) was performed on a Waters Associates Model 202 instrument using an analytical diphenylcorasil column (2 ft × 1/8 in.). Cotinine (V_e = 8.8 ml) was collected using MeOH–H_2O (1:9) as solvent at a flow rate of 1.0 ml/min, while nicotine, which had V_e = 40 ml in this solvent, was eluted with CH_3CN–0.1% $(NH_4)_2CO_3$ (1:4) at a flow rate of 1.0 ml/min (V_e = 4.8 ml). In their respective solvents, nicotine and cotinine were separated from desmethylcotinine, cotinine *N'*-oxide, γ-(3-pyridyl)-γ-oxo-*N*-methylbutyramide, γ-(3-pyridyl)-γ-oxobutyric acid, γ-(3-pyridyl)-γ-methylaminobutyric acid, nornicotine, and nicotine *N'*-oxide.

tive, the radioimmunoassays are also specific for the homologous antigen (Table I). Compared to nicotine, approximately 2000 times more cotinine are required to give 50% inhibition in the nicotinine–anti-nicotine reaction. Nicotine is an equally poor inhibitor of the cotinine–anti-cotinine system. The geometry of the five-membered ring may be a factor in determining the specificity of these antisera. Thus antibody combining sites may recognize the "envelope" conformation of the nicotine *N*-methylpyrrolidine ring or the more planar conformation of the cotinine *N*-methyl-2-pyrrolidone ring.

Several other metabolites that were tested are also poor inhibitors of these antigen–antibody reactions, most of them being three to four orders of magnitude less inhibitory than the homologous antigen (Table I). Nornicotine (*i.e.*, desmethylnicotine) and nicotine *N'*-oxide are about $^1/_{100}$–$^1/_{200}$ as effective as nicotine in inhibiting the nicotine–anti-nicotine reaction. *dl*-Desmethylcotinine is $^1/_{300}$ as effective as cotinine in inhibiting the cotinine–anti-cotinine reaction.

Nicotine contains two distinct ring systems, *i.e.*, the pyridine and *N*-methylpyrrolidine rings. The inhibition data indicate that the specificity of the antisera is directed toward both rings of the structure. The pyridine or pyrrolidine derivatives which possess only one of the rings give essentially no inhibition at the 50-nmol levels (Table I). The specificity of the anti-cotinine is also directed toward both ring systems, *i.e.*, the pyridine and *N*-methyl-2-pyrrolidone rings.

It is interesting to note that antibodies that were produced in this laboratory by immunizing rabbits with conjugates in which 2- or 6-aminonicotine was linked to macromolecules by their amino groups showed strong specificity for the *N*-methylpyrrolidine ring. In fact, *N*-methylpyrrolidine and nicotine inhibited the 2-aminonicotine immune system to the same extent. Furthermore, 2- and 6-aminonicotine are not satisfactory functionalized haptens, since the conditions under which they are prepared lead to racemization at C-2′ (Tschitschibabin and Kirssanow, 1924).

Anabasine, the piperidine analog of nicotine (which like nornicotine is found in tobacco) inhibits the nicotine anti-nicotine reaction 100 times less effectively than nicotine, again

TABLE III: Cotinine and Nicotine Levels in Physiological Fluids of Cigarette Smokers.

		M. S.[a]			K. G.[b]		
	Day	ng of Cotinine/ ml of Sera	mg of Cotinine (In 24-hr Urine)	mg of Nicotine	ng of Cotinine/ ml of Sera	mg of Cotinine (In 24-hr Urine)	mg of Nicotine
Days *before* subject stopped smoking	3	134	2.2	0.7	200	5.1	1.0
	2	172	3.0	1.1	170	6.1	1.2
	1	170	2.4	0.3	260	5.2	2.7
Days *after* subject stopped smoking	1	150	2.8	0.2	170	3.2	0.42
	2	60	1.6	0.1	75	2.4	0.28
	3	38	1.0	<0.1	30	1.6	0.6
	4	12	0.2		20	0.8	0.6
	5	5			15		
	8	<2	<0.1	<0.1	<2	<0.1	<0.1

