The Metabolism of [^{14}C]Nicotine in the Cat

By D. M. TURNER

Tobacco Research Council Laboratories, Harrogate, Yorks.

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The metabolism of [$2'\text{-}^{14}\text{C}$]nicotine given as an intravenous injection in small doses to anaesthetized and unanaesthetized cats has been studied. A method is described for the quantitative determination of [^{14}C]nicotine and [^{14}C]cotinine in tissues and body fluids. Nanogram amounts of these compounds have been detected. After a single dose of $40\text{ }\mu\text{g.}$ of [^{14}C]nicotine/kg., 55% of the injected radioactivity was excreted in the urine within 24 hr., but only 1% of this radioactivity was unchanged nicotine. [^{14}C]Nicotine is metabolized extremely rapidly, [^{14}C]cotinine appearing in the blood within 2.5 min. of intravenous injection. [^{14}C]Nicotine accumulates rapidly in the brain and 15 min. after injection 90% of the radioactivity still represents [^{14}C]nicotine. Metabolites of [^{14}C]nicotine have been identified in liver and urine extracts. [^{14}C]Nicotine-1'-oxide has been detected in both liver and urine.

The major metabolic pathways and detoxification products of nicotine have been elucidated for a number of species by McKennis and co-workers (McKennis, 1965; Papadopoulos & Kinzios, 1963; Papadopoulos, 1964*a,b*). However, the time-course of accumulation and disappearance of nicotine in tissues and body fluids has not been extensively studied, except by Schmitterlw and colleagues (Appelgren, Hansson & Schmitterlw, 1962; Hansson & Schmitterlw, 1962; and Schmitterlw, Hansson, Andersson, Appelgren & Hoffman, 1967). These workers used the technique of whole-body radioautography, so that results were only obtained from each animal at one particular time after administration of nicotine.

Accurate measurement of small amounts of nicotine in blood and tissues has not until recently been possible. An indirect estimate can be obtained, however, by measuring the pharmacological response to a puff of cigarette smoke introduced into the lungs of a cat and comparing this with an intravenous injection of nicotine (Armitage, 1965). Some of the effects of tobacco smoke may be matched very closely by a suitable intravenous injection of nicotine, and it has been calculated that a cigarette smoker gets a dose of nicotine, equivalent to 1 or $2\text{ }\mu\text{g./kg.}$ administered intravenously, every time he inhales a puff of smoke. Such small amounts of nicotine have been shown to have effects on the central nervous system of cats and rats (Armitage, Hall & Morrison, 1968).

The object of the present investigations was to study the metabolism of nicotine in cats after a single intravenous injection and in particular to measure

blood concentrations of nicotine and how long these concentrations were maintained. A method was therefore developed for the measurement of small amounts of nicotine and cotinine in body fluids and tissues after administration of [$2'\text{-}^{14}\text{C}$]nicotine.

MATERIALS AND METHODS

(-)-[^{14}C]Nicotine hydrogen tartrate labelled in the 2'-position of the pyrrolidine ring was synthesized in these laboratories by Dr H. Roderick by the method of Decker & Sammeck (1964). Specific radioactivity of the product was 20 mc/m-mole. Radiochemical purity was constantly checked by t.l.c. and paper chromatography, and by radioautography; the [^{14}C]nicotine showed evidence of radiochemical decomposition to the extent of 1.9% over a 3 month period. All samples of radioactive material, as aqueous solutions, were stored frozen in small plastic containers.

A small sample of (-)-[$2'\text{-}^{14}\text{C}$]cotinine (1.4 nc) was obtained by chromatography of a methylene dichloride-methanol extract from the liver of a cat that had received an intravenous dose of $40\text{ }\mu\text{g.}$ [^{14}C]nicotine/kg. ($13.5\text{ }\mu\text{C}$). Two solvent systems were used, as described below, and active material was eluted with methylene dichloride. T.l.c. of a sample of eluate followed by radioautography revealed a single spot of radioactivity.

Samples of demethylcotinine and γ -oxo- γ -(3-pyridyl)-methylbutyramide were generously given by Professor H. McKennis jun., Richmond, Va., U.S.A. Nicotine 1'-oxide was synthesized by Dr H. Roderick and a sample of (-)-cotinine was obtained from Dr Chesterfield of the Imperial Tobacco Group Ltd., Bristol. Nicotinic acid was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6. Nornicotine was obtained from Fluka A. G. Chemische Fabrik, Buchs, Switzerland. All other reagents and solvents were of A.R. grade.

