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METABOLISM AND EXCRETION OF ^{14}C-LABELLED DIETHYLCARBAMAZINE

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There has been no literature on the metabolism of piperazines since Hanzlik's paper in 1917 declaring piperazine to be valueless in gout. Hanzlik mentioned the general view that most of an oral dose of piperazine was rapidly excreted unchanged in the urine, but that some was oxidized in the body.

No further mention was made of piperazines in therapeutics until Hewitt and his co-workers (1947, 1948) investigated over one hundred piperazine derivatives for filaricidal activity on *Litomosoides carinii* infections in cotton rats. The outcome of this work was the introduction of diethylcarbamazine (1-diethylcarbamyl-4-methylpiperazine), which as a salt (Hetrazan, Banocide, Notezine) has been used extensively in the treatment of human filariasis.

While the mode of action of this drug was being investigated it was found that microfilariae are apparently unaffected when placed in a 1% solution at 37° C. for as long as 24 hr., yet if a dose of about 20 mg. base/kg. is injected into a cotton rat the number of circulating microfilariae is reduced by about 80% within 2–3 min. (Hawking, Sewell, and Thurston, 1950; Bangham, 1955). This contrast of an extremely rapid action *in vivo* with little or no apparent effect *in vitro* is unique among chemotherapeutic agents. As a first step in investigating it, the metabolism of this compound has been studied. The colour reaction described by Lubran (1950) has proved useful in studying the general behaviour of the drug in man; it is, however, not specific—there is a variable blank value for blood and urine estimations in untreated patients—and the technique does not estimate metabolites. Since there are no known sensitive specific and accurate chemical means of estimating piperazine compounds, material labelled with ^{14}C has been used in the experiments to be described.

Materials and Methods

Labelled Drug.—Diethylcarbamazine labelled with ^{14}C in the 3 and 5 positions of the piperazine ring was kindly synthesized by Dr. B. H. Chase and Mr. A. M. Downes (1953). A small amount of methylpiperazine similarly labelled was also available. Another batch of diethylcarbamazine kindly synthesized by Dr. H. R. V. Arnstein and labelled with ^{14}C in the methyl radical was used to study the metabolism of this radical. The specific activity of each of these batches of diethylcarbamazine was 1.5 μc./mg.

In neutral or alkaline solution diethylcarbamazine undergoes slow hydrolysis to methylpiperazine. A stock solution used for experimental purposes was kept at *p*H 6: this remained chromatographically pure for 4 months.

Doses.—All doses are expressed as mg. base/kg. body weight. The usual human oral dose is 2–4 mg./kg.; monkeys and rats were given doses varying from 2 to 25 mg./kg. as stated in each experiment. The radioactivity of each dose was adjusted so that each animal received 7–12 μc. as a single dose.

When given intravenously, the stock solution was neutralized with sodium bicarbonate immediately before injection, which was performed under light anaesthesia. Oral administration was by stomach tube.

Animals.—Male and female hooded rats (*Rattus*) and cotton rats (*Sigmodon*) weighing 150–250 g. were used. The male and female monkeys (*Macaca mulatta* and *Cercopithecus aethiops*) which were used weighed 3.8–6.5 kg.

Collection of Samples

Urine.—Urine from monkeys was collected at timed intervals by an indwelling catheter; a steady urine flow during the 12 hr. after intravenous doses was ensured by a concurrent slow intravenous saline drip. To facilitate more accurate collection of urine from rats the lower half of the body was depilated the day before use.

Expired CO_2.—For periods of observation of more than 30 min. rats were kept in a metabolism cage (capacity 1 l.). Air was sucked through this at 1 l./min. and then through a system of 3 bubbling tubes containing 10 N-NaOH connected in series with a

sentinel tube containing saturated barium hydroxide. For shorter periods of observation the rat was lightly anaesthetized with ether and its nose held in a small glass mask fitting round the neck. A steady swift suction was maintained through a tube from the mask to a glass spiral kept in liquid air. At the end of the observation period the liquid air spiral was isolated by taps and connected to the set of NaOH absorption tubes. The spiral was allowed to warm slowly and the released CO_2 was absorbed in the alkali. Carrier CO_2 was added by gently blowing through the spiral, so washing out the remaining expired CO_2. For short rapid successive collection-periods, two absorption spiral sets in parallel were used alternately. For monkeys a similar double absorption spiral set was used in conjunction with a mask fitting closely round the face.

The CO_2 was precipitated as $BaCO_3$ and washed with boiling distilled water, acetone, and ether. It was oven-dried, weighed, and an aliquot then plated and counted.

Estimation of Radioactivity

Samples were plated as infinitely thick preparations on 0.3, 1.0, 2.0, or 5 cm.^{2} discs. They were usually counted on an automatic sample changer under a thin-window GM tube. A perspex Harwell standard of 1 μc./g. giving approx. 1,000 c./min./cm.^{2} and a background (8–10 c./min.) were counted within at most 8 hr. of each sample. With the facilities that became available later they were counted alternately with each sample. All samples were counted for at least 1,000 counts. Samples giving less than twice the background were checked against the background count before and after.

By combustion of known amounts of labelled drug it was found that the $BaCO_3$ gave 11% more counts than calculated. This was assumed to be due to back-scatter by the heavy barium atom. Where possible, however, in calculating percentage recoveries the dose and the recovered sample were counted in the same chemical form, either as the organic compound or as $BaCO_3$.

