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ABSORPTION, DISTRIBUTION AND METABOLISM OF THE ANTHELMINTIC METHYRIDINE

J. BURNS, W. A. M. DUNCAN* and B. SCALES

Imperial Chemical Industries Limited, Pharmaceuticals Division,
Alderley Park, Macclesfield, Cheshire

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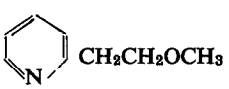
Abstract—The absorption of methyridine has been examined following oral, s.c. and i.p. dosing to sheep, calves and cows and percutaneous administration to rabbits. The drug is rapidly absorbed and distributed throughout the body tissues; it is readily metabolized to the major metabolite, pyrid-2-yl acetic acid which is excreted unchanged or as its glycine conjugate, with a half-life of 3–8 hr. Hepatic, NADPH dependent microsomal enzymes are responsible for the initial oxidation of methyridine to formaldehyde and a primary alcohol, which is further metabolised by the action of a soluble alcohol dehydrogenase. The trace residues of ^{14}C found in animal tissues at 5 or more days after dosing are shown to be due, in all probability, to the incorporation of $^{14}CO_2$ into the total carbon pool.

METHYRIDINE [2-(2′-methoxyethyl)-pyridine, "Promintic" injection, "Mintic" drench*] has been shown to possess anthelmintic activity, particularly for the nematodes of the alimentary canal, in both sheep and cattle,[1,2] and to be equally effective when dosed orally,



Methyridine

subcutaneously or intra-peritoneally. Preliminary investigations[3] on the distribution and metabolism of methyridine in five animal species indicated that the drug was rapidly distributed throughout the body and that a maximum of 1·5 per cent of the administered dose could be detected in the urine as unchanged drug. The metabolism of methyridine and its uptake into, and excretion from tissues has now been examined in considerable detail in sheep and cattle using radioisotope labelled drug. The results of these studies and also some *in vitro* studies are described.

MATERIALS AND METHODS

All reagents were of analytical reagent quality. The cyclohexane was further purified by successive washings with sodium hydroxide (1.ON), hydrochloric acid (1.ON) and three times with glass-distilled water. Dioxane was purified by the method of Vogel.[4] Methyridine, pyrid-2-yl ethanol, 2-vinyl pyridine, polyvinyl pyridine and the

* Present Address: Research Institute, Smith Kline and French Laboratories Limited, Welwyn Garden City, Herts.

silicone base barrier cream "Siopel"* (10% polydimethyl silorases) were obtained from the Pharmaceuticals Division, Imperial Chemical Industries Limited. Pyrid-2-yl acetic acid was prepared by a colleague, Dr. N. Greenhalgh. Tritium gas and ^{14}C-paraformaldehyde were purchased from the Radio-chemical Centre, Amersham, Bucks, and uranium metal turnings from the U.K.A.E.A., Springfield Works, Preston, Lancs. 2:5-Diphenyl oxazole (PPO), 1:4-bis-2(5-phenyloxazolyl)-benzene (POPOP) and Hydroxide of Hamine-IOX were purchased from Packard Instrument Ltd., Wembley. Scintillation-grade naphthalene was purchased from Thorn Electronics Ltd., and colloidal silica (Aerosil) from Bush, Beach, Segner, Bayley Ltd. The Chromax column, manufactured by LKB Productor, Sweden, was purchased from Messrs. Gallenkamp Towers. The photographic film used for autoradiography was Ilfex X-ray film (Ilford Ltd., Essex, England).

Nicotinamide-adenine dinucleotide (NAD), nicotinamide-adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase were purchased from Boehringer and Soehne GmbH., Mannheim, Germany.

Synthesis of ^{3}H-labelled methyridine

Tritiated 2-picoline. Pyrophoric uranium was used as a store for tritium gas. Two samples of 2-picoline (3·6 g and 4·3 g) were each generally labelled, using the Wilzbach technique, by exposure to tritium gas (4c) for 7 days. The irradiated samples were frozen in solid carbon dioxide–acetone mixture, the excess tritium removed and the crude ^{3}H-2-picoline in the combined samples (total activity 20 mc) freed from labile tritium and non-basic volatile impurities by dissolving in water (50 ml), acidifying with conc. hydrochloric acid (15 ml) and evaporating to dryness *in vacuo* on a water bath at 60°; six further portions (40 ml each) of water were added to the crude hydrochloride, with evaporation to dryness under reduced pressure between each addition. The aqueous-acidic distillate (300 ml) contained 9·3 mc of tritium oxide.

A solution of the crude ^{3}H-2-picoline hydrochloride (10·7 g) in water (10 ml) was cooled in ice, basified with potassium hydroxide pellets (6·0 g) and extracted with ether (3 × 20 ml). The combined ether extracts were dried over anhydrous sodium sulphate and filtered. The sodium sulphate was washed with 2-picoline (3 ml) in ether (3 ml) and the combined solutions, after concentration to about 15 ml, were fractionally distilled at atmospheric pressure.

The first fraction (9·0 ml) consisted of ether, fraction 2 (b.p. 53–126°; 4·5 ml) contained ether, 2-picoline and some basic impurity, which was detected by vapour phase chromatography. Fraction 3 (b.p. 126–126·5°; 12 ml) was ^{3}H-2-picoline. To minimize the loss of ^{3}H-2-picoline due to column retention, a portion (3 ml) of 2-picoline was added to the flask and the distillation repeated to give a further fraction (4) of ^{3}H-2-picoline (b.p. 126°; 2·5 ml). Fractions 3 and 4 were combined (12·65 g, 8·03 mc). The absence of tritium in the side-chain of the purified ^{3}H-2-picoline was demonstrated by comparing the molar specific activity of the ^{3}H-2-picoline with that of ^{3}H-pyridine-2-carboxylic acid derived from it. A portion (15 mg) of the ^{3}H-2-picoline was diluted to 0·500 g (spec. act. 428 μc/mole) with 2-picoline and oxidized with potassium permanganate, to pyridine-2-carboxylic acid.[5] The product, which was isolated as the hydrochloride m.p. 225°, had a specific activity of 430 μc/mole.

* Promintic, Mintic and Siopel are trade marks, the property of Imperial Chemical Industries Limited.

Tritiated methyridine. Pure ^{3}H-2-picoline (9 g, 6·0 mc) was heated with aqueous formaldehyde (40%, 3·6 g) for 6 hr at 150° in a sealed tube. The contents of the tube were washed out with methyl alcohol and water, and methyl alcohol and unreacted ^{3}H-2-picoline were removed by evaporation under reduced pressure at 65° (the distillate, which was discarded, contained 1·3 mc of tritium).

The residue of crude ^{3}H-2-(2′-hydroxyethyl)-pyridine was methylated by heating at 120–130° for 16 hr, with methyl alcohol (9 ml), sodium hydroxide (0·72 g) and water (0·72 ml) in a sealed tube. The dark brown solution, after acidification with concentrated hydrochloric acid, was evaporated at 95° under reduced pressure to give a residue which was dissolved in water (5 ml), cooled in an ice-water mixture and basified with excess solid potassium hydroxide. The aqueous mixture was extracted with ether (3 × 30 ml) and the combined ether extracts freed from any vinyl pyridine by extracting with saturated sodium bisulphite solution (1 vol) for 20 hr and then by stirring for a further 5 hr with a fresh portion (0·5 vol) of bisulphite solution. The bisulphite liquors were combined and extracted by stirring with two portions of ether for 30 min. The combined ether solutions were dried over anhydrous sodium sulphate, concentrated, and then fractionated under reduced pressure (12 mm mercury); at this pressure the ^{3}H-methyridine distilled at 89–90°. Methyridine (2 ml) was added towards the end of the distillation to increase the recovery of ^{3}H-methyridine.