Measurement of radioactivity. A Packard Tri-Carb

model 3003 liquid-scintillation spectrometer (Packard Instruments Ltd., Wembley, Middlesex) was used for measurement of sample radioactivity. Unless otherwise stated the liquid scintillator used was that described by Evans (1961), namely dioxan-ethanol-xylene (500 ml. of each) containing naphthalene (120 g.), 2,5-diphenyloxazole (7.5 g.) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (75 mg.). In this system ^{14}C was measured with a maximum efficiency of 70%. Efficiency of radioactivity measurements was determined by the channels-ratio method, samples being counted to at least 1% accuracy.

Paper chromatography. Labelled compounds in solvent extracts of urine and tissues were separated by descending chromatography on Whatman no. 1 paper at room temperature. One- and two-dimensional chromatography was employed, with an alkaline solvent system [*n*-butanol-ethanol-0.5M-NH₃ (4:1:1, by vol.)] or an acidic system [90% formic acid-*sec*-butanol-water (14:7:11, by vol.)].

Thin-layer chromatography. Kieselgel G (E. Merck A.-G., Darmstadt, Germany) was layered on standard glass plates (20 cm. × 20 cm. or 10 cm. × 10 cm.). The thickness of the layer was 0.25 mm. Before use the plates were heated at 110° for 30 min. (Stahl, 1962). Ascending chromatography was performed with two solvent systems, benzene-ethanol-acetone-aq. NH₃ (sp.gr. 0.88) (50:5:40:5, by vol.) (TLC1) and *n*-butanol-ethanol-2.5M-NH₃ (4:1:1, by vol.) (TLC2).

After development, paper and thin-layer chromatograms were dried and Koenig-positive zones of radioactivity detected by spraying with ethanolic 1% (w/v) *p*-amino-benzoic acid followed by exposure to cyanogen bromide vapour.

Radioautography. Paper and thin-layer chromatograms were overlaid on Kodirex X-ray film (Kodak Ltd., London W.C.2). Chromatograms and film were kept pressed together at room temperature for up to 4 weeks.

Animals. Experiments were performed on cats of either sex weighing 2-3 kg. and bred in these laboratories. Except when specifically stated three cats were used for each determination of tissue and body-fluid radioactivity. For studies on excretion of radioactivity, unanaesthetized cats were fitted with a small valve (Hall, Gomersall & Heneage, 1968) which was attached to the skull. The valve was connected to a cannula inserted into the left jugular vein, thus allowing an injection of [^{14}C]nicotine. Animals were allowed to recover for one week and housed in a stainless-steel metabolism cage for collection of urine and faeces.

In experiments on anaesthetized cats, chloralose (80 mg./kg.) (Koch-Light Laboratories Ltd., Colnbrook, Bucks) was injected intravenously after induction with ether. Blood samples were obtained from a polythene T cannula inserted in the left carotid artery. [^{14}C]Nicotine, as the hydrogen tartrate, in saline solution was injected via a cannula inserted in the right femoral vein. Urine was obtained continuously from a catheter inserted into the bladder via the urethra. Samples of bile were obtained by cannulation of the common bile duct. Samples of cerebrospinal fluid were obtained by perfusing the cerebral ventricles with artificial cerebrospinal fluid (Merlis, 1940) at a rate of 0.1 ml./min. via a Collison cannula (Feldberg & Sherwood, 1953), inserted into the left lateral ventricle. Perfusate was collected from a needle inserted through the cisternal membrane (Bhattacharya & Feldberg, 1958). Gastric juice was obtained at the end of experiments from animals starved during the previous day.

The radioactivity of certain body fluids could be counted directly without prior extraction. Samples of urine or artificial cerebrospinal fluid (250 μl .), bile (50 μl .) or trichloroacetic acid extracts of tissues (100 μl .) were added directly to the vial, which contained 15 ml. of scintillator. Powdered silica from thin-layer plate scrapings was added directly to the vial containing scintillator and 4% (w/v) Cab-O-Sil (Packard) (Snyder & Stephens, 1962; Snyder, 1964).

Total radioactivity of blood samples and tissue homogenates was determined by oxygen-flask combustion by a modification of the Kalberer & Rutschmann (1961) method. $^{14}\text{CO}_2$ was taken up into ethanolamine-methanol (1:19, v/v) and 10 ml. of the resulting solution added to 10 ml. of scintillator (4 g. of 2,5-diphenyloxazole/l. of toluene). Counting efficiencies were within the range 22-26%. Corrections for quenching were made by use of *n*-[^{14}C]hexadecane dissolved in toluene as internal standard. A standard of [^{14}C]nicotine was added to 100 μl . of blood or an inactive liver extract and burned with each set of samples.

