hibition in growth of nasopharyngeal carcinoma cells in culture by cardenolides is due to inhibition of amino acid accumulation.

In any event, the fact that similar structural features are important for inhibition of transport ATPase, KB cytotoxic activity, and atrial muscle inotropic activity lends support to the view that the receptors for the cardenolides in the three systems may be structurally very similar.

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Metabolism of (±)-Cotinine-2-^{14}C in the Rat[1a,2]

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A route to the synthesis of a number of nicotine metabolites bearing a ^{14}C label adjacent to the pyridine ring is described. This synthesis, which starts with the condensation of ethyl nicotinate-7-^{14}C and diethyl succinate, provides γ-(3-pyridyl)-γ-oxobutyric acid, γ-(3-pyridyl)hydroxybutyric acid, γ-(3-pyridyl)-γ-methylaminobutyric acid, cotinine, and demethylcotinine, as well as the two alkaloids nicotine and nornicotine. After administration of (±)-cotinine-2-^{14}C to the rat, the urine was examined chromatographically for radioactive and Koenig-positive (pyridine) substances. The general pattern of excretion of the radioactive Koenig-positive substances resembled those previously encountered in some other species and paralleled that found earlier with nonisotopic material. The radioactivity of administered (±)-cotinine was eliminated with a high degree of efficiency (90–97% of the administered dose), predominantly by way of the urine. Virtually no radioactivity was encountered in expired air, suggesting little or no conversion to nicotinic acid. Demethylcotinine and γ-(3-pyridyl)-γ-oxo-N-methylbutyramide were identified in the urine by isotopic dilution. Similar experiments where carrier nicotine was employed failed to provide evidence for the reversibility of the metabolic reaction nicotine → cotinine.

In the metabolism of (−)-nicotine in many mammalian species,[3] (−)-cotinine is one of the early metabolites that appears during the course of a long series of progressive oxidations leading eventually to the urinary excretion of 3-pyridylacetic acid. The experimental administration of large quantities of (−)-cotinine to the dog gives rise to (−)-demethylcotinine, one of the later intermediates in the series. Chromatographic studies on human urine after administration of (−)-nicotine suggest[3d] the presence of demethylcotinine, while administration of cotinine gives rise[4] to no, or insignificant, urinary excretion of the demethyl compound.

In view of the possible mammalian conversion of nicotine to nornicotine, a reaction[5] that was later to receive positive experimental support[3f,6] with rabbit liver and dog liver preparations, Wada, *et al.*,[7] administered nornicotine to dogs and studied the urinary metabolites. Among these, demethylcotinine was present. From these findings and additional data, there thus arose the possible alternate routes (Scheme I) to the formation of demethylcotinine in the dog.

Administration of (−)-nicotine-methyl-^{14}C to the rat gives[8] rise to significant quantities of ^{14}C activity in the respiratory CO_2. This excretion could presumably reflect several alternate routes of degradation, including (a) conversion of nicotine to nornicotine, (b) conversion of cotinine to demethylcotinine, and (c) oxidative attacks proceeding initially by way of the pyridine ring of nicotine.

Conversion of (−)-cotinine to (−)-demethylcotinine in the rat has already been established[9] on a semiquantitative basis using nonisotopic material.

We were thus led to consider and develop methods for the synthesis of pyrrolidone-ring-labeled cotinine-^{14}C and to determine some aspects of its metabolism and intermediary role as a nicotine metabolite in the rat.

Experimental Section

General Procedures.—Paper chromatograms were prepared on Whatman No. 1 paper and developed in solvent B,[10] 0.5 *N* NH_4OH–95% EtOH–*n*-BuOH (1:1:4 by volume), and solvent C,[10] 90% formic acid–*sec*-BuOH–H_2O (14:75:11 by volume). Thin-layer chromatograms were prepared on silica gel (Eastman Chromogram Sheet, Type K 301R) and developed in solvent K,[11,12] MeOH–$CHCl_3$ (15:85 by volume). Koenig-positive zones were disclosed as previously described.[7] Melting points were determined on the hot stage.

(1) (a) These studies were aided by grants from the American Medical Association Education and Research Foundation, The Council for Tobacco Research—U.S.A., and The American Tobacco Company. Presented in part at the 44th Annual Meeting of the Virginia Academy of Science, May 5, 1966. A preliminary report has appeared.[2] (b) To whom inquiries should be directed.

