plaque-inhibition test, were found to inhibit plaque formation of DNA containing vaccinia and herpes simplex viruses but not RNA containing West Nile and Newcastle disease viruses. A number of other compounds that might be candidates for the disruption of normal nucleic acid biosynthesis, including aminopterin and 5-fluoro-2'-deoxyuridine, also were tested and found inactive in this test system. Thymidine readily reversed plaque inhibition by both 5-iodo- and 5-bromo-2'-deoxyuridine.

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Mammalian Degradation of (−)-Nicotine to 3-Pyridylacetic Acid and Other Compounds.* (26561)

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Data obtained from administration of randomly labelled nicotine-C^{14} in the rat(1) and the dog(2) have indicated that over 90% of the administered radioactivity is excreted in the urine as metabolites and as unchanged nicotine. Metabolic studies(3-5) in the dog with non-isotopic (−)-nicotine, and the metabolic intermediate (−)-cotinine have led to the isolation and characterization of metabolites which account for approximately 30% of the administered dose, *viz.,* (−)-cotinine, (−)-desmethylcotinine, hydroxycotinine, (+) - γ - (3 - pyridyl) - γ - methylaminobutyric acid, and γ-(3-pyridyl)-β-oxo-N-methylbutyramide. In addition, experiments in the rat(6) with nicotine-C^{14}-methyl have clearly shown a metabolism of the methyl group of nicotine to respiratory carbon dioxide and urea.

The foregoing, which suggest a complexity in the metabolism of nicotine far in excess of estimates by other authors(7), have led to further investigation of the metabolism of randomly labelled (−)-nicotine-C^{14}. It has now been demonstrated that a sequence of metabolic reactions leads from (−)-nicotine to 3-pyridylacetic acid.

Materials and methods. Two male mongrel dogs, 11.7 and 13.8 kg under pentobarbital anesthesia received randomly labelled (−)-nicotine-C^{14}‡, 4.25 mg/kg, total radioactivity 1.07×10^6 CPM intravenously over an 8-hour period. The urine was collected from the bladder by an indwelling catheter during infusion and a subsequent 19-hour period. Radioactive determinations were made on infinitely thick samples which were dried on talc. Urine samples were processed by modifications of previously described procedures(3-5) and descending paper chromatograms were prepared on Whatman No. 1 paper as previously described(3-5).

Studies on the metabolism of (−)-cotinine were conducted on an adult male mongrel dog. (−)-Cotinine was administered orally (100 mg/kg). The animal received commercial pellets and water *ad libitum,* and urine (preserved with sodium fluoride) was collected as run-off from the metabolism cage.

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The daily urine samples were combined and frozen.

Results. The combined bladder urine from the 2 dogs which had received the radioactive nicotine contained 8.4×10^5 CPM, 79% of the administered radioactivity. The urine was then passed through a column of Dowex 50 (H^+) which served to retain basic material. The combined effluent and a water wash contained 3.7×10^4 CPM. The column was eluted with 4 M ammonia water to give a solution (fraction B) which contained 5.5×10^5 CPM, 65% of administered radioactivity. The column retained 3.03×10^5 CPM. This latter fraction was eluted with hydrochloric acid. Studies of the nature of the metabolites in this acidic eluate have been recorded elsewhere(8).

The ammoniacal solution (fraction B) was subjected to exhaustive extraction with chloroform to remove cotinine and other basic metabolites. The chloroform solution upon paper chromatography in the ammonia system showed Koenig positive radioactive zones corresponding to (−)-nicotine, (−)-cotinine, desmethylcotinine, hydroxycotinine, and some uncharacterized material. The aqueous solution, after extraction, showed by paper chromatography with both the formic acid and ammonia systems, radioactive and Koenig positive material corresponding by cochromatography to γ-(3-pyridyl)-γ-methylaminobutyric acid. A small amount of material corresponding in Rf value to cotinine, which could have arisen from spontaneous lactamization of the methylamino acid, was also disclosed. Other radioactive zones were not subjected to identification. One, however, corresponded in Rf value to authentic 3-pyridylacetic acid, authentic samples of which separated readily at Rf 0.12 from γ-(3-pyridyl)-γ-methylaminobutyric acid (Rf 0.15) in the ammonia system when concentrations of either of the 2 components were not excessive.