Extraction of nicotine and cotinine from blood. To 1 ml. of oxalated arterial or venous blood was added 1 ml. of an aqueous solution containing nicotine hydrogen tartrate (2 mg./ml.) and 0.5 ml. of trypsin (4 mg./ml.). The mixture was incubated for 60 min. at 37°, made alkaline with 50 μl . of 20% NaOH and extracted with 3 × 3 ml. of methylene dichloride. After addition of each volume of methylene dichloride the tubes were gently shaken for 2 min., centrifuged for 5 min. at 4000 rev./min. and the lower organic layer was removed. A blank and two blood samples to which had been added 10 μl . and 100 μl . of [^{14}C]nicotine respectively were extracted at the same time.

Urine and other body fluids were similarly extracted, with the exception that trypsin digestion was omitted and 0.5 ml. of water added to make up the volume. Tissues, removed *post mortem*, were washed with ice-cold saline, blotted, weighed and deep-frozen until required. Each tissue was homogenized in 5 vol. of saline containing 2 mg. of nicotine/ml., in a Griffiths glass-tube homogenizer at room temperature. To a sample of homogenate (2 ml.) was added an equal volume of 10% trichloroacetic acid slowly with shaking. Standards were made by adding [^{14}C]nicotine to a liver and brain homogenate and extracting similarly. The tubes were centrifuged for 5 min. at 4000 rev./min. and the clear supernatants removed. The radioactivity of 200 μl . portions was counted directly by adding them to the dioxan scintillator (15 ml.). Portions of homogenates burned to $^{14}\text{CO}_2$ were not significantly different in radioactivity from the trichloroacetic acid extracts. Portions (1 ml.) of the trichloroacetic acid supernatants were extracted by a method identical with that used for urine.

Pooled methylene dichloride extracts from each tissue or blood sample, acidified with dil. HCl (25 μl .), were added to a counting vial and evaporated to small volume in a stream of N₂. Liquid scintillator (15 ml.) was added and the vial was tightly capped, shaken and counted. It was not possible to extract all the radioactivity from body fluids or tissue extracts. Some remained in the aqueous phase. The proportion of this unextracted radioactivity varied with the type of sample and the time at which the sample was taken after administration of [^{14}C]nicotine. Recoveries of standard amounts of [^{14}C]nicotine were in the range 97-102%. [^{14}C]Cotinine in standard amounts was recovered nearly quantitatively in the range 95-99%.

Quantitative assay of nicotine and cotinine. Samples were extracted into methylene dichloride as described above with the exception that nicotine (50 $\mu\text{g.}$) and cotinine (25 $\mu\text{g.}$) were added as carriers. Pooled organic extracts, after being made slightly acidic, were evaporated to small volume under N_2 in conical centrifuge tubes. During concentration the sides of the tubes were washed down with small amounts of methylene dichloride. Samples were finally adjusted to 100 $\mu\text{l.}$ with a methylene dichloride-methanol solution (2:1, v/v) and portions used for t.l.c. in solvent system TLCl. Plates were developed and stained as described above, [^{14}C]nicotine and [^{14}C]cotinine being identified by the position of the carrier. For each extract the zones corresponding to these two compounds were scraped off and added separately to counting vials containing 4% (w/v) Cab-O-Sil. The remaining silica layer, containing unidentified metabolites, was also scraped off and added to a vial. The radioactivity of samples was then counted in the usual way. Recoveries of standard amounts of [^{14}C]nicotine and [^{14}C]cotinine added to blood or tissue and carried through the whole procedure were within the range 80–90%. Radioautography of blood and tissue extracts revealed no radioactive material sufficiently near the [^{14}C]nicotine and [^{14}C]cotinine spots to contaminate them, the major portion of extractable, but unidentified radioactivity remaining at or near the origin.

Identification of metabolites of [^{14}C]nicotine. Livers from anaesthetized animals killed 2 hr. after intravenous injection of [^{14}C]nicotine (40 $\mu\text{g./kg.}$) were homogenized in 5 vol. of methylene dichloride-methanol (2:1, v/v) in a Waring Blender. The homogenate was filtered and the organic layer of the filtrate separated from a small aqueous layer. The former was evaporated to dryness and the residue shaken with 2 $\times$ 5 ml. of methanol. This fraction contained 52% of the tissue radioactivity. The clear solution was concentrated to 1 ml. and a 200 $\mu\text{l.}$ sample subjected to two-dimensional chromatography on paper with the solvent systems mentioned above. Unlabelled nicotine and certain metabolites were added to the same papers before development. After development, papers were stained for Koenig-positive material and subjected to radioautography. Labelled metabolites identified by radioautography alone were eluted from the paper with methylene dichloride-methanol (2:1, v/v) and the concentrated eluates subjected to t.l.c. For identification of labelled and unlabelled material the same technique was used as after paper chromatography.