This preparation resulted in the synthesis of 2·2 mc of ^{3}H-methyridine.

Radiochemical purity ^{3}H-methyridine. An aqueous acidified solution of ^{3}H-methyridine was freeze-dried, reconstituted, and the process repeated three times. The successive aqueous distillates contained a constant and very small amount (about 0·007 per cent) of the total radioactivity; this was presumably derived from carry-over of the ^{3}H-methyridine. These results indicated the absence of labile tritium.

The picrate of methyridine (a well-defined and stable salt) was prepared and crystallized to constant specific activity. With such a highly coloured material, the counting efficiency was low and the purity could only be determined to about 0·5 per cent. Within this limit of accuracy the specific activity remained constant through five crystallizations.

The ^{3}H-methyridine after dilution with unlabelled vinyl and polyvinyl pyridine was examined for the presence of ^{3}H-2-vinyl pyridine and its polymer, by electrophoresis on Whatman 3MM paper which was moistened with 0·2 M citrate-phosphate buffer pH 2·0 or 3·5 and a potential gradient of 100 V/cm applied for 45 min. The separated components were detected by inspection under u.v. light and their radioactive content was determined as described below. In no sample did the vinyl pyridine and polyvinyl pyridine content account for more than 0·2% of the total activity.

Vapour phase chromatography of the ^{3}H-methyridine on a diglyceryl column at 110° with hydrogen:nitrogen as carrier gas at a flow of 40 ml/min showed the presence of 2-vinyl pyridine (0·2–0·3 per cent) and the 6-methyl and 2-methyl derivatives of methyridine (together less than 0·02 per cent).

Synthesis and purification of ^{14}C-methyridine

2-(2′-^{14}*C*,2′-*hydroxyethyl*)-*pyridine.* 2-picoline (12·0 g), water (2·5 ml) and ^{14}C-paraformaldehyde (1·0 g, 2 mc) were heated together in a sealed tube at 145° for 7 hr. The product was distilled under reduced pressure to give a 55 per cent yield of ^{14}C-labelled 2-(2′-hydroxyethyl) pyridine, b.p. 122° at 25 mm mercury.

^{14}C-methyridine. The above product was diluted to 5·9 g with 2-(2'-hydroxyethyl) pyridine and heated with 50 per cent (w/w) aqueous sodium hydroxide (0·9 ml) and methyl alcohol (12 ml) in a sealed tube at 124° for 17 hr. The ^{14}C-methyridine was isolated using the method given above for ^{3}H-methyridine and was obtained with an overall radiochemical yield of 24 per cent.

The ^{14}C-methyridine, obtained after distillation, was dissolved and washed as before with sodium bisulphite in ether (10 vol). The combined ethereal solutions were dried over anhydrous sodium sulphate and the ether removed *in vacuo* below 20°. The ^{14}C-methyridine was then applied to a column of silica gel (Merck Silica Gel G. nach Stahl) from which it was eluted with a mixture of light petroleum (60–80°) and acetone (50% v/v). The eluate from the column was collected in fractions (15 ml) and the identity of the material in each fraction established by thin layer chromatography of samples (5 μl) on silica GF plates, which were developed with the light petroleum:acetone solvent mixture. The plates were examined under u.v. light and radioactivity detected by autoradiography. Fractions which were observed to contain only ^{14}C-methyridine were combined and the solvent was removed *in vacuo.*

Radiochemical purity of ^{14}C-methyridine. The chemical and radiochemical purity of the combined ^{14}C-methyridine fractions was examined by applying samples to silica GF plates, which were then developed with either 50 or 10% light petroleum in acetone. Autoradiography of the developed plates showed the presence of only one radioactive substance, and the radio-chemical purity was estimated to be at least 99·6 per cent. The chemical purity of the ^{14}C-methyridine was also examined by vapour phase chromatography: (a) on a dispersol-C.W.L. column at 110° with hydrogen:nitrogen as carrier gas at a flow of 50 ml/min; (b) on a diglyceryl column operated as above, the only impurities detected were 2-vinyl pyridine (0·01%) and an unidentified impurity (estimated to be not more than 0·1 per cent); the preparation was estimated to be not less than 99·89 per cent pure.

Synthesis of pyrid-2-yl acetyl glycine

To a mixture of pyrid-2-yl acetic acid hydrochloride (3·5 g) and glycine ethyl ester hydrochloride (2·8 g) in sodium hydroxide (IN, 40 ml) was added dicyclohexyl carbodiimide (3·9 g) in acetone (40 ml). The solution was shaken for 3 hr at 20° and left overnight. The dicyclohexyl urea, which precipitated, was removed and the filtrate evaporated to dryness *in vacuo* to give an oil (4·1 g). This oil was hydrolysed by dissolving an aliquot (1 g) in methanol (10 ml) adding sodium hydroxide (1 N, 4·5 ml), and leaving at 20° overnight. Water (10 ml) was added and the solution extracted with chloroform before and after adjustment to pH 7·0. The aqueous phase was separated and the freeze-dried solid extracted with ethanol (15 ml), centrifuged and the supernatant evaporated to dryness *in vacuo.* The residue was treated with ethereal hydrogen chloride and the hydrochloride re-crystallized from aqueous acetone and from a methyl alcohol–ethyl acetate mixture to give a crystalline solid m.p. 203 to 5°. ($C_9H_{10}O_3N_2 . \frac{1}{2}$ HCl requires C 51·3, H 4·95, N 13·2: found C 51·9, H 4·9, N 13·5).

Chromatography

Qualitative chromatography. Descending chromatography was carried out at room-temperature on Whatman No. 1 paper using *n*-butanol saturated with 1·0 N ammonium hydroxide as solvent. Examination under u.v. light and spraying the

paper with 0·5% iodine in methanol were used for the non-specific detection of metabolic products. Irradiation for at least 30 min, with a lamp emitting light mainly at 255 mμ, was observed to produce a yellow colour with compounds having two carbon atoms in the 2-position of the intact pyridine nucleus and could be used quite reliably to detect sub-microgram quantities of metabolites retaining this structure. To detect aromatic hydroxylated metabolites the papers were sprayed with 5% sodium carbonate, dried at 50° and then resprayed with a freshly prepared reagent consisting of p-aminophenyl-β-diethylaminoethyl sulphone (0·5 g), hydrochloric acid (0·1 N, 50 ml) and sodium nitrite (5%, 2 ml).

Preparative chromatography. (i) The urine after acidification and concentration by freeze-drying was chromatographed on Whatman 3 MM paper with *n*-butanol saturated with 1·0 N ammonium hydroxide as solvent. The metabolites were located by one of the methods described above and the portion of the paper containing a metabolite was removed and the metabolite eluted with methanol.

(ii) Column chromatography was carried out on cellulose powder (Whatman), or on a Chromax column, with the same mobile phase as that used for the paper sheets. The metabolites in 5 ml of sheep urine could be satisfactorily separated on 30 g of cellulose powder. When the Chromax column was used, the metabolites were first separated from the bulk of inorganic material in the urine by extracting a freeze-dried urine sample (120 ml) with methanol. The methanol extract was evaporated at low temperature and the residue taken up in the eluting solvent (50 ml) which was then applied to the column which had been equilibrated with the mobile phase at a column pressure of 300 g/cm².