[a] M. S. is a 53-year-old male who has smoked one to two packs of cigarettes per day for 28 years. [b] K. G. is a 23-year-old male who has smoked one pack of cigarettes per day for 4 years. The physiological fluids were diluted in gel–Tris buffer and assayed as described in Methods and in Figure 4.

indicating the ability of the antibodies to recognize changes in the pyrrolidine ring. The greater effectiveness of 6-aminonicotine as an inhibitor relative to 6-hydroxynicotine (*i.e.*, 30 times as effective) probably reflects the preferred existence of this latter compound in the tautomeric pyridone form, analogous to 2-hydroxypyridine (Mason, 1959). The gross structural change of the pyridine ring would be expected to lower the ability of the anti-nicotine sera to bind the hydroxy compound relative to 6-aminonicotine.

Since the specificity of the cotinine antisera was sufficiently narrow to permit the determination of cotinine in the presence of nicotine and the other metabolites listed in Table I, it was used to follow the enzymatic oxidation of nicotine to cotinine in rabbit liver extracts.

The results obtained by radioimmunoassay were confirmed using high-pressure liquid chromatography under conditions where cotinine and nicotine were clearly separated from each other and standard samples of seven other nicotine metabolites (Table I). In expt 1 (Table II) where limited amounts of NADPH were used, approximately 20% of the nicotine was converted to continine. Nicotine accounted for 67% of the remaining radioactivity. In expt 2, in which a NADPH-regenerating system was used, approximately 40% of the nicotine was converted to cotinine, while 30% of the nicotine remained unchanged. Small amounts of radioactivity were also distributed under several peaks which are in the process of being identified.

Thus, the radioimmunoassay provides a simple and rapid quantitative procedure to monitor the enzymatic production of cotinine. The assay was performed directly on the enzymatic reaction mixtures. Extraction and chromatographic procedures which separate the compounds into different subgroups were not required. Other workers have shown that nicotine is oxidized *in vitro* to cotinine in liver extracts of several different species (Hucker *et al.*, 1960; Papadopoulos and Kintzios, 1963; Turner, 1969; Ståhlhandske, 1970; Booth and Boyland, 1971).

The radioimmunoassays for cotinine and nicotine were also used to analyze the sera and urine of two smokers before and after they stopped smoking. While smoking, the cotinine levels in the sera of these subjects ranged from 150 to 260 ng per ml of sera. After they had stopped smoking for 1 day, the cotinine levels were still within 80–90% of the values obtained during their smoking period. At the end of the 7th day, they had little or no detectable cotinine in their sera. While smoking, the nicotine excreted in a 24-hr urine sample ranged from a minimum of 0.2 mg/24 hr for M. S. to 2.6 mg/24 hr for K. G. Cotinine values ranged from 3 to 6 mg per 24 hr for the same subjects. After they stopped smoking, nicotine and cotinine levels steadily decreased.

The question of whether nicotine in smokers' urine represents unaltered nicotine or nicotine *N'*-oxide that had been converted to nicotine by reductase(s) (Booth and Boyland, 1971) remains to be answered. Nicotine (Turner, 1969) and cotinine (McKennis *et al.*, 1958a; Hucker *et al.*, 1960; Applegren *et al.*, 1962; Papadopoulos, 1964; Beckett and Triggs, 1966; Turner, 1969) have been found in the blood and urine of experimental animals and man. However, only recently has nicotine been determined quantitatively in the blood of smokers (Burrows *et al.*, 1971; Isaac and Rand, 1972).