Estimation of Total Radioactivity by Combustion.—(*a*) *In a liquid.* An accurate measured aliquot of the liquid (up to 0.1 ml.) was loaded on to a 2 cm.^{2} disc of Whatman No. 1 filter paper in a platinum combustion boat. This amount of paper gave 110–120 mg. $BaCO_3$, which is a convenient quantity for accurate weighing, easy handling, and plating. Smaller amounts of carrier were used if a low specific activity was expected. A simplified electrically driven and heated Pregl microanalytical line was used with a silica tube (11 mm. internal diameter) packed in the usual way. Dried CO_2-free oxygen was passed through the tube and the CO_2 produced by combustion was precipitated by bubbling through saturated barium hydroxide at 60–80° C. in sintered glass filter ampoules. Quantitative precipitation occurred, and the $BaCO_3$ could be washed, dried, and weighed in the same ampoule without atmospheric contamination. The specific activity of the known weight of $BaCO_3$ could then be used to estimate the radioactivity in the liquid sample taken. (*b*) *In a solid.* Weighed amounts of solid tissue which would give approximately 50–120 mg. $BaCO_3$ were combusted in the same way. Carrier carbon was added if necessary.

Accuracy of Combustion Method.—Calibration by combustion of known amounts of labelled crystalline substances showed a standard error of 3% for solid samples and 5% for liquid samples (including pipetting, weighing, and counting errors).

Separation of Metabolites

By Paper Chromatography.—Aliquots of urine or tissue homogenates were shaken thoroughly with 5–10 ml. 10 N-NaOH and 10–40 ml. chloroform. The chloroform was dried with sodium sulphate and concentrated by evaporation at room temperature. The concentrate was loaded on a strip of Whatman No. 1 chromatographic paper 2.5 cm. wide, and run as an ascending chromatogram using fresh distilled pyridine 350 parts, amyl alcohol 350 parts, and water 300 parts, as the mobile phase. A run lasting 16 hr. gave a solvent front at 30 cm. which provided adequate separation of spots. The chromatogram tank was kept in a thermostatically controlled room and the vapour inside maintained well saturated. Contamination with very small amounts of water or salt considerably altered the R_F values. Paper buffered at a high *p*H was found to give more reproducible R_F values.

Paper electrophoresis and numerous other chromatographic systems were tried, but none gave such good separation of spots.

All chromatograms were scanned for radioactivity by moving the strip automatically under a thin-window GM tube coupled to a ratemeter recording with a two-channel pen recorder.

All metabolites can be developed with iodine vapour, but neither this method nor the use of any other chromogenic reagents was specific or sufficiently sensitive for the minute amounts concerned.

Approximate R_F values were :

Piperazine base, 0.1; methylpiperazine hydrochloride, 0.1–0.2; unknown metabolite hydrochloride, 0.4; unknown metabolite base, 0.5; diethylcarbamylpiperazine, 0.60–0.65; diethylcarbamazine, 0.75; breakdown product of unknown metabolite, 0.8–0.9; methylpiperazine base, 0.8–0.9.

By Column Chromatography.—Zeocarb (200–400 mesh, 8–12% crosslinked) was prepared in the H form in the usual way. By successive decantation a grain size was obtained of such uniformity that there was no detectable layering after loading on the column.

On a pilot column of 80 g. resin (1.5 × 20 cm.) good separation of metabolites was achieved. Bases from the urine of a rat given 5 mg. of ring-labelled diethylcarbamazine intravenously were extracted into ether, which was then extracted with 5 ml. 1 N-HCl. (Although piperazine is not very soluble in ether, this solvent was used because of its low boiling point, since

some of the metabolites are both volatile and unstable as bases at higher temperatures.) The acid solution was loaded on the column and the bases eluted with 1 N-HCl. The eluate was tested for radioactivity at first by the conventional method of counting 0.1 ml. aliquots of fractions dried down on polythene discs, and later by passing it through a Helium-alcohol flow counter (Bangham, D. R., unpublished) which registered activity by means of a ratemeter coupled to a two-channel pen-recorder. This obviated much tedious sampling work and provided a continuous immediately available record of activity coming from the column.

Each peak of radioactivity was identified by paper chromatography and crystallization with carrier to constant activity. Methylpiperazine followed by diethylcarbamylpiperazine appeared as well-defined peaks; the unknown metabolite was well separated from the second peak, but was not quite separated from the fourth peak, diethylcarbamazine.

A large scale preparative column (600 g. Zeocarb, 2.8 × 85 cm.) was used with the intention of preparing some of the unknown metabolite for chemical identification. Five monkeys were injected intravenously with 50 mg. (≡14.1 μc.) ring-labelled diethylcarbamazine and their urine collected for the next 24 hr. in 10 ml. 1.0 N-HCl. The urine was freeze-dried in 12 bottles, 5 ml. 10 N-NaOH added to each bottle, and the metabolites extracted by shaking vigorously by hand with 6 × 100 ml. ether each. The ether was concentrated to 20 ml. under a 80 cm. fractionation column, and extracted with 10 × 2 ml. 0.5 N-HCl. This acid was loaded on the column, which was then eluted with 1.0 N-distilled HCl at a constant rate of 60 ml./hr. in fractions of 45 ml.