Thin layer chromatography. Thin layer plates of silica gel, with or without the addition of a fluorescent indicator, were prepared using the apparatus described by Stahl.[6]

Chemical methods of analysis

The estimation of methyridine: extraction from blood. The compound was extracted from blood (0·5 ml) by shaking for 5 min with water (0·5 ml), 0·1 N sodium hydroxide (0·2 ml), 0·2 M phosphate–citrate buffer pH 8·0 (0·8 ml) and cyclohexane (12 ml). An aliquot (10 ml) of the cyclohexane extract was separated and back-extracted with 0·2 M phosphate-citrate buffer pH 2·1 (2 ml).

Extraction from tissue. The tissue (1 g) was homogenized in 0·2 M phosphate-citrate buffer pH 8·0 (1 ml), cyclohexane (12 ml) was added and the drug extracted by shaking for 5 min. The extract was centrifuged and the drug extracted from the cyclohexane as described above.

Calculation. The optical density of the aqueous phase was measured at 262 mμ and 275 mμ against a reagent blank in a micro cuvette with a 40 mm light path. At pH 2·1 methyridine has an extinction coefficient of 7950 at 262 mμ and 1430 at 275 mμ. After allowance has been made for losses due to sampling, 74 per cent of the drug was extracted by the described procedure so that:

$$\mu\text{g drug/g tissue} = 17{\cdot}0\ (d_{262} - d_{275})$$
$$\mu\text{g drug/ml blood} = 34{\cdot}1\ (d_{262} - d_{275})$$

where d_{262} and d_{275} are the observed optical densities at 262 mμ and 275 mμ respectively. With blood and tissues from untreated animals the value for $d_{262} - d_{275}$

rarely exceeded 0·020 so that corrections were neglected except when the concentration of the drug was of the order of 1 μg/g, the lower limit of the method.

Estimation of formaldehyde. The method of Tanenbaum and Bricker[7] was used.

Estimation of 2-(2′-hydroxyethyl)-pyridine. Portions (0·5 ml) of the incubation mixtures were diluted to 2·0 ml with 0·2 M phosphate–citrate buffer (pH 8·0) and extracted with chloroform (12·0 ml). An aliquot of the chloroform extract was removed and back-extracted with 0·2 M phosphate–citrate buffer (pH 2·1). The aqueous extract was basified, extracted with chloroform and an aliquot of the chloroform back extracted into buffer (0·2 M, pH 2·1). This technique minimized interference due to nicotinamide. The optical density of the aqueous extract was measured at 262 mμ and 270 mμ in micro cuvettes of 10 mm or 40 mm light path, and the concentration of 2-(2′-hydroxyethyl)-pyridine calculated using calibration curves obtained by carrying different concentrations of the compound through the full extraction procedure. Under these conditions a 66–70 per cent recovery of 2-(2′-hydroxyethyl)-pyridine was obtained.

Estimation of pyrid-2-yl acetic acid. Urine (0·1 ml) and hydrochloric acid (0·1 N, 1·4 ml) were heated at 100° for 15 min. The volume was readjusted to 1·5 ml, if necessary, and a sample (1·0 ml) together with sodium hydroxide (1·0 N, 0·5 ml) transferred to a micro steam distillation apparatus. The product was steam distilled into hydrochloric acid (1·0 N, 10 ml) until the total volume was 100 ml. The optical density of the distillate, which contained the 2-picoline formed by decarboxylation, was measured at 262 mμ and 275 mμ in a 10 mm cuvette. The concentration of pyrid-2-yl acetic acid was calculated from calibration curves obtained by decarboxylating known quantities of pyrid-2-yl acetic acid by the above procedure.

Ethereal sulphates. The method of Sperber[8] was used to detect the presence of ethereal sulphates.

Codeine. This was determined by the methyl orange method of Axelrod.[9]

Morphine. The modification by Axelrod[10] of the procedure described in Snell and Snell[11] was used.

p-*Ethoxy acetanilide* (*acetophenetidin*). After hydrolysis to the free amine, the determination was carried out by the method of Axelrod.[12]

p-*Hydroxy acetanilide* (*N-acetyl-p-aminophenol*). This was analysed by the method of Brodie.[13]

Radioisotope methods of analysis

The radioactivity of all samples was measured using a Tri-Carb Automatic Liquid Scintillation Spectrometer.

The toluene phosphor system contained PPO (16 g) and POPOP (0·40 g) in toluene (1000 ml); this system was sometimes diluted 75 ml to 100 ml with toluene before use. The dioxane phosphor contained napthalene (104 g), PPO (10 g) and POPOP (0·25 g) in dioxane (1000 ml). "Diotol" contained dioxane (500 ml), methanol (300 ml), toluene (300 ml), napthalene (104 g), PPO (6·5 g) and POPOP (0·13 g).

Methyridine in milk, plasma and urine. The body fluid (4·0 ml) was diluted with borate buffer (0·2 M, pH 9·0, 6·0 ml) and extracted with toluene (20 ml). The toluene layer was clarified by centrifugation at 0° and an aliquot (15 ml equivalent to 3·0 ml of the body fluid) assayed, after the addition of the toluene phosphor (5 ml), at 70–72 per cent efficiency for ^{14}C- and at 20–32 per cent efficiency for ^{3}H-containing samples.

Methyridine in faeces and tissues. Faeces and tissue were homogenized in borate buffer (0·2 M, pH 8·5–9) to give a 25 per cent suspension. An aliquot (15 ml) was removed, shaken for 5 min with two portions of toluene (each 7·5 ml) and the two phases separated by centrifugation. The toluene extracts were combined (15 ml) and an aliquot (10 ml) assayed using the diluted toluene phosphor (5 ml). If the toluene extract was coloured, as was usually the case with faecal extracts, the whole of the toluene extract (15 ml) plus a further 5 ml of toluene, which was used to remove the last traces of the toluene from the centrifuge tube, was back extracted into dilute hydrochloric acid (0·1 N, 5 ml). The aqueous layer was then basified with sodium hydroxide (5 N, 0·2 ml), extracted with toluene (15 ml) and an aliquot (10 ml) was assayed as above.

All toluene solutions of ^{3}H-methyridine obtained by these methods were assayed with 28–31% efficiency. The count obtained for the 10 ml aliquot of the toluene extract was proportional to the amount of methyridine in 10 ml of the homogenate (2·5 g of tissue or faeces).

Total activity in plasma. The plasma samples were assayed using a slight modification of the "Hyamine" or "Diotol" method of Herberg.[14]

Total activity in tissues and whole blood. Three methods were used; the two used in the initial investigations were the tissue dissolution methods of Herberg;[14] these have been described previously, along with the method of determining the background count of variably quenched samples.[15] In order to improve the sensitivity with which the radioactivity in the tissues could be determined, a large-scale combustion furnace was used for the conversion of tissue carbon to carbon dioxide.

Weighed portions of whole tissue were dried in Petri dishes by irradiation overnight with i.r. lamps. The dried material was weighed, coarsely ground, and portions containing no more than 0·300 g of carbon were combusted using a semi-automatic combustion apparatus;[16] the risk of explosion was minimized by using carbon dioxide-free air instead of oxygen, and to prevent back diffusion and consequent incomplete combustion due to condensation of volatile material on the cooler parts of the silica combustion tubes, a plug of silica wool was placed in the combustion train behind the boat and contents. Water from the combustion was collected by condensation in a radiator trap cooled in solid carbon dioxide-chloroform mixture and the carbon dioxide was absorbed in sodium hydroxide solution (5·0 N, 10 ml) contained in a spiral absorption column, constructed to allow the circulation of absorption fluid.