Urine obtained from animals killed after 2 hr. was concentrated to small volume (approx. 5 ml.) and a sample of radioactive material was eluted from a small strip of Whatman no. 1 paper with the alkaline solvent system. The eluate was acidified with HCl and evaporated to dryness *in vacuo* at 60°. The residue was shaken with 2 $\times$ 4 ml. of methanol and after addition of 25 $\mu\text{l.}$ of m-NaOH, concentrated again to 1 ml. By this method 90% of the original urine radioactivity was recovered. Samples of the methanol extract were subjected to paper chromatography and t.l.c. in the presence of unlabelled nicotine and certain metabolites, as for liver extracts.

RESULTS

[^{14}C]Nicotine in blood. Fig. 1 shows the amounts of [^{14}C]nicotine and [^{14}C]cotinine appearing in blood over a 2 hr. period after intravenous injection of

Fig. 1. Carotid blood concentrations of [^{14}C]nicotine and [^{14}C]cotinine over a 2 hr. period after an intravenous injection of 40 $\mu\text{g.}$ of [^{14}C]nicotine/kg. (5.4 $\mu\text{C/kg.}$). Vertical bars represent the S.E.M. ○, Nicotine; ●, cotinine.

[^{14}C]nicotine. The fall in the concentration of nicotine was extremely rapid and after 5 min. the blood contained only 1% of the injected dose. Metabolism to cotinine was extremely rapid also, significant amounts of this compound appearing in the blood 2.5 min. after injection although high concentrations did not occur. A single experiment was performed on a cat whose liver had been removed from circulation by ligating the portal vein and hepatic artery close to the liver and cannulating the vein distal to the tie, so that venous return to the liver could be channelled into the jugular vein. Chromatographic and radioautographic studies showed that the rate of decline of radioactivity in the blood of this animal was somewhat slower and [^{14}C]cotinine was not detected until some 30 min. after injection.

A certain proportion of radioactivity remained in the extracted blood and although combustion analysis showed that the proportion of unextracted material was negligible over the first 10–15 min., after 2 hr. had elapsed some 40% of the radioactivity remained in the blood. Table 1 shows the proportions of [^{14}C]nicotine and [^{14}C]cotinine in blood. The proportion of unidentified material that was extractable remained at, or very close to, the origin on t.l.c.

Excretion of radioactivity in urine and faeces. Radioactivity in urine collected for 24 hr. periods during 3 days from three unanaesthetized animals given 40 $\mu\text{g.}$ of [^{14}C]nicotine/kg. (5.4 $\mu\text{C/kg.}$) was measured before extraction. Fig. 2 shows the output of radioactivity. In 3 days 70% of the injected dose had been excreted via urine, 55% being excreted in the first 24 hr. In contrast, however, the first 24 hr. collection of faeces contained only 0.5%

Table 1. *Radioactivity representing nicotine and cotinine in carotid blood of the cat*

Results are expressed as percentage of the total radioactivity ($\pm$ S.E.M.), after an intravenous injection of 40 μ g. of [14 C]nicotine/kg. (5.4 μ C/kg.)

	Time after injection (min.)								
	0.5	1	2.5	5	10	15	30	60	120
Nicotine	100.2 $\pm$ 0.7	98.7 $\pm$ 0.6	85.1 $\pm$ 0.7	64.6 $\pm$ 0.9	45.7 $\pm$ 1.4	42.9 $\pm$ 0.3	29.2 $\pm$ 0.6	23.6 $\pm$ 1.3	21.3 $\pm$ 1.3
Cotinine	0	0	4.8 $\pm$ 0.1	9.5 $\pm$ 0.2	16.4 $\pm$ 0.6	19.2 $\pm$ 0.3	23.3 $\pm$ 0.5	26.6 $\pm$ 1.5	25.5 $\pm$ 1.3

Fig. 2. Urinary excretion pattern of radioactivity over a 72hr. period after an intravenous injection of 40 μ g. of [14 C]nicotine/kg. (5.4 μ C/kg.). Vertical bars represent the S.E.M. ●, Anaesthetized animals; ○, unanaesthetized animals.

of the total dose and a second 24hr. sample contained negligible amounts of radioactivity.

Results obtained on urinary excretion of radioactivity for periods of 30min.–8hr. after injection in anaesthetized animals are included in Fig. 2, since the output of radioactivity for both unanaesthetized and anaesthetized animals appears to lie on the same curve. At the end of these experiments the bladder contents were collected and pooled with urine obtained through the catheter. In all experiments urinary pH was monitored and found to lie within the range 6.7–7.2. Attempts were made, by using intravenous infusions of sodium dihydrogen phosphate, to acidify the urine, so that the variation in urinary nicotine excretion with pH might be studied. However, no changes were observed with concentrations of the phosphate in the range 1–50mg./ml.