Fractions of each of the four peaks of radioactivity were pooled and run through a column of 600 g. Deacidite E in batches of 500 ml. The Deacidite had been prepared as recommended and washed with 2 l. warm methanol to remove colouring matter; it was regenerated between each cycle with 25 l. 2% $NaHCO_3$ and washed with 20 l. distilled water. The neutral pooled fractions were then made slightly acid again and freeze dried, before being made alkaline and extracted with 10 l. ether. This ether was finally concentrated under a 80 cm. fractionation column and the concentrate collected in a small weighed beaker.

The specific activities of these oily concentrates were less than calculated owing to contamination with colouring matter from the resins. The concentrates were therefore chromatographed on thick Whatman MMM paper in the pyridine-amyl alcohol-water system, and the specific peaks of radioactivity eluted with methanol.

Urine from a second group of six monkeys was dried similarly and the metabolites separated on a second column. Some improvement in the concentration of the last broad peak of radioactivity was achieved by changing the eluting acid to 2 N-HCl at the appearance of the first peak.

The treatment of the unknown metabolite is described below.

Estimation of Individual Metabolites by Carrier Techniques

Diethylcarbamazine.—Diethylcarbamazine hydrochloride and dihydrogen citrate were too hygroscopic when prepared for counting, since an automatic feed counter was used for radioactivity assay in which it was convenient to leave the specimen overnight.

Diethylcarbamazine gave a fine, stable non-hygroscopic white powder on precipitation with Kalignost $[(C_6H_5)_4B]^-Na^+$; Zeidler, 1952). One hundred milligrams carrier diethylcarbamazine (the dihydrogen citrate) was added to the solution—for example, urine—which was made strongly alkaline with sodium hydroxide and extracted with chloroform. This chloroform was filtered, concentrated, and extracted with 2 ml. 1.0 N-acetic acid. A filtered solution of Kalignost (150 mg./ml. water) was then added dropwise to the acetic acid extract until no more precipitation occurred. This precipitate was washed well with cold distilled water, dried in a vacuum desiccator, and plated for counting. The precipitate was recrystallized from acetone-petroleum (40–60°) to constant activity. Kalignost precipitates a wide range of organic bases, and several recrystallizations were necessary to produce constant activity in assays on samples of urine.

As an alternative method the chloroform was extracted with water and the drug precipitated with cold aqueous ammonium reineckate. Although this was much more specific for diethylcarbamazine, the conditions were difficult to reproduce and the salt tended to be unstable. The method was therefore discarded in favour of the one first described.

1-Diethylcarbamylpiperazine.—The urine sample was added to weighed amounts of carrier (80–100 mg. base) and the bases were extracted into chloroform as described for diethylcarbamazine. The chloroform was dried with sodium sulphate, filtered, and evaporated by warming gently. The residue was taken up in 2 ml. pure dry pyridine, about 500 mg. toluene-*p*-sulphonyl chloride was added, and the mixture heated to 100° C. for 15–20 min. After cooling it was poured into about 50 ml. cold distilled water. This gave a good yield of white crystals of the toluene-*p*-sulphonyl derivative, which could be recrystallized from hot ethanol-water. The method is relatively specific, and little reduction in count rate was observed after 2–4 crystallizations.

Attempts to form a dithiocarbamate with carbon disulphide were not regularly successful, and recrystallization sometimes gave yields too small for reliable counting. Theoretically the carbon disulphide precipitation method may be non-specific, and, in fact, after 6–7 recrystallizations with carrier, the count rate, although constant, was approximately three times that calculated from the toluene-*p*-sulphonate method. This contamination is so great as to suggest that the unknown metabolite may form a compound closely similar to the carrier.

Methylpiperazine.—Weighed amounts of carrier (about 150 mg. of the dihydrochloride monohydrate,

mol. wt. 191) were added to the urine sample. Extraction from acid solution, followed by extraction with chloroform from the alkaline solution, was carried out as above; the chloroform was filtered and alcoholic picric acid added dropwise. The crystalline dipicrate so formed (mol. wt. 558) was washed well with absolute ethanol and recrystallized to constant activity from dimethylformamide-absolute ethanol. About four recrystallizations were needed.

Piperazine.—Weighed amounts (about 200 mg.) of piperazine hexahydrate (mol. wt. 194) were added to the urine sample, which was made strongly alkaline with 10 N-sodium hydroxide and extracted with ether and then with chloroform. This chloroform was extracted thoroughly with 1.0 N-acetic acid and an aqueous solution of chromic acid added to the extract forming crystals of piperazine dichromate (mol. wt. 304). After standing for 30 min. in the cold these crystals were filtered off and washed with absolute ethanol. About five recrystallizations from hot water were needed to obtain constant activity.