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SCHEME I

NORNICOTINE I

NICOTINE II

COTININE III

γ-(3-PYRIDYL)-γ-OXOBUTYRAMIDE IV

DEMETHYLCOTININE V

γ-(3-PYRIDYL)-γ-OXO-N-METHYLBUTYRAMIDE VI

γ-(3-PYRIDYL)-γ-OXOBUTYRIC ACID VII

4-(3-PYRIDYL)-3-BUTENOIC ACID VIII

γ-(3-PYRIDYL)-γ-HYDROXYBUTYRIC ACID IX

5-(3-PYRIDYL)TETRAHYDROFURANONE-2 X

γ-(3-PYRIDYL)BUTYRIC ACID XI

3-PYRIDYLACETIC ACID XII

Radioactive determinations were made in a Nuclear-Chicago 720 Series liquid scintillation system. Isolated compounds, with the exception of highly colored picrates, were counted in scintillator solution I (butyl-PBD, 2-(4-*t*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxidiazole (7 g) in 1 l. of solution of toluene–ethylene glycol monomethyl ether (2:1 by volume)). Respiratory CO_2 in the animal experiments was collected in solution II[13] (ethanolamine–ethylene glycol monomethyl ether (1:2 by volume)). Aliquots (3 ml) were added to 15 ml of solution I for counting. Dried feces and picrates were burned by the Schöniger method in a modified Thomas-Ogg flask.[14] The resultant CO_2 was collected in solution III (15 ml of solution I and 3 ml of solution II). Confirmation of the radioactivity of the picrates (0.4–0.7 mg) was achieved by direct counting in scintillator solution IV (BBOT, 2,5-bis[2-(5-*t*-butylbenzoxazolyl)]thiophene (4 g), in 1 l. of toluene–ethylene glycol monomethyl ether (2:1 by volume)). Counts were corrected by the channel-ratio procedure and by internal standards.

Ethyl Nicotinate-7-^{14}C.—Nicotinic acid-7-^{14}C was esterified in EtOH–H_2SO_4 during 24 hr. The cooled solution was treated with an excess of $CHCl_3$, ice, and concentrated NH_4OH to pH 9. Evaporation of the $CHCl_3$ layer and two subsequent $CHCl_3$ extractions provided the ester, 1.46 g (R_f 0.73, solvent K), 79%.

Ethyl γ-(3-Pyridyl)γ-oxo-β-carbethoxybutyrate-γ-^{14}C.—A solution of ethyl nicotinate (above) in 25 ml of benzene was concentrated to 2 ml, treated with 290 mg of NaH (55% in mineral oil) under N_2, and then 2 drops of absolute EtOH. Freshly distilled ethyl succinate (870 mg) was added to the stirred mixture under reflux during 15 min. At the end of 1 additional hr, the stirred mixture was cooled to 0° and treated with 10 ml of 1 *N* HCl. After removal of an upper organic layer (I), the cooled aqueous phase was adjusted to pH 8 with Na_2CO_3 for extraction with three portions of ether (II). The organic layer (I) was extracted with 10 ml of cold 1 *N* HCl. The acidic solution was alkalinized

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TABLE I
EXCRETION OF RADIOACTIVITY IN 24 HR FOLLOWING INTRAPERITONEAL INJECTION OF (±)-COTININE-^{14}C TO FEMALE ALBINO RATS

Rat no.	Wt, g	Dose mg/kg	Dose dpm × 10^5	Dose recovered, % Feces	Respiratory air	Urine	Total	Recovery from processed urine, % CHCl$_3$ extract	Aq phase
1	245	10.4	6.9	1.1	0.0	97	98.1	79	15
2	250	10.2	6.9						
3	130	10.1	3.6	1.3	0.0	97	98.3	74	16
4	125	10.4	3.6						
5	174	15.7	7.4	0.3	0.0	98	98.3	78	16
6	170	9.6	4.4	1.9	0.0	90	91.9	72	14

and extracted with ether (III) as above. The residue from evaporation of the combined extracts (II and III) was fractionated at bath temperature, 110° (3 mm), to yield 500 mg of ethyl nicotinate as a distillate and 855 mg (33%, based on ethyl nicotinate) of ethyl γ-(3-pyridyl)-γ-oxo-β-carbethoxybutyrate as residue.