To assay $^{14}CO_2$, the carbonate content of the sodium hydroxide was adjusted by the passage of 5 per cent CO_2 in air until the pH of the solution was 8·0; under these conditions there was no loss of $^{14}CO_2$ from the absorption spiral. The volume of gas required was calculated by titration of aliquots of the hydroxide solution with hydrochloric acid (1·0 N). Duplicate samples (3·0 ml) of the solution were then assayed in 17·0 ml dioxane phosphor to which silica (Aerosil, 50 g/l. of phosphor) had been added; the solution of colloidal silica in dioxane gelled on addition of the aqueous solution and thus prevented settling of the finely divided sodium carbonate crystals. The counting efficiency of these suspensions (46–48 per cent) was determined by adding known amounts of ^{14}C-sodium carbonate solution to the 3·0 ml samples prior to mixing with the dioxane phosphor.

Muscle biopsy samples (about 2 g) were combusted without previous drying.

When used for the analysis of poorly quenching tissues such as fat or brain, the

combustion method was about five or six times more sensitive than the "Hyamine" method, but when used for samples with high quenching characteristics a 40 fold improvement was easily obtained.

Total activity in milk, urine and faeces. Milk was vigorously shaken to disperse the cream immediately prior to sampling. Faeces, which were frequently contaminated with urine, were homogenized with water and diluted to 1500 ml; suspended material was allowed to settle out before sampling.

Aliquots (2 or 3 ml) of milk, diluted urine or aqueous faecal extracts were diluted to 20 ml with the dioxane phosphor containing silica. Using this method ^{14}C was assayed at 58–68 per cent efficiency and ^{3}H at 8–10 per cent efficiency.

Radiochemical assay of the chromatographically separated metabolites. Radioisotope labelled metabolites on chromatograms were assayed by:

(i) Transverse strips, cut from the chromatograms, were immersed in "Diotol" (15 ml), to which was added ethanol (5 ml) and counted directly. This method sufficed to indicate the position of the metabolites on the paper.

(ii) A more quantititative procedure for tritiated material was to burn the strips by a modified oxygen-flask technique and to assay the condensed water.

(iii) The sheets were cut into strips from which the metabolites were eluted with methanol; after careful evaporation of the solvent the residue was dissolved in the scintillation medium and assayed with efficiencies of about 10 per cent for ^{3}H and 40–70 per cent for ^{14}C.

(iv) Eluate fractions from columns were assayed directly using the dioxane phosphor; the eluate (4·0 ml) was diluted to 20·0 ml with the phosphor. ^{14}C was assayed at about 60 per cent efficiency and ^{3}H at about 4 per cent efficiency.

The identification of metabolites produced in vivo

The isolation and determination of ^{14}C-urea in urine. Urea in urine was determined quantititatively by the method described by Cole.[17] Urine (200 ml) from a sheep dosed with ^{14}C-methyridine was evaporated on a steam bath and the urea extracted from the residue with acetone. The urea was re-crystallized three times from isopropyl alcohol, converted to the nitrate which was recrystallized twice from water and then converted back to urea using barium carbonate. Three further recrystallizations from isopropyl alcohol gave urea of a constant specific activity (maximum 4400 dpm/g). Some purified samples of urea were free from radioactivity.

The determination of ^{14}C-carbon dioxide in blood. Blood ^{14}C-bicarbonate was determined by diluting whole blood or plasma (20 ml) with physiological saline (20 ml) and acidifying with sulphuric acid (6 ml, 30 per cent). The evolved carbon dioxide was dissolved in "Hydroxide of Hyamine—10 X" (1·0 M solution in methyl alcohol, 3 ml) to which was added the toluene phosphor (5 ml), toluene (5 ml), absolute ethanol (5 ml) and concentrated hydrochloric acid (0·2 ml), ^{14}C was assayed at about 35 per cent efficiency.

The isolation and determination of tritiated water. Weighed amounts of tissues, or fluids, were freeze-dried to constant or known weights and the condensate, after thawing, was acidified with one drop (50 μl) of concentrated sulphuric acid and again freeze-dried; in one experiment water was added to the freeze-dried tissue and the freeze-drying repeated after several days equilibration at 6°, this cycle was repeated until the ^{3}H-content of the condensed water was substantially reduced. The aqueous

condensate was assayed using 2·0, 2·5 or 3·5 ml portions made up to 20 ml with the dioxane phosphor. Tritium was assayed in the above volumes of water at 10·5, 8·2 and 6·5 per cent efficiency respectively.

The isolation of pyrid-2-yl acetic acid hydrochloride. The concentrated urine from animals dosed with methyridine was applied to sheets of Whatman 3 MM for chromatography with *n*-butanol saturated with hydrochloric acid (IN) for 48 hr. The chromatograms were air-dried and the portion of the paper containing the most strongly u.v.-absorbing material was cut out and eluted with methyl alcohol. The eluates were combined and evaporated to dryness *in vacuo* to give a brown oily residue. Ethereal hydrochloric acid was added to the flask and solidification induced; the residue was washed free of mineral acid with small portions of ether and acetone. The amorphous material was crystallized from a methyl alcohol–ether mixture. The long needles had a m.p. 125–26° (uncorrected) which was not depressed on admixture with authentic pyrid-2-yl acetic acid hydrochloride (mp. 125–126°), an identical i.r. spectrum and on heating decomposed to give 2-picoline. ($C_7H_7O_2N.HCl$ requires C 48·4, H 4·6, N 8·1: found C 49·0, H 4·7, N 8·2).

The identification of pyrid-2-yl acetyl glycine. Prolonged irradiation of paper chromatograms developed using butanol-ammonia as the solvent, indicated the presence of a second metabolite having an R_f smaller than that of pyrid-2-yl acetic acid. Fractions corresponding to this material were obtained using column chromatography (Fig. 3) and the homogeneity of fractions ascertained by running portions of each fraction on paper and examining under u.v. light. Fractions rich in the required compound were combined and evaporated to dryness *in vacuo*. The brown gum was treated with ethereal hydrochloric acid, but attempts to induce solidification were unsuccessful. A portion of the gum was hydrolysed by heating with sodium hydroxide (1·0 N) and an aliquot was assayed for amino acids by chromatography on paper (Whatman No. 1) using both isopropanol and liquid phenol as eluents. The gum had an identical i.r. spectrum and chromatographic mobility to synthetic pyrid-2-yl acetyl glycine. Final confirmation of identity was obtained using standard reverse-isotopic dilution techniques in which the synthetic pyrid-2-yl acetyl glycine hydrochloride was crystallized to constant specific activity from aqueous acetone solutions of the ^{14}C-labelled gum.

The identification of 2-(2′-*hydroxyethyl*)-*pyridine.* Homogenous fractions from a column chromatogram (Fig. 3) which corresponded in R_f value to that of authentic 2-(2′-hydroxyethyl)-pyridine were combined and evaporated to dryness *in vacuo*. The oily residue was mixed with an ether solution of authentic 2-(2′-hydroxyethyl)-pyridine (0·20 g) and the whole converted to the hydrochloride by treating with ethereal hydrochloric acid. The insoluble oil was crystallized from methyl alcohol-acetone and methyl alcohol–ethyl acetate to give deliquescent crystals of constant specific activity (28,000 dpm/g).