The residue obtained by evaporation of the foregoing chloroform solution was treated with water and the aqueous mixture was placed upon Dowex 1 (OH^-). The effluent contained basic components, cotinine, hydroxycotinine and desmethylcotinine. The column retained a variety of Koenig positive components, Rf 0.27, 0.33, 0.46, 0.60, 0.74 (ammonia system) and Rf 0.05, 0.21, 0.34, 0.46 (formic acid system). These substances were readily eluted from the column with dilute acetic acid. The zone at Rf 0.74 (ammonia system), Rf 0.34 (formic acid system) cochromatographed with authentic γ-(3-pyridyl)-β-oxo-N-methylbutyramide(4).

The combined urine (6-1) from the dog receiving 100 mg/kg of (−)-cotinine per day for 14 days was adjusted to pH 8-9 by addition of ammonia water and then continuously extracted with chloroform. The chloroform solution yielded by published procedures(3-5): (−)-desmethylcotinine (1.2 g, 5.3 molar % of the dose of (−)-cotinine), γ-(3-pyridyl)-β-oxo-N-methylbutyramide (1.06 g, 4.04%) and unchanged (−)-cotinine (2 g, 8.33%). Hydroxycotinine of the urine was converted by treatment with acetic anhydride-pyridine to the acetyl derivative(5) for isolation (4.64 molar %).

The extracted urine was then placed upon a column of Dowex 21K (OH^-) (4-1). The combined effluent and water wash contained Koenig positive material, disclosed with *p*-aminobenzoic acid and cyanogen bromide, which was not further investigated. The column was then eluted with 1 M acetic acid which served to remove Koenig positive components (Rf 0.02 and Rf 0.12, ammonia system; Rf 0.05 and Rf 0.41, formic acid system). Following treatment with decolorizing carbon, the eluate was evaporated to dryness. The residue was triturated with 25 ml of absolute ethanol and then filtered. After addition of acetone (25 ml), the solution was cooled and filtered. The residue from evaporation of the solvent was dissolved in dilute hydrochloric acid and at pH 2 placed upon a column of Dowex 50 (H^+) (1 × 10 cm). After a water wash, the column was eluted with 1 M ammonia water. The ammoniacal solution was placed on Dowex 21K (OH^-). After a water wash, the column was eluted with 25 ml of 1 M acetic acid. The residue from evaporation of the solvent was reprocessed on the 2 exchange resins as described

above to give 300 mg of light-colored viscous oil, which gave Koenig positive zones at Rf 0.12 and 0.02 (ammonia system) and Rf 0.41 and 0.05 (formic acid system). The major zone (Rf 0.12 and Rf 0.41, respectively) corresponded in value to that of authentic 3-pyridylacetic acid, prepared according to Malon and Dean(9). The oil, upon treatment with picric acid in ethanol(4), yielded 250 mg of 3-carboxymethylpyridinium picrate, m.p. 167-168° after recrystallization from methanol. The analytical sample was dried at 60° and 1 mm over potassium hydroxide. Calculated for $C_{13}H_{10}N_4O_9$: C, 42.63; H, 2.75; N, 15.30. Found: C, 42.51; H, 2.93; N, 15.42.§ The melting point was not depressed upon admixture with authentic material(4), m.p. 167-168°.

The foregoing picric acid salt from the metabolic experiment was dissolved in water and placed on Dowex 50 (H^+). After removal of picric acid by a water wash, the column was eluted with 25 ml of 4 M ammonia water. The ammoniacal solution was placed on a column of Dowex 21K (OH^-). After a water wash, the column was eluted with 1 M acetic acid. The acidic solution yielded pure crystalline 3-pyridylacetic acid, m.p. 144-146°, upon evaporation of the solvent. For analysis, the compound was recrystallized from methanol and dried under diminished pressure, m.p. 144-146°. Calculated for $C_7H_7NO_2$: C, 61.32; H, 5.15; N, 10.22. Found: C, 61.45; H, 5.18; N, 10.24. The melting point was not depressed by admixture with an authentic sample, m.p. 144-146°.

Discussion. The pattern of excretion of radioactive metabolites of (−)-nicotine affords a confirmation and extension of earlier studies in which a high degree of urinary excretion of nicotine and metabolites has been noted. The screening procedure employed in the present study, which was based upon methods developed for isolation and identification of metabolites of nicotine (3-5) also reveals that the number of metabolites in urine far exceeds the original estimates(7) which pointed to 3 major and 4 minor metabolites. Moreover, the structural relationships of the metabolites isolated in the present and previous studies are in accord with a metabolic sequence which involves participation of pyridine compounds in excess of 7. In addition to these, the urine contains a variety of labelled compounds resulting from demethylation of (−)-cotinine and production of one-carbon metabolites as well as those which contain C^{14} as the result of loss of 2 of the labelled carbons in the pyrrolidine ring during degradation of (−)-nicotine to 3-pyridylacetic acid.