γ-(3 Pyridyl)-γ-oxobutyric Acid-γ-^{14}C.—The residual carbethoxybutyrate (above) was processed[15] to yield 347 mg (63%) of the oxo acid, mp 160–163°.

(±)-γ-(3-Pyridyl)-γ-methylaminobutyric Acid-γ-^{14}C.—The oxo-acid (above) in 3.0 g of methylamine and 30 ml of absolute EtOH was allowed to stand overnight. The mixture was hydrogenated[15] at 60° and atmospheric pressure in the presence of 5% Pt–$BaSO_4$ (150 mg) until cessation of H_2 uptake (6 hr). The residue from evaporation of the solvent at room temperature was dissolved in 1 ml of absolute EtOH. The cooled solution was treated with acetone to a persistent turbidity and scratched to yield 200 mg (53%) of product, mp 120–123°, after recrystallization from alcohol–acetone (R_f 0.15, solvent B; R_f 0.54, solvent A).

(±)-Cotinine-2-^{14}C.—The residue from evaporation of the combined mother liquors from the methylamino acid (above) was heated to 120° under N_2 for 1 hr to give cotinine (R_f 0.62, solvent K), which was recrystallized from 2-propanol–hexane, mp 52–54°.

Metabolism of (±)-Cotinine-2-^{14}C.—Female albino Wistar-strain rats (Albino Farms, Red Bank, N. J.) that were previously maintained on a diet of water and Purina Rat Chow received (Table I) intraperitoneal injections of aqueous (±)-cotinine-2-^{14}C. The animals were then housed in glass metabolism cages with water, but no food. Respiratory CO_2 was entrained in a stream of air which passed through a concentrated H_2SO_4 trap and finally into a tower of ethanolamine–ethylene glycol monomethyl ether (1:2 by volume).[13] Urine was separated from feces by a wire screen which permitted some cross contamination. Terminal bladder urine was obtained by aspiration after anesthetization of the animals with ether.

Nicotine.—An aliquot from the $CHCl_3$ extract (rats 1 and 2) containing 2.4 × 10^5 dpm was treated with 53 mg of finely powdered (−)-nicotine dipicrate. The mixture was brought slowly to dryness in a rotating evaporator and the residue was dissolved in acetone–alcohol for reevaporation of the solvent. The residue was dissolved in a small quantity of alcohol. Upon addition of water and cooling, nicotine dipicrate deposited from the mixture. The product, mp 219–220° (undepressed by admixture with an authentic sample), contained no radioactivity above background and all of the initially present radioactivity was accounted for in the mother liquors. In a similar experiment (rats 3 and 4) an aliquot (1.0 × 10^5 dpm) from the $CHCl_3$ extract was similarly treated with (−)-nicotine dipicrate (40 mg). No radioactivity above background was recovered in the carrier. Examination of an additional urine (rat 5) produced the same results.

Nornicotine.—A solution of (−)-nornicotine dipicrate (31 mg in ethanol–acetone) was added to a $CHCl_3$ aliquot (2.34 × 10^5 dpm from rats 1 and 2). The solution was processed essentially as described above and the (−)-nornicotine picrate, mp 189–191° (undepressed by admixture with an authentic sample), showed no radioactivity above background. In a similar experiment (rats 3 and 4) with 72 mg of carrier and a $CHCl_3$ aliquot (8.5 × 10^4 dpm), the carrier after four recrystallizations showed no radioactivity above background. Examination of an additional urine (rat 5) provided the same results.

Demethylcotinine.—A $CHCl_3$ aliquot (2.02 × 10^5 dpm from rats 3 and 4) was concentrated to dryness under diminished pressure. The residue was dissolved in acetone and an aliquot (1.57 × 10^5 dpm) was chromatographed on Whatman No. 1 paper (solvent B) to give four radioactive zones: R_f 0.50 (11.9% of the activity of the $CHCl_3$ fraction), R_f 0.62 (2.5% and Koenig positive), R_f 0.69 (16.8% and Koenig positive), R_f 0.75 (42.8% and Koenig positive). The remaining radioactivity was more or less randomly distributed throughout the chromatogram and the zones at R_f 0.62 and R_f 0.69 corresponding in R_f value to hydroxycotinine and demethylcotinine, respectively, failed to show complete separation.[16]