Formulation of methyridine for administration

The formulation of labelled methyridine for oral, intraperitoneal and subcutaneous dosing were equivalent to the commercial formulations of methyridine. Percutaneous absorption studies were carried out using aqueous solutions of the free base (90 per cent), and aqueous solutions containing the free base (71 per cent) in which the pH had been adjusted to 4·06 by the addition of sulphuric acid.

Animals, dosing and collection of samples for analysis

Sheep, calves and cows were of varying breed. Male albino mice (20 g) and rats (120–150 g) were of the Alderley Park, I.C.I. Ltd. strain. Rabbits, "Sandy-lops" of either sex were obtained from a commercial breeder.

Animals were dosed either orally, subcutaneously or intra-peritoneally with 200 mg methyridine/kg. Blood samples were obtained by venepuncture. Muscle biopsy samples (about 2 g) were taken from the rump under local anaesthesia (Novacaine).

For percutaneous absorption studies, the dorsal fur of rabbits was removed and shaped polythene cylinders placed on the skin. These were held in position by elastic bands attached to the sides of the rabbit box and an adhesive was applied to reduce leakage of the applied formulation between the skin and cylinder. The extent and rate of absorption of methyridine, which was applied to the area of the skin contained within the cylinder (15·2 cm^2), was measured by estimating the drug content of the blood at varying intervals after dosing and also by washing out the methyridine remaining in the cup at the end of the experiment, the drug absorbed then being estimated by difference.

In the experiments using silicone grease (Edwards High Vacuum grease) and "Siopel" cream a thin layer of the grease or cream was applied to the skin before applying the formulation.

Measurement of in vitro *enzyme activity*

Rabbits, rats and mice were stunned and exsanguinated, the liver or other tissues immediately removed and placed in ice-cold 1·5 % KCl solution. Tissue slices (0·5 mm thickness) were prepared using a Stadie–Riggs tissue slicer. Tissue homogenates were prepared by the method of Gillette.[19]

A typical complete incubation mixture (5·0 ml) contained the filtered liver homogenate or derived fractions, equivalent to 0·5–1·0 g liver, nicotinamide 100 μmoles, $MgCl_2$ 75 μmoles, G-6-P 100 μmoles, NAD 0·8 μmoles, NADP 0·6 μmoles, neutralized semicarbazide 100 μmoles and sodium phosphate buffer 200 μmoles pH 7·3. After a brief pre-incubation period at 37° in a Duboff metabolic shaking water bath the reaction was started by adding methyridine or 2-(2′-hydroxyethyl)-pyridine (5 μmoles), codeine or *p*-ethoxy acetanilide (2 μmoles). Inhibition studies were carried out using β-diethylaminoethyl diphenylpropylacetate (SKF 525A) 0·5 μmole, and *p*-methoxybenzylamine (PMB) 20 μmoles.

RESULTS AND DISCUSSION

Investigations with ^{3}H-methyridine

Initial investigations were carried out using ^{3}H-methyridine and the tissue dissolution methods of analysis. It soon became apparent that a small amount of tritium was being converted to tritiated water which was distributed throughout all tissues. This was of little consequence when unchanged drug was being assayed, but proved to be a serious problem when the residual concentration of metabolites was being measured. Thus in plasma samples taken from eight sheep, 4 days after subcutaneous dosing (Fig. 1) the average level of tritiated water accounted for at least 80 per cent of the total radioactivity present. An attempt was made to obtain an accurate measure of the tritiated water content of tissues and it was thought that this could be done

quite accurately by assaying up to 3·0 ml of water obtained from tissues using the dioxane phosphor system.

Preliminary experiments were carried out on the tissues of an animal dosed with triated water. Four days after subcutaneous dosing the animal was sacrificed and samples of wet tissue assayed for total tritium content and tritiated water (Table 1). Plasma was the only body component from which the whole of the triated water could be removed by one complete freeze-drying procedure; the amount removed from tissue ranged from 65 per cent in the liver to 47 per cent in the spleen. Further attempts were made to measure the total tritiated water in the tissues by removing the total exchangeable tritium after equilibrating freeze-dried tissues with water. A minimum of three dried-tissue-water equilibration stages was required before all the labile tritium was removed from muscle, and further stages would be required with liver.

TABLE 1. TOTAL RADIOACTIVITY AND THAT OF WATER DERIVED FROM TISSUES OF A SHEEP DOSED WITH ^3H-H_2O

Tissue	Total tritium dpm/g tissue	Tritiated water		
		dpm/ml obtained from tissue by a stepwise freeze-drying procedure		dpm/g tissue obtained by summation
		(a)*	(b)	(a) + (b)
Muscle	6940 ± 250	3600 ± 100	4650 ± 50	3400 ± 100
Liver	4270 ± 175	3840 ± 50	4260 ± 50	2800 ± 120
Kidney	6300 ± 245	2280 ± 100	4280 ± 50	3260 ± 100
Spleen	7850 ± 290	2360 ± 45	4260 ± 50	3700 ± 100
Plasma	3940 ± 160	4000 ± 50	5000 ± 50	4370 ± 80

Figures show radioactivity (±S.D.) in the tissues of a sheep, 4 days after s.c. dosing with a 10% solution of ^3H-H_2O (50 μc) in unlabelled methyridine.

* (a) Radioactivity in first portion of water condensed during the freeze drying procedure.
(b) Radioactivity in the remainder of the water condensed whilst taking the tissue to dryness.

These results are in accordance with those of Siri,[18] who found an increase of 50 per cent in the specific activity of water collected in stages from tissues of animals dosed with tritiated water. Siri also showed that when the dried tissues were rehydrated with non-radioactive water, 90 per cent of the tritium exchanged occurred with a half-time of 1 hr, and the remainder with a half-time of 10 hr.

The small amount of labile tritium observed under our *in vivo* conditions, amounted to about 2% of the dosed ^3H-methyridine and must have originated from the pyridine nucleus. The difficulty in assessing accurately what proportion of the tissue residue of tritium was due to the presence of the intact pyridine nucleus and how much was due to the presence of tritiated water could not be resolved and the later studies were carried out with ^{14}C-labelled methyridine.

Investigations with ^{14}C-labelled methyridine

The possibility of metabolic breakdown of the pyridine ring was considered unlikely. This, and the prohibitive cost of preparing sufficient material labelled with ^{14}C in the nucleus prompted the synthesis of sidechain labelled methyridine. Preliminary examination of the *in vivo* stability in sheep and calves showed that the ^{14}C-bicarbonate

in the plasma on no occasion accounted for more than 1 per cent of the total radioactivity present at 4 days after dosing (equivalent to about 0·0003 per cent of administered drug) and sometimes no ^{14}C-bicarbonate could be detected. Also the maximum amount of ^{14}C-urea isolated from the urine accounted for only 0·0018 per cent of the excreted radioactivity. These results indicated that no significant amount of the labelled carbon atom on the methyridine side-chain was being oxidized to CO_2 and that such labelling should give true figures for the tissue residues of drug and its metabolites.

The concentration of methyridine and metabolites in the blood and plasma of sheep and calves

The concentrations of methyridine and of its metabolites in the plasma of sheep at various times after oral, subcutaneous and intraperitoneal dosing are shown in Fig. 1. The concentration of methyridine in the blood was maximal 1–2 hr after dosing and the maximum concentration of metabolites occurred 1 hr later. Methyridine disappeared rapidly from the blood and plasma so that the concentration of the drug was 1 μg/ml or less at 24 hr. The overall rate of excretion of the metabolites was slower but their concentration in the plasma had decreased to less than 5 μg/ml within 2 days. As observed from an examination of Fig. 1 the excretion half-life for methyridine from the plasma is about 2 hr and for the major and minor metabolic products is 3–6 hr and 4–10 days respectively.