Qualitative studies on the excretion of [14 C]nicotine and its metabolites were performed on pooled urine samples from anaesthetized cats. Nine radioactive compounds were found, of which six have been identified, namely nicotine, cotinine, nornicotine, demethylcotinine, pyridylacetic acid

and nicotine 1'-oxide. Labelled metabolites were identified by radioautography after paper chromatography in two dimensions, followed by elution and t.l.c. in two solvent systems. Non-radioactive carriers were identified by spraying with Koenig reagent. Table 2 shows the R_F values and other relevant data for the identified compounds.

Quantitative studies on the urinary excretion of unchanged [14 C]nicotine and its metabolite [14 C]cotinine were also performed. Results obtained from two animals at each time are shown in Table 3, which shows that the amounts of nicotine and cotinine excreted are very small and even in 24hr. only 1% of the unchanged [14 C]nicotine is excreted. A large proportion of the radioactivity in the urine samples was not extractable into methylene dichloride. Radioautograms, obtained after t.l.c. in system TLC1, of extracted urine samples revealed at least eight radioactive spots, four of which were identifiable as nicotine, cotinine, nornicotine and demethylcotinine. [14 C]Nicotine and [14 C]cotinine were well separated and in all extracted samples the bulk of the radioactivity remained at the origin.

Excretion of radioactivity into bile, cerebrospinal fluid and gastric juice. Bile samples, collected over a 4hr. period, showed an increase in radioactivity that was maximal 30min. after injection (Fig. 3). Qualitative chromatographic studies on extracted bile revealed [14 C]nicotine and [14 C]cotinine in all samples. Over the period of experiment 0.5% of the injected dose was released into the bile. In two experiments a significant but small amount of radioactivity was released into the artificial cerebrospinal fluid perfusing the cerebral ventricles (Fig. 3). Maximum output occurred during the first 10min. of collection. Chromatography and radioautography revealed that the radioactivity was largely due to [14 C]nicotine. Other metabolites, if present, were not sufficiently radioactive to be detected.

Gastric juice collected 2hr. after injection contained a relatively large amount of radioactivity ($5.0 \pm 1.7\%$). Quantitative studies performed on portions of gastric juice revealed [14 C]nicotine ($56.8 \pm 4.2\%$) and [14 C]cotinine ($18.7 \pm 4.1\%$). Other unidentified metabolites were also detected.

[14 C]Nicotine and its metabolites in tissues. Contents of nicotine and cotinine in various tissues were

Table 2. R_F values on t.l.c. and paper chromatography of [^{14}C]nicotine and some labelled metabolites extracted from urine and livers of cats

The colours developed over a period of 30 min. after exposure to cyanogen bromide and faded after 24 hr.

	R_F				
	Alkaline system	Acid system	TLC1	TLC2	Colour*
Nicotine	0.87	0.36	0.88	0.75	Yellow
Cotinine	0.78	0.43	0.65	0.67	Orange
Nornicotine	0.76	0.39	0.52	0.46	Orange
Demethylcotinine	0.68	0.32	0.43	0.64	Red-brown
γ -Oxo- γ -(3-pyridyl)- <i>N</i> -methylbutyramide	0.80	0.45	0.43	—	Pink
Nicotine 1'-oxide	0.48	0.42	0	0.29	Red-orange
Pyridylacetic acid	0.23	0.39	0	0.30	Rose red

Table 3. Excretion of radioactivity in urine of cats after an intravenous injection of 40 μg . of [^{14}C]nicotine/kg. (5.4 $\mu\text{C/kg}$.)

The 2 hr. and 4 hr. values refer to anaesthetized animals, the 24 hr. values to unanaesthetized animals. Results are quoted as the mean $\pm$ S.E.M.

	Time after injection		
	2 hr.	4 hr.	24 hr.
% as [^{14}C]nicotine	0.66 $\pm$ 0.04	0.77 $\pm$ 0.03	1.23 $\pm$ 0.24
% as [^{14}C]cotinine	0.27 $\pm$ 0.04	0.48 $\pm$ 0.03	0.43 $\pm$ 0.04

Fig. 3. ^{14}C Radioactivity in bile and cerebrospinal fluid perfusate over a 4 hr. period after an intravenous injection of 40 μg . of [^{14}C]nicotine/kg. (0.49 $\mu\text{C/kg}$.). Artificial cerebrospinal fluid was perfused through the cerebral ventricles at a rate of 0.1 ml./min. Vertical bars represent the S.E.M. Two cats were used for the studies on cerebrospinal fluid radioactivity. ●, Bile; ○, cerebrospinal fluid perfusate.