A 48-hr continuous extraction of the paper in the vicinity of the R_f 0.69 zone with ethanol removed 83% of the radioactivity there present. After removal of EtOH by evaporation, the residue was dissolved in 5 ml of acetone and warmed to dissolve 52 mg of added carrier (±)-demethylcotinine.[16] The solution was cooled and a few drops of hexane were added to hasten the precipitation of demethylcotinine. The resultant demethylcotinine, mp 110–113°, showed a constant specific activity, 328 dpm/mg, after six recrystallizations (R_f 0.47 in solvent K, corresponding in R_f value to authentic material). The recovered radioactivity corresponded to 6% of the administered dose, or 8% of the radioactivity in the $CHCl_3$ fraction.

The combined mother liquors from the recrystallization of demethylcotinine were treated with 27 mg of picric acid in ethanol. The mixture was cooled and concentrated to obtain crystalline (±)-demethylcotinine picrate. After five recrystallizations (EtOH), the product, mp 161–164° (undepressed by admixture with an authentic sample[15b]), showed a constant specific activity 142 dpm/mg, again corresponding to 6% of the administered dose.

γ-(3-Pyridyl)-γ-oxo-N-methylbutyramide.—An aliquot of the $CHCl_3$ solution from rats 1 and 2 (3.23 × 10^5 dpm) was treated with 85 mg of γ-(3-pyridyl)-γ-oxo-N-methylbutyramide in 5 ml of acetone. The solution was evaporated to dryness under diminished pressure. The residue was dissolved in a minimal quantity of acetone and cooled. Upon addition of a few drops of hexane, crystals of γ-(3-pyridyl)-γ-oxo-N-methylbutyramide formed. The product, mp 118–120° (undepressed by admixture with an authentic sample), was recrystallized five times to a constant specific activity, 97 dpm/mg, R_f 0.60 in solvent K, representing 2.5% of the radioactivity of the administered dose. In another experiment from the same group of rats, the recovery was 2.5% of the administered radioactivity. The activity recovered in the form of the oxoamide from rats 3 and 4 was 13% of the administered dose. The recovery from rat 5 was 9.0% of the administered dose, while the carrier experiment with rat 6 accounted for 9.5% of the administered dose.

γ-(3-Pyridyl)-γ-oxobutyric Acid.—The aqueous phase (rats 3 and 4) remaining from the $CHCl_3$ extraction was concentrated to dryness under diminished pressure. A solution of 50 mg of γ-(3-pyridyl)-γ-oxobutyric acid in 10 ml of alcohol was added and the mixture was heated to reflux. After removal of the solvent under diminished pressure, the residue was treated with EtOH (15 ml). After filtration, the solution was concentrated

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to 2.3 ml and cooled. Crystalline material, mp 125–130° and essentially devoid of radioactivity, was removed and discarded. Further concentration and cooling provided γ-(3-pyridyl)-γ-oxobutyric acid, mp 160–162° (undepressed by authentic material). After four recrystallizations, the product showed a constant specific activity of 210 dpm/mg (R_f 0.25 in solvent K) which was equivalent to 1.4% of the administered radioactivity. In a similar experiment with rat 6, the radioactivity recovered amounted to 1.2% of the administered radioactivity.

Results and Discussion

In previous experiments,[9] administration of (−)-cotinine to the rat led to the urinary excretion of demethylcotinine, hydroxycotinine, and γ-(3-pyridyl)-γ-oxo-N-methylbutyramide and provided paper chromatographic evidence for the presence of other metabolites. In the present series of experiments, in which (±)-cotinine-^{14}C was administered to the same species, general confirmation and extension of some earlier findings was achieved and data were obtained which indicated (Tables I and II) a very effective elimination of cotinine, as itself and in the form of numerous metabolites, consistent in R_f value with previous studies and predominantly by way of the urine.