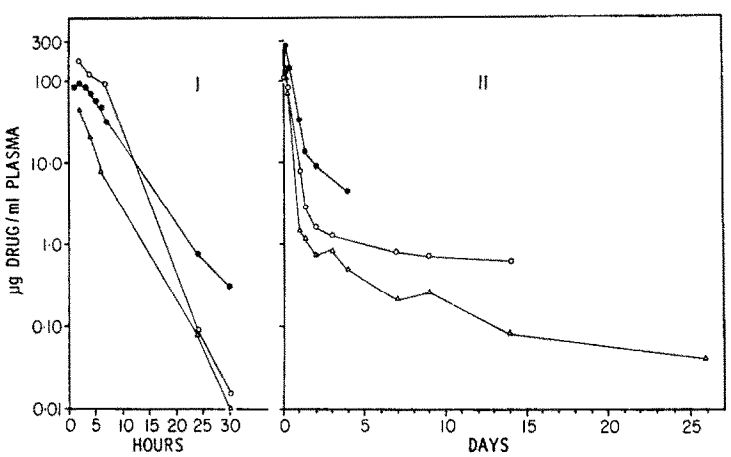


FIG. 1. Average plasma levels of methyridine (I) and its metabolites (II) as μg of drug from which they were derived. Four sheep were dosed p.o. △——△, and two i.p. ○——○, with ^{14}C-methyridine at 200 mg/kg. Eight sheep were dosed s.c. ●——●, with ^{3}H-methyridine at 200 mg/kg.

In calves after oral and intraperitoneal dosing, the plasma levels reached a maximum concentration in 1–3 hr (Fig. 2). Although there was a two to three fold variation in the maximum concentration of methyridine found in the plasma of individual animals, there appeared to be no substantial difference in the maxima and the rate of disappearance between the two routes of dosing. This was confirmed in a cross-over experiment to eliminate individual variation, in which three animals were each dosed

orally and then intraperitoneally with unlabelled drug; results of spectrophotometric determinations of the unlabelled drug up to 24 hr after dosing are shown in Fig. 2. The excretion half-life of methyridine from the plasma of calves is about 4 hr; that of the ^{14}C-labelled metabolites is about 6–8 hr until 2–3 days after dosing, and thereafter is about 10–13 days. Extrapolation to the time of dosing of the steeper portion of the

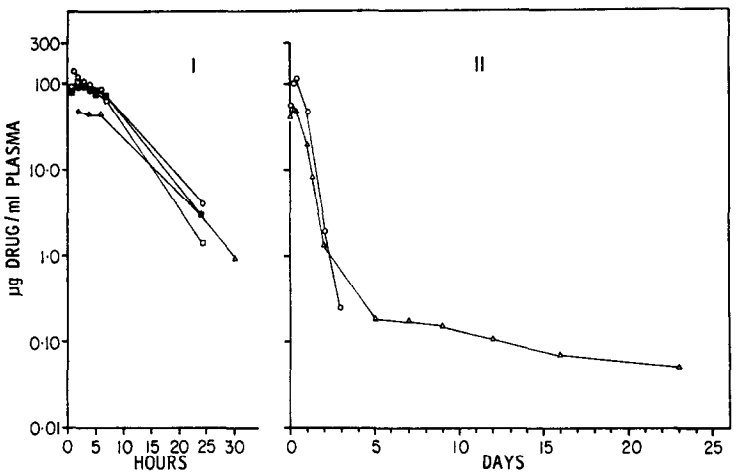


FIG. 2. Average plasma levels of methyridine (I) and its metabolites (II) as μg of drug from which they were derived. Four calves were dosed p.o. △——△, and eight i.p. ○——○, at 200 mg/kg with ^{14}C-methyridine. Three calves were dosed both p.o. ■——■ and i.p. □——□, with unlabelled drug and the methyridine was determined spectrophotometrically.

metabolite "plasma level"/time curves for both orally and intraperitoneally dosed calves, gave intercepts at about 200 μg/ml plasma (cf. dose of 200 mg/kg). This value indicated that possibly nearly all the methyridine was being metabolized to rapidly eliminated compounds. Extrapolation of the other part of the plasma level/time curves to the time of dosing gave an intercept at about 0·3 μg/ml plasma which indicated that this radioactive residue with a half-life of 10–13 days, represented no more than 0·01 per cent of the administered dose in the total circulating blood volume; 20 days after dosing its concentration in the plasma was less than 0·1 μg/ml.

The concentration of methyridine and its metabolites in the tissues of sheep and calves

Methyridine was rapidly distributed throughout the tissues of sheep and calves, but within 24 hr of dosing the concentration had dropped to 1 μg/g or less. The concentration of metabolites in the tissues after attaining a maximum also decreased rapidly to low values, but the presence of traces of a more persistent radioactive residue was clearly indicated at 20 days (Table 2), when very low, but significant amounts of radioactivity were present in most of the tissues examined; on average at 20 days this was equivalent to about 0·6 μg/g tissue in sheep and 0·3 μg/g tissue in calves.

Although the radioactivity present in the tissues of sheep decreased with time the ratio of the concentration of radioactive residue in the tissue to that in the plasma increased from day 3 (2·6 for liver, 0·8 for muscle) to day 8 (8·0 for liver, 3·5 for muscle)

TABLE 2. CONCENTRATION AND EXCRETION HALF-LIFE OF ^{14}C IN THE TISSUES OF SHEEP AND CALVES AFTER DOSING ORALLY OR INTRAPERITONEALLY WITH ^{14}C-METHYRIDINE

Tissue	Sheep				Calves				
	Excretion half-life (days)	Days after dosing			Excretion half-life (days)	Days after dosing			
		5	20	40		5	20	40	60
Heart	7	2·10	0·430	0·056	18	0·590	0·330	0·161	0·070
Liver	7	6·90	1·30	0·210	17	1·360	0·740	0·330	0·150
Kidney	5	4·00	0·580	0·046	13	0·660	0·310	0·110	0·040
Spleen	6	2·70	0·550	0·066	14	0·680	0·330	0·130	0·049
Brain	5	2·70	0·420	0·038	15	0·560	0·260	0·095	0·034
Muscle	6	2·30	0·440	0·052	15	0·610	0·320	0·138	0·058
Intestine	—	—	—	—	17	0·450	0·250	0·114	0·050
Fat	—	—	—	—	14	0·500	0·240	0·090	0·034
Bone marrow	—	—	—	—	15	0·580	0·280	0·108	0·041

The results expressed as μg ^{14}C-methyridine/g tissue were calculated from the smoothed curves drawn through the scattered individual observations on the appropriate tissues of seven sheep and twelve calves. The individual observations were obtained as a mean of six assays on each tissue. These values are not corrected for the ^{14}C-content of contemporary carbon.

and then appeared to remain constant (7·5 for liver, 3·0 for muscle) from day 16 onwards. The concentration of methyridine and its metabolites in plasma of sheep decreased most rapidly after oral dosing and more rapidly after intraperitoneal than after subcutaneous dosing (Fig. 1). If this difference is meaningful, then extrapolation of the plasma levels to zero time would indicate that the maximum concentration of the most persistent radioactive residue after intraperitoneal dosing would be equivalent to about 7 μg methyridine/g in the liver and 3 μg/g in the muscle, whereas after oral dosing the maximum levels possible would be 1·5 μg/g in the liver and 0·5 μg/g in muscle. However, because the rate of excretion differed in the groups of sheep, it is difficult to speculate on the magnitude of the apparent difference between the three methods of dosing, but in sheep the total amount of these more persistent residues could never represent more than 0·03 per cent of the total administered radioactivity in the whole liver or 0·12 per cent of the administered radioactivity in the total muscle mass.