Although the subject of another communication, nicotine and cotinine levels in the sera of approximately 600 subjects (about 40% smokers and 60% nonsmokers) have been determined by the radioimmunoassay procedure. While no significant amounts of either compound were detected in the sera of nonsmokers, up to 73 ng/ml of nicotine and between 73 and 650 ng per ml of cotinine was detected in the sera of smokers. From the limited number of sera, and amniotic fluids thus far assayed, it appears that there is poor or no correlation between the smoking history, *i.e.*, the number of cigarettes smoked, the number of years an individual has been smoking, etc., and the cotinine and nicotine levels in the fluids of smokers. The intake of nicotine is variable among smokers, since they inhale differently, take a different number of puffs from a cigarette and may detoxify nicotine to different degrees depending on the enzymes present in their lungs, livers, kidneys, etc. Since nicotine is so quickly detoxified, an assay for a longer lived metabolite such as cotinine could prove to be more important in epidemiological studies than an assay for the parent compound.

Acknowledgment

The authors are grateful to Dr. Lawrence Levine for advice

and constructive criticism, to Dr. Stanley Cernosek, Jr., for the initial succinylation of the *trans*-3′-hydroxymethylnicotine and to Mrs. Lory Peta for excellent technical assistance. Some of the enzymatic experiments were performed by Mr. Alan Leichtner.

References

Applegren, L. E., Hansson, E., and Schmitterlöw, C. G. (1962), *Acta Physiol. Scand. 56*, 249.

Beckett, A. H., and Triggs, E. J. (1966), *Nature (London) 211*, 1415.

Berson, S. A., and Yalow, R. S. (1971), *in* Immunobiology, Good, R. A., and Fisher, D. W., Ed., Stamford, Conn., Sinauer Associates, Inc., p 287.

Booth, J., and Boyland, E. (1971), *Biochem. Pharmacol. 20*, 407.

Bowman, E. R., and McKennis, Jr., H. (1963), *Biochem. Prep. 10*, 36.

Burrows, I. E., Corp, P. J., Jackson, G. C., and Page, B. F. J. (1971), *Analyst 96*, 81.

Craig, J. C., and Purushothaman, K. K. (1970), *J. Org. Chem. 35*, 1721.

Cushman, M., and Castagnoli, Jr., N. (1972), *J. Org. Chem. 37*, 1268.

Dagne, E., and Castagnoli, Jr., N. (1972a), *J. Med. Chem. 15*, 840.

Dagne, E., and Castagnoli, Jr., N. (1972b), *J. Med. Chem. 15*, 356.

Goodfriend, T. L., Fasman, G. D., Kemp, D., and Levine, L. (1966), *Immunochemistry 3*, 223.

Greenwood, F., Hunter, W., and Glover, J. (1963), *Biochem. J. 89*, 114.

Hucker, H. B., Gillette, J. R., and Brodie, B. B. (1960), *J. Pharmacol. Exp. Ther. 129*, 94.

Isaac, P. F., and Rand, M. J. (1972), *Nature (London) 236*, 308.

Landsteiner, K. (1945), The Specificity of Serological Reactions, Cambridge, Harvard Univ. Press.

Mason, S. F. (1959), *J. Chem. Soc.*, 1253.

McKennis, Jr., H. (1965), *in* Tobacco Alkaloids and Related Compounds, von Euler, U. S., Ed., Oxford, Pergamon Press, p 53.

McKennis, Jr., H., Turnball, L. B., and Bowman, E. R. (1958a), *J. Amer. Chem. Soc. 80*, 6957.

McKennis, Jr., H., Turnbull, L. B., Bowman, E. R., and Tamacki, E. (1963), *J. Org. Chem. 28*, 383.

McKennis, Jr., H., Turnbull, L. B., Wingfield, Jr., H. N., and Dewey, L. J. (1958b), *J. Amer. Chem. Soc. 80*, 1634.

Papadopoulos, N. M. (1964), *Can. J. Biochem. 42*, 435.

Papadopoulos, N. M., and Kintzios, J. A. (1963), *J. Pharmacol. Exp. Ther. 140*, 269.

Ståhlhandske, T. (1970), *Acta Physiol. Scand. 78*, 236.

Tschitschibabin, A. E., and Kirssanow, A. W. (1924), *Ber. 57*, 1163.

Turner, D. M. (1969), *Biochem. J. 115*, 889.