TABLE II

PAPER CHROMATOGRAPHY OF PROCESSED URINE OF RATS AFTER INTRAPERITONEAL ADMINISTRATION OF (±)-COTININE-2-^{14}C

R_f value of zones developed in solvent B	
$CHCl_3$ extract	Remaining aq phase
0.44[a]	0.14[b]
0.66[c]	0.22[d]
0.75[e]	0.31[f]
0.83[g]	

[a] Radioactive and Koenig negative. [b] Radioactive and Koenig positive: corresponding in R_f value to γ-(3-pyridyl)-γ-methylaminobutyric acid. [c] Radioactive and Koenig positive; corresponding in R_f value to demethylcotinine and hydroxycotinine. [d] Radioactive and Koenig positive; corresponding in R_f value to γ-(3-pyridyl)-γ-oxobutyric acid. [e] Radioactive and Koenig positive; corresponding in R_f value to cotinine and γ-(3-pyridyl)-γ-oxo-N-methylbutyramide. [f] A Koenig-positive and nonradioactive zone which appeared only in chromatography of the processed urine of rat 4. [g] Koenig positive and nonradioactive.

For the radioactive studies, (±)-cotinine-2-^{14}C was prepared synthetically for the first time. The particular location of the label was selected in view of the commerical availability of nicotinic acid-7-^{14}C as starting material for the synthesis[15] of γ-(3-pyridyl)-γ-oxobutyric acid-γ-^{14}C. The latter substance, in addition to being a mammalian metabolite of nicotine, provides chemical access[7,15b,c,16] to a wide variety of metabolites of nicotine, including[15b,17,18] the alkaloid itself.[19]

In the present report details are given for the small-scale synthesis of ethyl γ-(3-pyridyl)-γ-oxo-β-carbethoxybutyrate-γ-^{14}C from ethyl nicotinate-7-^{14}C and the conversion of the carbethoxy compound to γ-(3-pyridyl)-γ-oxobutyric acid-γ-^{14}C. The subsequent reductive N-methylamination of the oxo acid to obtain (±)-γ-(3-pyridyl)-γ-methylaminobutyric acid-γ-^{14}C and (±)-cotinine-2-^{14}C was accomplished by a modification of previously described procedures[15b,16] and afforded the two desired nicotine metabolites essentially devoid of the side product γ-(3-pyridyl)-γ-hydroxybutyric acid, which accompanied the products obtained by the original methods.

In view of the long-standing discussion[20] on the possible metabolic conversion of nicotine to nicotinic acid in mammals, it was of interest to seek evidence for radioactivity in the respiratory CO_2 in rats that had received (±)-cotinine-2-^{14}C. From the 24-hr period of collection, little or no radioactivity was found in the expired air. These results, in the light of the demonstrated magnitude of the metabolism[21] of the carboxyl carbon of nicotinic acid to respiratory CO_2, suggest that the oxidation of (±)-cotinine to nicotinic acid plays no role of any great magnitude in the metabolic degradation of (±)-cotinine.

In all of the animals studied the aqueous phase remaining after $CHCl_3$ extraction of the alkalinized urine contained γ-(3-pyridyl)-γ-oxobutyric acid, indicated by the chromatograms, and amounting to 1.2–1.4% of the administered radioactivity by the isotopic dilution studied in two cases. Evidence was obtained in all of the experiments, by chromatograms and by isotopic dilution, for the presence of γ-(3-pyridyl)-γ-oxo-N-methylbutyramide and demethylcotinine. The oxo acid may have arisen as a result of the metabolic hydrolysis of the oxoamide, as previously noted,[22] or alternatively from the metabolism of demethylcotinine as previously described.[23]

Although the metabolism of nicotine (II) to demethylcotinine (V) by two alternate routes (Scheme I), one *via* the demethylation of the intermediate cotinine (III) and the other *via* the demethylation of nicotine to nornicotine (I) which is subsequently oxidized to demethylcotinine, is apparent from numerous mammalian studies *in vivo* and *in vitro*, little attention has been given to the possible reconversion of cotinine to nicotine, a reaction which can be accomplished readily by chemical reductions.[19] Since a metabolic reaction of this type and a subsequent demethylation to give nornicotine, a precursor of demethylcotinine, would provide an additional route to γ-(3-pyridyl)-γ-oxobutyric acid, evidence was sought for the presence of nicotine and nornicotine in the urine of the cotinine-treated animals. Neither the chromatograms nor the isotopic dilution experiments afforded any evidence for the presence of the two alkaloids. Since numerous restrictions apply to the interpretation of the current data which were obtained with (±)-cotinine-2-^{14}C, a detailed study of the metabolism of the appropriately labeled, naturally occurring L-(−) form appears to be warranted.

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