Disappearance curves for the ^{14}C concentration in each of the tissues of the calves (Table 2) showed that the ^{14}C present more than 9 days after dosing, had an excretion half-life of 13–18 days.

Extrapolation to the time of dosing indicated that this product represented about 0·2 per cent of the administered dose in the total muscle mass of the calves, 0·08 per cent in the whole liver and less than 0·01 per cent in any other edible tissue. There was no apparent difference between the concentration in the tissues of this ^{14}C-labelled metabolite after oral and intraperitoneal dosing. Forty days after dosing the calculated concentration of ^{14}C in all tissues, except liver, was equivalent to that derived from about 0·1 μg ^{14}C-methyridine/g tissue (Table 2).

All tissue and plasma levels were calculated using a background count value of 34·1 cpm obtained using freshly prepared solutions of sodium carbonate in which the carbon dioxide was derived from fossilized carbon. They are uncorrected for the apparent ^{14}C- content of the tissues of animals which had not been dosed with ^{14}C-methyridine, or for the apparent ^{14}C-content of contemporary carbon. Atmospheric carbon dioxide contains 30 dpm ^{14}C/g carbon; this is sufficient to raise the background count of the sodium carbonate solutions by 2·5 cpm. The average count of sodium carbonate samples allowed to equilibrate with atmospheric CO_2, and those prepared by combustion of untreated control calves was 36·5 $\pm$ 1·6 cpm. The individual results averaged in Table 2 for the tissue of ^{14}C-methyridine dosed animals are therefore the upper limit values and may be as much as 0·1 μg/g tissue too high. This suggests that at 40 days, the true tissue level of trace amounts of radioactivity in calves will probably be almost zero, except in the liver where some residue still persists.

The nature of the persistent ^{14}C-residue in tissues of sheep and calves

Although the excretion half-life of this residual material did not differ significantly from one tissue to another within one species (Table 2), it was apparent that the liver of both sheep and calves contained a higher level of radioactivity than any other tissue. Sixty days after dosing with ^{14}C-methyridine the amount of radioactive residue in the liver of calves after correction for the apparent ^{14}C-content of contemporary carbon (which is equivalent to about 0·1 μg methyridine/g), had fallen to about 0·05 μg/g. Two possibilities arose which would explain the existence of this trace ^{14}C-residue;

the first was that the ^{14}C-impurity present in the synthetic material was bound to tissue and only very slowly excreted; the second was that $^{14}CO_2$ was incorporated into the tissues. The impurity, 2-vinyl pyridine was the most likely impurity to combine with protein, but the ^{14}C-methyridine used contained no more than 0·01 per cent of 2-vinyl pyridine and this amount of radioactivity was not sufficient to account for the amount of the persistent residue calculated to be present at zero time. The excretion half-life of the ^{14}C-material was similar to that reported for ^{15}N-labelled protein in liver and indicates that the second possibility was the most likely one: that traces of ^{14}C-methyridine may have been oxidised to $^{14}CO_2$ which resulted in general labelling of the carbon pool. This is supported by the occurrence in the urine of some animals of traces of ^{14}C-urea. Using the liver from one animal, selected because it contained the highest observed concentration of ^{14}C in all the tissues examined after disappearance of known metabolites (47 dpm/g above background) it was shown that the residual ^{14}C was distributed throughout the sub-cellular fractions and that it was not dialysable. Because of the very low concentration of ^{14}C in the liver it was not possible to investigate it further. These results however are consistent with the view expressed above that this residual ^{14}C material is due to general labelling of the carbon pool with $^{14}CO_2$ produced most likely by decarboxylation of the major metabolite.

The percutaneous absorption of methyridine

When methyridine, formulation pH 8·0 ,was applied continuously to the shaved skin (15·2 cm^2) of eleven rabbits, the concentration in the blood rose rapidly, and within 1 hr after application had reached levels as high as 550 μg/ml. Within $5\frac{1}{2}$ hr, four animals had died; they had absorbed from 12 to 60 ml methyridine up to the time of death and at death had blood levels of 550–650 μg/ml. An attempt was made to reduce the rate of absorption by lowering the pH of the formulation. At pH 4·06, the blood levels increased more slowly; the maximum observed in six rabbits, 1 hr after application was 21 μg/ml and after 7 hr the maximum was 264 μg/ml. A protective covering of silicone grease or a film of a silicone base barrier cream had a similar effect to lowering the pH of the formulation. The range of blood levels observed after 5 hr continuous application being 23–93 μg/ml for the silicone grease and 14–196 μg/ml for the barrier cream. These results clearly indicate that lowering the pH, or the application of a protective film of a silicone preparation significantly lowers the rate of absorption and therefore decreases the concentration of methyridine in the blood.

The excretion of methyridine in the urine, faeces and milk

The quantitative recovery of urine and faeces from large animals presents some technical difficulties but the results obtained with ^{3}H-methyridine and ^{14}C-methyridine (Table 3) indicate that methyridine and its major metabolites are rapidly excreted in the urine of sheep, calves and small laboratory animals irrespective of the route of dosing. The faeces frequently contained radioactive material but this is considered to be due to contamination with urine, as in cases where no contamination occurred no radioactivity was detected in the faeces.

Less than 2 per cent of the dosed methyridine was excreted unchanged in the urine of sheep and calves; this value was similar to those observed in small laboratory animals. No attempt was made to collect urine over short-time intervals but the results of 12-hourly collections indicate that the mean rate of excretion of the administered

TABLE 3. THE AVERAGE RECOVERY OF METHYRIDINE AND METABOLITES FROM THE URINE OF DIFFERENT SPECIES

Species	Route	Compound dosed	Percentage of dose recovered within 2 days after administration						
				Metabolites					
			Methyridine	A*, B	C	D	EF	G	^{14}C-Urea
Sheep	i.p., p.o., s.c.	Methyridine	0·5–2	3	5–10	80–90	5–10	0·1	0·0018
Calf	i.p., p.o.	Methyridine	0·5–2	3	2–8	85–95	5–10	—	0·0015
Rabbit	p.o.	Methyridine	0·2	—	—	80	—	—	—
Mouse	p.o.	Methyridine	0·2	—	—	100	—	—	—
Rat	p.o.	Methyridine	0·4	—	—	85	—	—	—
Rat	p.o.	2-(2′-hydroxyethyl)-pyridine	—	—	—	82	—	—	—
Rat	p.o.	Pyrid-2-yl acetic acid	—	—	—	70	—	—	—

Compounds were administered at 200 mg/kg.
* For identification of the metabolites, see Fig. 3.

^{14}C was maximal during the first 24-hr period following dosing and thereafter decreased exponentially so that the excretion rate fell by 50 per cent every 7 hr (range 3–12 hr): this agrees very well with the half-life of about 6–8 hr which was observed for the major metabolites of ^{14}C-methyridine in the plasma of calves.

The results for the excretion of methyridine and metabolites in the milk of cows show that this is a minor route of excretion, no more than 0·55 per cent of the total administered dose being excreted by this route during 8 days.