RESULTS

Metabolism and Excretion of Ring-labelled Diethylcarbamazine

Table I shows the percentage recovery of an intravenous dose of diethylcarbamazine excreted in the urine in terms of total radioactivity (using ring-labelled material), of unchanged drug, and of

TABLE I

RECOVERY OF ^{14}C-LABELLED DIETHYLCARBAMAZINE FROM URINE

Animal	Position of ^{14}C in Drug Molecule	Dose (mg./kg.)	Route of Administration	% Excreted in the Urine in 24 Hr.		
				Total Radioactivity	As Diethylcarbamazine	As Methylpiperazine
Rat ..	Piperazine ring	24·0	i.v.	97%	18%	
,,	,,	27·0	,,	90%	20%	
Monkey	,,	2·8	,,	90%	11%	3·3%
,,	,,	2·9	,,	94%	12%	1·6%
,,	,,	15·0	,,	83%	18%	
,,	,,	7·4	Oral	70%	15%	
,,	,,	8·8	,,	78%	16%	
,,	Methyl radical	2·0	i.v.		11%	5·3%
,,	,,	2·0	,,		12%	3·5%
Rat ..	,,	20·0	,,	72%	10%	3·4%
,,	,,	20·0	,,	82%	14%	5·1%

methylpiperazine (using both ring- and methyl-labelled material). Fig. 1 shows cumulative curves of the percentage of total radioactivity and unchanged drug excreted in the urine (using ring-labelled material) and $^{14}CO_2$ in expired air (using methyl-labelled material, see below). In Fig. 2 the logarithm of the percentage of the administered dose excreted in 12 min. periods—based on the curves shown in Fig. 1—is plotted against a linear time scale. The log rate of excretion of both unchanged drug and expired $^{14}CO_2$ is approximately on the same straight line, which intercepts the abscissa at just over 3 hr. The slow excretion of $^{14}CO_2$ which occurs thereafter may be due to slow clearing of plasma bicarbonate and possibly some slow demethylation of another metabolite, but it is obvious that the major part of the expired $^{14}CO_2$ follows the excretion of unchanged drug. The slope of the log rate of excretion of total radioactivity has at least two components. During the first 3 hr. there is a rapid component,

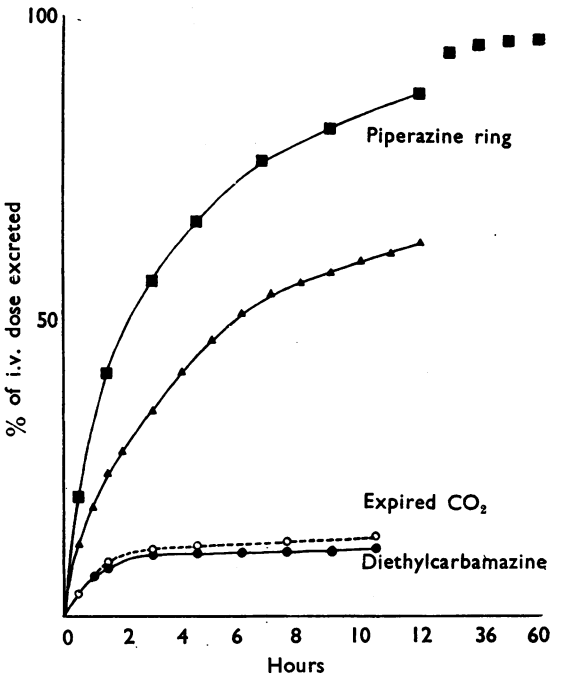

FIG. 1.—Graph showing cumulative excretion of: (1) ■ piperazine ring containing compounds in the urine; (2) ● unchanged diethylcarbamazine in the urine; (3) ○ expired $^{14}CO_2$; (4) ▲ the sum of (2) and (3) subtracted from (1). This is believed to represent the excretion of the unknown metabolite. Note change in time scale after 12 hr. Average of 4 rats given 20 mg./kg. diethylcarbamazine i.v. See Figs. 2 and 3 for further analysis of these curves.

which on extrapolation crosses the zero line at 6 hr. There is then a slow component from 3 hr. to approximately 10 hr. which on extrapolation crosses the zero at about 15 hr. If the sum of the percentage excreted as unchanged drug and $^{14}CO_2$ is subtracted from the total radioactivity excreted, the curve obtained is shown in Fig. 1 and the log rate of excretion of this is plotted in Fig. 2. This shows a slope with at least two components, an initial steep slope until approximately 2 hr. which would cross the base line at about 6 hr. and a

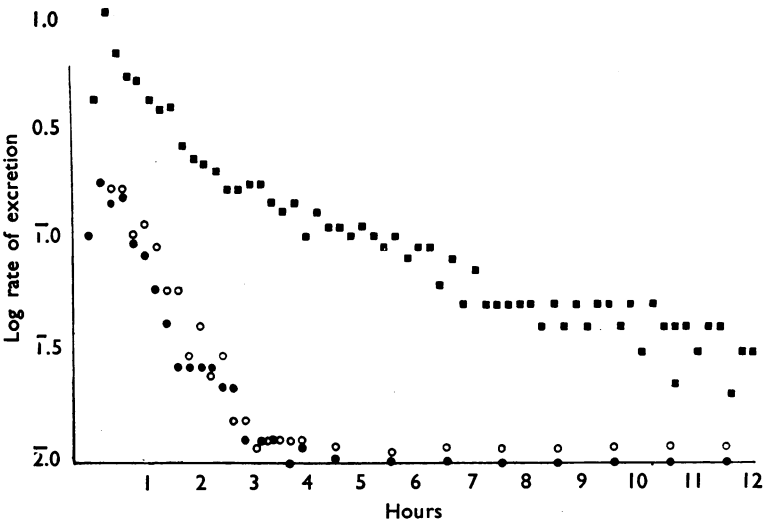

FIG. 2.—Graph showing the log of the percentage of the administered dose excreted in 12 min. periods, i.e. the log of the rate of excretion plotted from the curves shown in Fig. 1. The excretion rate of $^{14}CO_2$ (marked ○) closely follows the excretion rate of unchanged diethylcarbamazine (●) until 3 hr. after the dose.

second long component from 2 to 10 hr. which is believed to represent the rate of excretion of the unknown metabolite.