determined over a 2 hr. period after a single intravenous injection of 40 μg . of [^{14}C]nicotine/kg. Table 4 shows the results obtained, [^{14}C]nicotine and [^{14}C]cotinine contents being expressed as ng./g. wet weight of tissue. All tissues showed a maximum [^{14}C]nicotine content 5 min. after injection and

thereafter radioactivity declined. [^{14}C]Cotinine contents rose very slowly in most tissues over the 2 hr. period, but in liver there was a significant increase in cotinine, reaching a maximum after 15 min. Adrenal tissue also showed a smaller maximum concentration of cotinine after 15 min. At the end of the experiment most tissues contained small amounts (approx. 5 ng./g.) of cotinine. Stomach and adrenal tissues however contained high concentrations. Although amounts of total radioactivity/organ were all maximal 5 min. after injection, the proportion of that radioactivity representing nicotine varied markedly, as Table 5 shows. In brain at least 90% of the radioactivity was due to nicotine over a period of 15 min. Liver, kidney and lung, however, contained much smaller proportions of nicotine even 5 min. after injection, lung tissue containing a very small proportion of [^{14}C]nicotine indeed. The other tissues showed contents intermediate between those of brain and kidney.

Paper chromatography and t.l.c. coupled with radioautography revealed five identifiable metabolites of nicotine in liver extracts obtained 2 hr. after injection. These metabolites were identical with those previously observed in urine. The radioactive compounds co-chromatographed with authentic standards in four different solvent systems. R_F values of these compounds, shown in Table 1, were consistent with those reported in the literature (Papadopoulos, 1964a). In addition, at least five as yet unidentified radioactive materials were found in liver, two being present also in urine extracts. No evidence of [^{14}C]nicotinamide or [^{14}C]nicotinic acid was found.

Nicotine 1'-oxide has recently been identified in the urine of cigarette smokers and, *in vitro*, as a metabolite of nicotine in animal liver homogenates (J. Booth & E. Boyland, personal communication). Their investigation also demonstrated that the oxide could be resolved into two optical isomers on paper chromatography in a butanol-propanol-ammonia solvent system.

Table 4. [^{14}C]Nicotine and [^{14}C]cotinine in tissues of the cat, expressed as ng./g. wet weight of tissue ($\pm$ S.E.M.), after intravenous injection of 40 μg . of [^{14}C]nicotine/kg. (5.4 μC /kg.)

		Time after injection (min.)				
		5	15	30	60	120
Adrenals	Nicotine	66.3 $\pm$ 1.8	43.4 $\pm$ 1.2	16.8 $\pm$ 3.2	10.2 $\pm$ 0.1	4.9 $\pm$ 0.3
	Cotinine	6.6 $\pm$ 0.6	10.1 $\pm$ 1.0	7.0 $\pm$ 0.5	8.5 $\pm$ 0.6	12.6 $\pm$ 1.1
Brain	Nicotine	96.9 $\pm$ 0.5	34.5 $\pm$ 0.9	17.0 $\pm$ 0.1	11.1 $\pm$ 0.1	3.8 $\pm$ 0.2
	Cotinine	0.8 $\pm$ 0.1	0.9 $\pm$ 0.1	2.5 $\pm$ 0.8	3.6 $\pm$ 0.6	3.6 $\pm$ 0.1
Liver	Nicotine	76.9 $\pm$ 3.3	44.1 $\pm$ 1.3	33.2 $\pm$ 2.2	20.3 $\pm$ 1.2	3.2 $\pm$ 0.3
	Cotinine	13.7 $\pm$ 2.6	29.0 $\pm$ 2.6	10.8 $\pm$ 2.0	10.8 $\pm$ 0.2	4.8 $\pm$ 0.2
Kidney	Nicotine	111.0 $\pm$ 7.8	59.6 $\pm$ 4.3	44.7 $\pm$ 1.6	33.2 $\pm$ 1.1	11.7 $\pm$ 3.8
	Cotinine	5.2 $\pm$ 0.2	1.4 $\pm$ 0.1	9.6 $\pm$ 0.8	8.0 $\pm$ 0.1	5.5 $\pm$ 0.9
Lung	Nicotine	47.5 $\pm$ 4.1	31.4 $\pm$ 3.0	21.9 $\pm$ 2.6	8.6 $\pm$ 0.4	4.8 $\pm$ 1.1
	Cotinine	2.0 $\pm$ 0.4	1.5 $\pm$ 0.6	4.1 $\pm$ 0.1	4.0 $\pm$ 0.1	2.7 $\pm$ 0.5
Skeletal muscle	Nicotine	8.2 $\pm$ 0.5	6.7 $\pm$ 0.1	6.2 $\pm$ 1.0	5.9 $\pm$ 0.2	1.9 $\pm$ 0.4
	Cotinine	0.0	0.4 $\pm$ 0.1	1.2 $\pm$ 0.4	1.6 $\pm$ 0.0	2.3 $\pm$ 0.2
Stomach	Nicotine	76.5 $\pm$ 5.5	35.7 $\pm$ 6.8	23.3 $\pm$ 5.3	9.5 $\pm$ 0.6	6.7 $\pm$ 0.3
	Cotinine	1.8 $\pm$ 0.0	2.3 $\pm$ 0.4	2.2 $\pm$ 0.1	6.6 $\pm$ 0.3	8.9 $\pm$ 0.3

Table 5. Radioactivity representing [^{14}C]nicotine in tissues of the cat

Results are expressed as a percentage of the total radioactivity ($\pm$ S.E.M.), after an intravenous injection of 40 μg . of [^{14}C]nicotine/kg. (5.4 μC /kg.).