The evidence presented below indicates that about 99·5 per cent of a dose of methyridine is metabolized to products with short metabolic half-lives and this, in conjunction with the rate of excretion of the metabolites in the urine and the data on residues in the tissues (Table 2), is consistent with the view that all the administered methyridine is excreted in the urine as unchanged drug or metabolites.

The in vivo *metabolism of methyridine*

The use of the direct assay technique, and oxygen flask combustions enabled five radioactive spots to be detected on paper chromatograms. These metabolites were

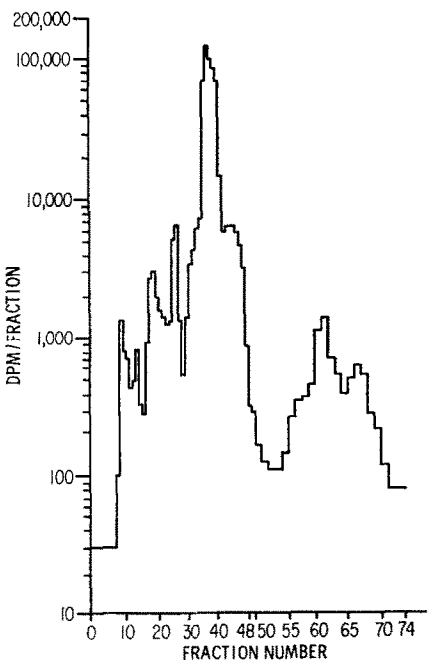


FIG. 3. Chromax column chromatographic separation of ^{3}H-methyridine metabolites. Fractions 1 to 48 were 150 ml each; others were 300 ml each. Methyridine, fractions 7–11. Metabolite G, 2-(2′-hydroxyethyl)-pyridine, fraction 14. Metabolite F, fractions 17-20. Metabolite E, fractions 26–28. Metabolite D, pyrid-2-yl acetic acid, fractions 36–40. Metabolite C, pyrid-2-yl acetyl glycine fractions 43–48. Metabolite B, fractions 59–63. Metabolite A, fractions 66–70.

satisfactorily separated on a Chromax paper roll column, and in all, eight radioactive peaks were obtained (Fig. 3) from the column.

Metabolites A and B. These two metabolites of lowest R_f were observed in the urine of sheep and calves when dosed with either ^{3}H or ^{14}C-labelled methyridine. They were stable to acid hydrolysis and did not give a yellow colour on prolonged irradiation with u.v. light. It would appear that these compounds both have the two

carbons of the methyridine side chain intact, but possibly have an altered pyridine nucleus. This fraction accounted for about 3 per cent of the administered dose.

Metabolite C. Prolonged irradiation of paper chromatograms with u.v. light, produced a yellow colour with this compound which suggested the presence of a metabolite containing two carbon atoms in the 2-position of the intact pyridine nucleus. This metabolite had an R_f a little lower than that of the major metabolite; lt was isolated by column chromatography (Fig. 3) but could not be obtained crystalline; it gave glycine on alkaline hydrolysis and was shown to be pyrid-2-yl acetyl glycine by reverse-isotope dilution techniques. This metabolite was estimated, by radioactive assay of paper sheet and column chromatograms, and by semi-quantitative estimates of the glycine liberated by hydrolysis to account for 5–10 per cent of the administered dose in sheep and from 2–8 per cent in calves.

Metabolite D. This metabolite was isolated from preparative paper chromatograms of non-radioactive urine as a hydrochloride and identified unequivocally as pyrid-2-yl acetic acid hydrochloride by melting-point, i.r. spectroscopy and physical properties. In all the species examined the excreted pyrid-2-yl acetic acid accounts for 80–100 per cent of the administered drug within 2 days of dosing.

Metabolites E and F. These two metabolites were found in the urine of sheep and calves dosed with ^{3}H or ^{14}C-labelled methyridine. They had similar R_f values which were approximately twice that of pyrid-2-yl acetic acid in the butanol-ammonia solvent system. These two metabolites have not been identified. Together they account for 5–10 per cent of the administered dose.

Metabolite G. This fraction was observed in the urine of sheep dosed with ^{3}H-methyridine; after concentration and paper chromatography it was shown to be homogenous and to have an R_f similar to that of 2-(2′-hydroxyethyl)-pyridine. It gave a yellow coloured spot on irradiation with u.v. light and was identified as 2-(2′hydroxyethyl)-pyridine by reverse-isotope dilution with inactive 2-(2′-hydroxyethyl)-pyridine hydrochloride. This material accounted for 0·1 per cent of the administered dose.

The first peak obtained on elution of the column was unchanged methyridine. The results are summarized in Table 3.

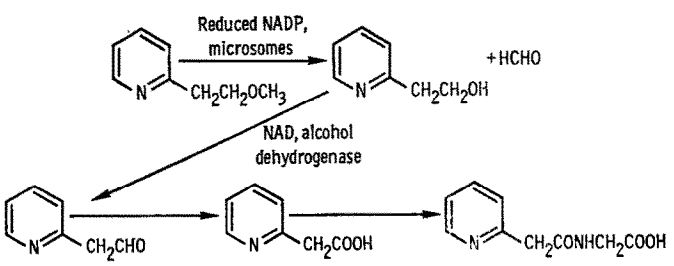


FIG. 4. Postulated metabolic pathway of methyridine.

The presence of the above metabolites suggests that metabolism takes place via the pathway shown in Fig. 4. Further evidence that 2-(2′-hydroxyethyl)-pyridine is an intermediate in the metabolism was obtained on dosing the compound to rats, when 2 days after dosing 82 per cent of the dose was recovered as pyrid-2-yl acetic acid (Table 3). When pyrid-2-yl acetic acid was dosed subcutaneously to rats 70 per cent of the dose was excreted unchanged in the urine within 16 hr (Table 3).

The in vitro *metabolism of methyridine*

In rabbits, rats and mice it was shown that the liver was the organ primarily responsible for at least the primary metabolic degradation of methyridine. Under optimum conditions, liver homogenates would demethylate 1·5 μmoles of methyridine per hr per g tissue with the concomitant production of 0·6 μmoles formaldehyde. This rate of metabolism was some eight times greater than that observed with any other tissue. The demethylation enzyme system was found in the microsomal rich centrifugate of liver homogenates and required NADPH and oxygen.

The metabolism of 2-(2′-hydroxyethyl)-pyridine required an alcohol dehydrogenase present in the microsomal supernatant (78*s*), and NAD. The supernatant (78*s*) derived from 1 g of liver metabolized 2·0 μmoles of substrate per hr. Although we were unable to demonstrate the production of the aldehyde or of the final oxidation product, pyrid-2-yl acetic acid *in vitro*, the results support the suggestion that the major metabolic route is as shown in Fig. 4.

Preliminary investigations of the inhibition of ether cleaving microsomal enzymes showed that the cleavage of both codeine and methyridine by microsomal enzymes was 95–100 per cent inhibited by PMB at 4×10^{-3} M, whereas the presence of SKF 525A at 10^{-4} M caused an 80 per cent inhibition of codeine metabolism, a 30 per cent inhibition of methyridine metabolism but had no inhibitory effect on the metabolism of *p*-ethoxy acetanilide to *p*-hydroxy acetanilide. These results obtained with codeine and *p*-ethoxy acetanilide are in close agreement with those of Axelrod,[12] and they suggest that the ether splitting microsomal enzyme necessary for the metabolism of codeine may also be that which metabolises methyridine to 2-(2′-hydroxyethyl)-pyridine.

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