The isolation of 3-pyridylacetic acid, which has apparently never been previously recorded as a metabolic or natural product, serves to suggest a sequence: (−)-nicotine → (−)-cotinine → γ-(3-pyridyl)-β-oxo-N-methylbutyramide → γ-(3-pyridyl)-β-oxobutyrate + methylamine → 3-pyridylacetate + acetate. This interpretation is apparently consistent with the experiments of Werle and Meyer (10) in which nicotine was converted to methylamine in the presence of liver slices. Metabolic studies with γ-(3-pyridyl)-β-oxo-N-methylbutyramide and the corresponding acid are, however, required for substantiation or rejection of the hypothetical metabolic route.

Little is known of the metabolism of 3-pyridylacetic acid in the dog. The compound has been studied in the rat and the human (11). In both species following oral administration of 3-pyridylacetic acid a high degree of urinary excretion of the compound was reported. Ratti and De Fina reported(12) success in depressing serum cholesterol values in man by sustained oral administration of gram quantities of 3-pyridylacetic acid.

Summary. 1. The pattern of urinary excretion of C^{14} in the dog following intravenous administration of (−)-nicotine-C^{14} has been examined chromatographically by improved procedures. 2. The fractionated urine showed C^{14} activity corresponding chromatographically to the known metabolites: cotinine, γ-(3-pyridyl)-γ-methylaminobutyric acid, desmethylcotinine-hydroxycotinine, and γ-(3-pyridyl)-β-oxo-N-methylbutyramide. 3. Among many unidentified radioactive components, the urine contained material cor-

§ Microanalyses by Spang Micronalytical Laboratory and by Weiler and Strauss.

responding chromatographically to 3-pyridylacetic acid. 4. Following oral administration of (−)-cotinine to the dog, 3-pyridylacetic acid was isolated from urine and characterized by analysis, melting point, mixed melting point and as its picric acid salt. 5. A precursor of 3-pyridylacetic acid is on formal grounds γ-(3-pyridyl)-β-oxobutyric acid, derivable from the known metabolite γ-(3-pyridyl)-β-oxo-N-methylbutyramide.

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Immunoassay of Insulin Content of Crystalline Glucagon Preparations. (26562)

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Purification and crystallization of glucagon (1) have resulted in a preparation of exceedingly high purity. However, since no specific procedure for inactivation of insulin is included in the purification procedure, a slight insulin contamination is virtually unavoidable. It has therefore been suggested(2) that results obtained in biological experiments with glucagon should be interpreted with caution particularly when large doses of glucagon are used. McNaught(3), by measuring glucose uptake and net gas output by slices of lactating rat mammary gland estimated the insulin content of a glucagon preparation to be about 1.0%. Randle(4) determined glucose uptake by rat diaphragm and found the insulin content of an amorphous glucagon preparation to be 1.2%. One crystalline preparation contained less insulin but another (Lot #258-234 B-54-2) produced an even greater increase in glucose uptake than did the amorphous glucagon at the same concentration. J. R-Candela and R. R-Candela (5) reported that glucagon reduced the effect of insulin on glucose uptake and Snedecor *et al.*(6) found a similar inhibition of insulin-induced glycogen deposition. In contrast, Narahara and Williams(7) reported that glucagon potentiates the action of small doses of insulin on diaphragm. Recently, Lee and associates(8) estimated the insulin content of several crystalline glucagon preparations to range from 0.007% to 0.08% by measurement of $C^{14}O_2$ production from glucose-1-C^{14} in the rat epididymal fat pad preparation.

In view of the uncertain effects of glucagon on the response of tissues to insulin and the marked differences in estimated insulin content of crystalline glucagon preparations reported by various investigators an immunologic assay for insulin in 2 glucagon preparations (lots #258-234 B-54-2 and lot #258-234 B-167-1)* was undertaken, the results of which are recorded herein.

Methods. The immunoassay employed here depends on the ability of insulin in unknown solutions to inhibit, competitively, the

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