		Time after injection (min.)				
		5	15	30	60	120
Adrenals		83.9 $\pm$ 4.9	66.5 $\pm$ 1.5	42.0 $\pm$ 5.4	31.7 $\pm$ 0.6	17.2 $\pm$ 0.8
Brain		95.3 $\pm$ 0.8	89.6 $\pm$ 0.3	69.2 $\pm$ 5.2	59.6 $\pm$ 0.4	31.5 $\pm$ 0.5
Liver		43.3 $\pm$ 0.2	25.8 $\pm$ 0.2	20.9 $\pm$ 1.7	14.3 $\pm$ 1.1	4.3 $\pm$ 0.1
Kidney		57.0 $\pm$ 2.7	44.2 $\pm$ 4.0	30.4 $\pm$ 1.2	24.5 $\pm$ 0.2	13.8 $\pm$ 3.9
Lung		25.5 $\pm$ 1.0	27.8 $\pm$ 2.8	17.7 $\pm$ 1.6	9.8 $\pm$ 0.3	5.7 $\pm$ 1.4
Skeletal muscle		87.6 $\pm$ 1.6	66.5 $\pm$ 0.8	45.5 $\pm$ 2.0	45.8 $\pm$ 3.6	13.9 $\pm$ 5.2
Stomach		86.0 $\pm$ 0.5	73.8 $\pm$ 0.3	62.1 $\pm$ 2.7	41.7 $\pm$ 2.0	18.3 $\pm$ 1.7

Examination of a sample of [^{14}C]nicotine 1'-oxide extracted from cat liver in the present experiments under the above conditions revealed two radioactive spots of R_F 0.52 and 0.44. Authentic nicotine 1'-oxide also separated into two spots, which co-chromatographed with the labelled materials. These unlabelled spots were stained a red-orange colour with the Koenig reagent and appeared black under u.v. light. The two radioactive materials were present in approximately equal amounts but in the authentic materials the spot with R_F 0.52 appeared to be present in greater amounts than that with R_F 0.44.

DISCUSSION

As a contribution to a study of the effects of nicotine on the central nervous system a sensitive method was required to determine the amounts of nicotine and its metabolites in tissues and body fluids of the cat and other animals. The dose of nicotine administered in these experiments was 40 μg /kg. This represents the total amount of

nicotine that a human smoker might be expected to receive during approximately 10 min. when inhaling deeply from a single cigarette. Methods previously reported in the literature for the quantitative assay of nicotine and cotinine (Hucker, Gillette & Brodie, 1960; Beckett & Triggs, 1966; Harke, Frahm & Schultz, 1969) were less sensitive. A modification of the Hucker method (Tjälve, Hansson & Schmiterlöw, 1968) has been used to measure [^{14}C]nicotine in animal tissues, after injection of relatively large doses of [$Me-^{14}\text{C}$]nicotine. The method could conceivably be used to measure smaller concentrations of nicotine but it is not applicable to studies with [$2'-^{14}\text{C}$]nicotine, owing to the presence of many more radioactive metabolites. This labelling position for nicotine was chosen in order to study as many metabolites of [^{14}C]nicotine in cat tissues as possible.

The results presented provided quantitative evidence of the time-course of the distribution and fate of nicotine in animals, information about which until recently has been largely qualitative (Appelgren *et al.* 1962). The blood concentration of