Total Excretion.—After intravenous administration of ring-labelled diethylcarbamazine dihydrogen citrate to either rats or monkeys, 80–100% of the injected radioactivity was found in the urine in 24 hr.—for example, two rats given 24 and 27.5 mg./kg. i.v. excreted 97 and 90% respectively; two monkeys given 7.7 and 15.0 mg./kg. i.v. excreted 100 and 83% respectively in 24 hr. After oral doses two monkeys given 7.4 and 8.8 mg./kg. excreted 70 and 78% respectively in 24 hr. Within the limits of accuracy of the methods used for collection of urine, combustion, and counting, the whole dose in terms of radioactivity appeared in the urine within 30 hr. Half the total activity injected was excreted in the urine in 3 hr. $\pm$ 20% both in rats and in monkeys. Samples of faeces which had been combusted contained negligible radioactivity.

Excretion as Diethylcarbamazine.—In rats and in monkeys 10–20% of an intravenous dose of diethylcarbamazine was excreted as unchanged drug in 24 hr. There was some suggestive evidence that after a larger dose more unchanged drug is excreted in the urine (Table I). In 4 rats given doses of 25–40 mg./kg. i.v. half of this amount was excreted in the urine in 50 $\pm$ 10 min. The maximum rate of excretion was 8.5%/hour during the period of 12–24 min. after the dose, which corresponds closely with the maximum rate of excretion (9%/hr.) of labelled methyl group as expiratory carbon dioxide during the same period (Figs. 1 and 2).

There was no evidence for retention of the drug, and after 9 hr. less than 1% of the dose was excreted as unchanged drug in the urine. After repeated injections more was excreted unchanged: a rat which had been given 24, 25, 23, 26 mg./kg. of methyl-labelled base (see below) intraperitoneally on four successive days excreted 7.4, 12, and 14% on the first, second, and fourth days while the drug was being administered, and only 0.5% on the fifth day. However, the $^{14}CO_2$ expired in the first 6 hr. after the first and last dose was 15.6% and 15.0% respectively of the previous dose given. This suggests that demethylation takes place to the same extent despite the increased excretion of unchanged drug.

Excretion as 1-diethylcarbamylpiperazine.—Monkeys and rats excreted between 5 and 15% of the dose as this substance. It was first detected in the urine about 20 min. after an intravenous dose.

Excretion as Methylpiperazine.—About 2–5% of the dose was usually excreted as methylpiperazine after intravenous doses. Under 1% was found in the urine as this substance in the first 9 hr. This probably represents a maximum, since diethylcarbamazine undergoes hydrolysis in alkaline solution, and urine was therefore maintained neutral or acid to prevent hydrolysis.

Excretion as Piperazine.—This is also excreted in small amounts; about 1–6% was identified as piperazine, and most of this appeared more than 6 hr. after the dose.

Excretion as the Unknown Metabolite.—As this has not been identified it cannot be directly estimated with carrier. Judged by the difference of the sum of the other metabolites and the total dose recovered it accounted for at least 60% of an intravenous or oral dose of 7–10 mg./kg. in the rat and of 2–30 mg./kg. in the monkey (Figs. 1 and 3). With lower doses it appeared to account for a larger proportion of the dose. This metabolite is discussed in more detail below.

Metabolism and Excretion of Methyl-labelled Diethylcarbamazine

Excretion as Expired $^{14}CO_2$.—Rats which had been given 13–15 mg./kg. of base intravenously expired 10–15% of the dose as $^{14}CO_2$. The time and rate of excretion in expired air closely followed that of the unchanged drug in the urine (Fig. 2). The slopes of the excretion curves were parallel until 3.2 hr. after the dose; thereafter a small, very slow release of $^{14}CO_2$ continued until 12 hr. after the dose. This might be accounted for by a slow demethylation of a metabolite taking place, together with the clearing of isotopically labelled plasma bicarbonate.

Rapidity of Demethylation and Excretion as $^{14}CO_2$.—If the action of a metabolite is invoked to explain the rapid biological effects of the drug, then there should be proof of the appearance of such a metabolite within the appropriate time. This cannot be done directly until the metabolite has been identified so that the carrier method of estimation can be used. Failing this, therefore, the excretion of the carbon of the methyl radical as expired $^{14}CO_2$ was studied, since it provides a simple if indirect indication of one stage of metabolism. The experiment is summarized in Table II. Four rats were given methyl-labelled drug intravenously and the excretion of $^{14}CO_2$ followed during the ensuing 0–2 and 2–5 min. periods. It can be seen from the results that demethylation is in fact rapid, if not extensive, during this time.

TABLE II

EVIDENCE FOR METABOLISM OF THE METHYL GROUP IN THE FIRST 5 MIN. AFTER AN INTRAVENOUS DOSE OF DIETHYLCARBAMAZINE LABELLED WITH ^{14}C IN THE METHYL RADICAL

	Dose (mg./kg. Base)	Radio-activity (μc. i.v.)	Period After Dose (min.)	% of Dose Expired as $^{14}CO_2$	Rate of Excretion (%/hr.)	Sample Counts Above Back-ground
Rat 1	24	7·6	0–5	0·128	1·54	57·5
„ 2	22	6·9	0–5	0·154	1·84	24·3
„ 3	18	7·0	0–2	0·0177	0·53	15·9
			2–5	0·050	1·0	30·7
„ 4	20	7·0	0–2	0·0178	0·53	12·0
			2–5	0·040	0·8	29·6

Rats 1 and 2 were given the drug without neutralization with sodium bicarbonate.
Rats 3 and 4 were given 10 mg. sodium bicarbonate with the dose.

Excretion in the Urine.—(*a*) *Total.* Two rats which had been given 20 mg./kg. i.v. excreted 72% and 82% of the total radioactivity in the urine in 24 hr. About 10–15% of this was unchanged drug and about 5% was methylpiperazine. (*b*) *As unknown metabolite.* Chromatography of urine extracts also showed a large amount of radioactivity at the same R_F as the unknown metabolite of ring-labelled drug. This was confirmed by running chromatograms of each of these extracts separately, and one loaded with some of each extract. Each of the three chromatograms showed a single large peak at the same R_F. It seems probable, therefore, that the methyl group is also present on the unknown metabolite. (*c*) *As*

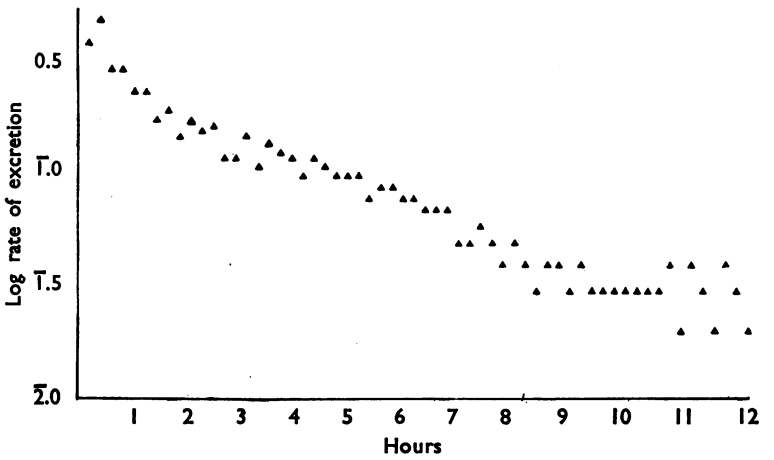

FIG. 3.—Graph showing the log of the excretion rate of the curve (marked ▲) in Fig. 1, which represents excretion of the unknown metabolite, plotted in the same way as in Fig. 2. A straight line could be drawn through the points from 2 hr. to 10 hr.

urine formate. The isolation of radioactive formate in the urine with the technique of Weinhouse and Friedmann (1952) also suggests that this methyl group carbon equilibrates with blood bicarbonate. Two rats which had been given 20 and 25 mg./kg. of drug i.v. excreted 0.3% in 4 hr., and 0.14% in 2 hr., and 5.17% in 2–4 hr. after the dose, respectively, as urine formate.

Entry of the Methyl Carbon into the Metabolic "Methyl Pool."—To see whether the methyl carbon also enters the metabolic pool the method of Mackenzie and du Vigneaud (1952) was used. They gave a rat 50 mg. choline hydrochloride by stomach tube daily for 8 days and again 50 mg. 30 min. before the test dose. In this way the methyl pool was kept saturated, and the carbon of any further methyl groups introduced (for example, i.v. administered drug) would be promptly excreted as respiratory $^{14}CO_2$. In their experiments using ^{14}C methionine as the test methyl group they found a 2–3-fold increase in CO_2 activity over 24 hr. and 10-fold increase in activity during the first hour.

No increase was found in two such experiments when rats were given 21.5 and 12.1 mg. base/kg. of methyl-labelled diethylcarbamazine.

Hydrolysis of Urine

After Ring-labelled Drug.—Two rats (200 g.) were given i.v. 20 mg./kg. of ring-labelled drug. Their urine was collected over 24 hr. in 2.0 ml. 1 N-HCl. Duplicate combustions of an aliquot showed that this urine contained 97 and 98% of the dose. Twenty millilitres of this urine were refluxed with 20 ml. 10 N-HCl for 10 hr. Duplicate analyses on this hydrolysate were carried out for piperazine and methylpiperazine, each being recrystallized and counted five times, as the dichromate and dipicrate respectively.

The results of two such hydrolyses showed 74.0 and 73.5% of the hydrolysate to be methylpiperazine and 22.3 and 20.6% to be piperazine. The remaining 5% may be accounted for by experimental error.