nicotine drops sharply after injection, presumably as the compound distributes itself in the various tissues. Metabolism of nicotine occurs principally in the liver, as is well established, and is extremely rapid since [^{14}C]cotinine appears in the blood within a few minutes. The oxidation of nicotine to cotinine is believed to be important in terminating the pharmacological actions of the former (Bowman, Hansson, Turnbull & McKennis, 1964). Metabolism of nicotine to other compounds also appears to occur rapidly since varying but significant amounts of other metabolites were found in all tissues examined, even after 5 min. Although it is unlikely that metabolism occurs in all tissues, there is nevertheless a marked variation between tissues in the amounts of these compounds. This may be due to a variation in the binding capacity of the tissues. Lung tissue is the most obvious example, since even 5 min. after injection the proportion of radioactivity representing nicotine is even less than that occurring in the liver. Kidney and lung tissues of the rat have been shown to have a small capacity *in vitro* for metabolizing [^{14}C]nicotine (Hansson, Hoffman & Schmitterlw, 1964). Unless corresponding organs of the cat differ in their capacity to metabolize nicotine, the results in Tables 3 and 4 suggest that lung tissue particularly can concentrate nicotine metabolites. Brain tissue appears to be unusual in that although the content of nicotine declines with time, the proportion of radioactivity representing nicotine remains at a higher value when compared with other tissues, at least over the first 15 min. The observation of [^{14}C]nicotine as the only significant compound present in the cerebrospinal fluid perfusate is consistent with the relatively high brain-tissue content of nicotine. Presumably this high proportion of [^{14}C]nicotine in brain tissue reflects the difference in the ability of nicotine to pass through the blood-brain barrier compared with its metabolites. The amounts of radioactivity in the tissues of the adult cat over the period of time correlate well with the observations of Appelgren *et al.* (1962) and Schmitterlw *et al.* (1967) who have used the technique of whole-body radioautography to plot the distribution of radioactivity in mouse, rat and kitten tissues after injection of much larger doses of [^{14}C]nicotine. Their results suggest a rapid accumulation of radioactivity in the brain and other tissues, notably the salivary glands, and a gradual increase in radioactivity in gastric juice and stomach contents. The present observations show that about 5% of the injected radioactivity is present in gastric juice after 2 hr., the major portion of the activity representing nicotine and cotinine. High concentrations of [^3H]cotinine have been found in mouse stomach 60 min. after intravenous injection of the labelled substance (Bowman *et al.* 1964) and in the present experiments some 20% of the radio-

activity in the gastric juice was due to [^{14}C]cotinine after 2 hr. Most of this nicotine and cotinine must, together with that excreted via the bile, be reabsorbed in the intestine, where pH conditions favouring an increase in the ratio of free base to ionized material occur, since only a small fraction of injected activity appears in the faeces over a 24 hr. period.

Though the majority of injected radioactivity was excreted via the urine only small amounts of unchanged [^{14}C]nicotine and [^{14}C]cotinine appeared. This probably in part reflects the rapidity with which nicotine is metabolized. Since the pH of the urine was within the range 6.7–7.2 a proportion of nicotine reaching the kidney would be reabsorbed from the renal tubules. Over this pH range about 10–20% of the nicotine would be present as free base (Travell, 1960) which would thus aid reabsorption by the kidney. The observation that the rates of urinary excretion of radioactivity in both anaesthetized and unanaesthetized cats appear to lie on the same curve, together with the observations on the excretion rates for [^{14}C]nicotine and [^{14}C]cotinine, suggests the possibility that the anaesthetic does not have much effect on the metabolism of nicotine.

No studies on the possible release of $^{14}\text{CO}_2$ in expired air were undertaken although previous evidence suggests that little or no $^{14}\text{CO}_2$ is expired in rats and dogs after injection of randomly labelled nicotine (Ganz, Kelsey & Geiling, 1951; Fishman, 1963; Morselli, Ong, Bowman & McKennis, 1967). In contrast, however, some 6% of $^{14}\text{CO}_2$ is present in expired air if [Me- ^{14}C]nicotine is used (McKennis, Turnbull, Schwartz, Tamaki & Bowman, 1962). For $^{14}\text{CO}_2$ to be produced by metabolism of [^{14}C]nicotine in the present experiments, [^{14}C]nicotinic acid would have to be formed via side-chain oxidation from [^{14}C]pyridylacetic acid. No [^{14}C]nicotinic acid was detected in liver extracts, which supports the view that the metabolic conversion of nicotine into nicotinic acid is not a significant route in the cat (Larson, Haag & Silvette, 1961). Evidence for the presence of γ -oxo- γ -pyridyl-*N*-methylbutyramide was not obtained since it had closely similar R_F values to cotinine on paper chromatograms and an identical R_F with demethylcotinine on t.l.c.

Oxidative *N*-dealkylation of tertiary amines has been demonstrated in microsomal preparations from animal tissues fortified with NADPH and oxygen (Parke, 1968). Studies *in vitro* on rabbit liver (Hucker *et al.* 1960) showed that the conversion of nicotine into cotinine required NADPH and oxygen, and more recently nicotine 1'-oxide has been identified, *in vitro* and *in vivo*, in rabbit liver (Papadopoulos, 1964b, 1965). It was therefore of great interest to see if [^{14}C]nicotine 1'-oxide was a metabolite *in vivo* of [^{14}C]nicotine in the cat. The

studies reported here have confirmed its presence in the liver and urine of the anaesthetized cat after injection of [^{14}C]nicotine although a quantitative assay has not as yet been performed. All the other metabolites identified in both urine and liver extracts from the experiments have been reported as metabolites of nicotine in other species including man.

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