After Methyl-labelled Drug.—A similar hydrolysis of urine from another pair of rats treated with the same dose of methyl-labelled material showed that 80 and 86% of the dose was excreted in the urine, and 97% and 104% (experimental error = $\pm$ 5%) of the hydrolysate was methylpiperazine in two exactly similar experiments, each with duplicate analyses. This suggests that practically all of the ^{14}C in the urine is present as one or more substances which can be hydrolysed to methylpiperazine.

The Unknown Metabolite

This is the first metabolite to appear in the urine, and a substance with a similar R_F value appears in the blood within 2 min. after an intravenous dose. It has proved difficult to isolate because of its instability, and so far it has defied identification. Because of this it cannot be estimated directly by carrier, but judged by the difference between the sum of the other metabolites and the total amount excreted it seems to account for about 60% of the dose (Figs. 1 and 3, and see Discussion).

Isolation and Properties.—The attempt to isolate by column chromatography enough for chemical identification was disappointing on account of its instability. The few milligrams isolated by elution of the peak from the final thick paper chromatogram showed it to be an odourless, pale yellow, oily, basic material. This material is soluble in chloroform and ether but is more soluble in methanol. It is stable to acid (5 N-HCl for 5 hr. at 100° C. gave only partial hydrolysis), and to oxidizing agents (20 vol. hydrogen peroxide), and it can be boiled for 30 min. in dilute solution at *p*H 6 with little decomposition. It is, however, unstable in weakly alkaline solutions and is hydrolysed at *p*H 8 at 50° C. to a substance of R_F 0.8. The intermediate breakdown product (R_F 0.8) is less polar and more soluble in organic liquids (particularly methanol) than in water.

An attempt at a simple counter-current separation of these substances illustrates the instability of the unknown, and the relative non-polarity of its breakdown product. Shaking 29 mg. of the oily material obtained from the ether extract of the column-separated unknown—still contaminated with colouring matter from the resins Zeocarb 225 and Deacidite—in a 6 x 50 ml. mutually saturated butanol-distilled water system, converted two-thirds of it to the breakdown product, 90% of which stayed in the butanol phase.

At no time has more than 90% purity (radioactive) been achieved with the unknown substance since there is always some breakdown product with it. Further efforts at purification were made, but the yield from the column preparation was so small that it was not possible to characterize it chemically. There was, however, sufficient to obtain an infra-red spectrograph on the unknown substance as a base embedded in potassium bromide. The breakdown product crystallized from dimethylformamide as a few small amorphous polarizing crystals after 2 wk. at room temperature in a

desiccator. I am indebted to Dr. R. K. Callow for the following report on infra-red absorption spectra:

"Infra-red absorption spectra were measured in potassium bromide on a Perkin–Elmer Model 21 double-beam spectrometer with a rock-salt prism.

"Descriptions of infra-red absorption spectra for this group of compounds have not been traced in the literature. Piperazine, 1-methylpiperazine, 1 : 4-dimethylpiperazine, 1-ethoxycarbamylpiperazine, 1-diethylcarbamylpiperazine, and diethylcarbamazine were examined for comparison, all as liquid films. Simple relations between spectra and composition were difficult to find.

"The unknown metabolite and its breakdown product (as a smear) showed an absorption, with two peaks at 2,910 and 2,840 cm.$^{-1}$ (Ch stretching) forming a narrow band between 3,000 and 2,800 cm.$^{-1}$ This is characteristic of the unmethylated piperazine compounds examined. The N-methyl derivatives showed strong absorption extending to 2,700 cm.$^{-1}$ The unknown and its breakdown product (smear) also showed absorption at 1,725 cm.$^{-1}$ as a shoulder to a broad band at 1,650 cm.$^{-1}$ This suggests the presence of $-CO_2H$ or CO_2Et. 1-Ethoxycarbamylpiperazine is the only one of the examined substances which had absorption in this region. A band at 1,275 cm.$^{-1}$ in the spectrum of the unknown product gives some support to this suggestion. The change from the unknown to its breakdown product involves a change in the value of the maximum of the broad band between 1,700 and 1,600 cm^{-1} from 1,625 to 1,650 cm.$^{-1}$ No suggestion can be made as to the cause of this change.

"Crystals isolated from dimethylformamide were extracted with a little chloroform and the extract was evaporated on potassium bromide. The infra-red absorption of this material suggested that the material might be one component of the unknown, assuming the sample of the latter examined to be a mixture. There was a peak at 2,900 cm.$^{-1}$, a blunt peak at 1,660 (but no indication of a 1,725 band), and some absorption in the 1,450–1,350 region, including a peculiar spike at 1,385 cm.$^{-1}$ present in the unknown and doubtfully in its disintegration product.

"To sum up, although the infra-red spectrum of the preparation of the unknown metabolite suggests inhomogeneity, it may be consistent with the loss of the methyl group, and the possible presence of CO_2Et. Of the compounds tested it is most similar to that of 1-ethoxycarbonyl-4-methylpiperazine."

Other Possible Derivatives. — Various other possible compounds were added as carriers to urine containing the unknown substance: 1 : 4-dimethylpiperazine (R_F 0.4–0.5), diethylcarbamazine as a quaternary compound (methiodide crystallized as the picrate), 1-ethoxycarbonyl-4-methylpiperazine (as dihydrogen citrate), creatinine (as picrate), urea, the *N*-acetyl derivative of diethylcarbamylpiperazine (R_F 0.7–0.75), iminodiacetic and methyliminodiacetic acid, and 4-methyl-2 : 6-diketopiperazine were all tested, but no radioactivity was found on recrystallization. Furthermore, chromatograms of acid hydrolysates showed no ninhydrin spots characteristic of amino acids. It is concluded, therefore, that the unknown metabolite is not any of the above compounds, and is not attached to an amino acid.

Discussion

By the use of ^{14}C ring-labelled diethylcarbamazine it has been shown that the piperazine ring is almost entirely excreted in the urine in 24–30 hr. This is not surprising, since the piperazine ring does not normally occur in nature and it is unlikely that the body would break down or use such a ring structure. After intravenous administration 10–20% of the dose was excreted as unchanged drug, 8–15% as diethylcarbamylpiperazine, 2–5% as methylpiperazine, and 1–6% as piperazine. The remaining 60% was excreted as another unidentified metabolite. When methyl-labelled drug was given, about 10–15% of radioactivity appeared in expired carbon dioxide and 5% in the urine as formate. This suggests that the unknown metabolite must thus have retained its methyl group. It can be seen that the sum of the two demethylated compounds, diethylcarbamylpiperazine (10–15%) plus piperazine (1–6%), approximately equals the sum of $^{14}CO_2$ expired (10–15%) plus the amount excreted as formate (1–5%).

This is confirmed by the acid urine hydrolysis which yielded 20% as piperazine and 75% as methylpiperazine. Moreover, about 80% of activity appeared in the urine 24 hr. after injecting methyl-labelled drug, and this with the expired $^{14}CO_2$ amounts to the usual activity excreted 24 hr. after injecting ring-labelled drug.

The entry of methyl carbon into the metabolic pool depends very much on what molecule it comes from and on the dose of that substance. Whereas the methyl radicals of choline (Arnstein and Neuberger, 1953) and methionine (Mackenzie, Chandler, Keller, Rachele, Cross, and du Vigneaud, 1949) partake rapidly and extensively in the metabolic turnover of protein and other amino acids, it cannot be assumed that the methyl radicals of other molecules, e.g., 1-diethylcarbamyl-4-methylpiperazine, or *N*-methyl-labelled morphine (Elliott, Tolbert, Adler, and Anderson, 1954), necessarily take part readily in this methyl pool. The dose administered also considerably affects the amount expired as carbon dioxide. The excessive amounts of drug given in the above experiments probably saturated the methyl pool, and therefore excretion as expiratory $^{14}CO_2$ was at a maximum, so that

if any methyl group did enter into the protein and amino-acid cycle the amount would represent an insignificant percentage of the dose.

The expired carbon dioxide and urinary formate probably represent the total amount of demethylation, and together with the results of urine hydrolysis it suggests that the unknown metabolite retains the methyl radical. This suggestion is at variance with the results of the infra-red spectrum on the small amount of the unknown metabolite and its breakdown product (R_F 0.8). Both these have absorption peaks at 2,910 and 2,840 cm.$^{-1}$, which is characteristic of the unmethylated piperazine compounds so far examined, whereas the methylated piperazine compounds show strong absorption extending to 2,700 cm.$^{-1}$ Both the unknown substances show absorption spectra which are markedly different from that of methylpiperazine inasmuch as each has absorption peaks which the other has not.

Until the conditions for stability of the unknown metabolite are more clearly defined it may prove difficult to isolate enough for chemical characterization, particularly in view of the difficulty in crystallizing piperazine compounds. The method of isolation described proved wasteful on account of the extraction procedures necessary before and after separation on the column which involved exposure to a high *p*H and a certain length of time as the unstable free base, and the resin itself contaminated the concentrate with considerable amounts of colouring matter.

Summary

1. The metabolism of the antifilarial drug diethylcarbamazine has been studied using drug labelled with ^{14}C in the 3 and 5 positions in the piperazine ring. Separation of metabolites by paper and column chromatography, and their estimation by carrier methods, are described.

2. More than 95% of a dose of 2–25 mg./kg. given intravenously to rats or monkeys is excreted in 30 hr. in the urine as compounds containing an intact piperazine ring.

3. Of such an intravenous dose, 10–20% is excreted in the urine as unchanged drug, most of which is excreted in the first 3 hr. From 5 to 15% is demethylated and excreted in the urine as diethylcarbamylpiperazine. From 2 to 5% is excreted as methylpiperazine, and from 1 to 6% as piperazine; both these appear in the urine 9 hr. after the dose.

4. A fourth metabolite accounts for at least 60% of the dose. This substance has been isolated in crude form, but, because of its instability as a free base, has not yet been identified. (Infra-red spectrography of this substance suggests that it is similar to ethoxycarbonylpiperazine.)

5. Experiments with drug labelled with ^{14}C in the methyl radical indicate that 10–15% of the radioactivity of an intravenous dose (at the same dose levels) is excreted as $^{14}CO_2$, and 2–5% as urine formate. Radioactivity can be detected in expired carbon dioxide within 2 min. after an i.v. dose. Analysis of excretion curves of $^{14}CO_2$ and unchanged drug indicates that only the unchanged drug is demethylated to any great extent.

6. Hydrolysis of urine containing metabolites of drug labelled in the piperazine ring and in the methyl radical suggests that the unknown metabolite retains its methyl group.

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