CARBON-14 RING LABELLING OF SOME METHYLXANTHINES OF PHARMACOLOGICAL IMPORTANCE

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SUMMARY

Preparative, separative and analytical methods are described for the following labelled compounds: 4-amino[5-^{14}C]formamidouracil (II), 1,3-dimethyl-4-amino[5-^{14}C]formamidouracil (III), 1,3-dimethyl[8-^{14}C]xanthine ([8-^{14}C]theophylline) (IV), 1,3,7-trimethyl[8-^{14}C]xanthine ([8-^{14}C]caffeine) (V), 4-amino[2-^{14}C]uracil (VI), 4-amino-5-nitroso[2-^{14}C]uracil (VII), 4,5-diamino[2-^{14}C]uracil (VIII), [2-^{14}C]xanthine (IX), 3-methyl[2-^{14}C]xanthine (X), [2-^{14}C]theobromine (XI), [2-^{14}C]theophylline (XII) and [2-^{14}C]caffeine (XIII).

INTRODUCTION

Several of the naturally occurring derivatives of xanthine (1,2,3,6-tetrahydro-2,6-dioxopurine) present in plants of the tea, coffee, cocoa and cola species have pharmacological activity. These include caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine). They are of

importance in medicine because of their stimulant and diuretic effects. They are also used illegally as stimulants to improve the performance of racing animals and athletes. In view of this widespread usage both in medicines and common drinks, remarkably little is known about the metabolic fate of these drugs. This may often prevent detection of their illegal use by analysis of body fluids.

This paper describes the radiochemical synthesis of xanthine and some of its more important derivatives and their precursors for use in some further metabolism experiments in animals.

DISCUSSION

Position of Radioactive Label

For the N-methylxanthines a carbon-14 atom in the purine ring is the only radiolabel likely to be metabolically stable. Published work to date indicates that demethylation is one metabolic process, and oxidation to uric acid derivatives another.[1-5] Choice of labelling position should also take account of the possibility of ring opening to give metabolites of the type of alloxan and allantoin. For a complete metabolism study therefore both imidazole-ring labelled xanthines and pyrimidine-ring labelled xanthines were required, and in the latter case the placing of the label at $C_{(6)}$ was to be avoided.

Previous Isotopic Syntheses of Xanthines

Xanthine itself has previously been labelled with carbon-14 at $C_{(8)}$.[6] Methylxanthines labelled with carbon-14 in the purine ring have not been reported, with the exception of [2-^{14}C]caffeine, which was prepared by methylation of commercially obtained [2-^{14}C]xanthine.[7]

Choice of Synthetic Method

The available methods for synthesis for N-methylated xanthines were reviewed and are reported in full elsewhere.[8]

A biological synthesis using cultures of tea callus tissue[9,10] with incorporation of [^{14}C]carbon dioxide or [^{14}C]methionine was considered but rejected because of the likely low yields and low incorporation, lack of certainty about the position of the label and the experimental difficulties involved.

Chemical synthesis can be carried out from either imidazole or pyrimidine precursors[11] and in both cases the methyl groups can be introduced either through the use of a suitably methylated precursor or by direct methylation of an intermediate or a xanthine with fewer methyl groups.

Imidazoles are useful mainly for the synthesis of $N_{(7)}$ and $N_{(9)}$ methylated xanthines; formation of $N_{(3)}$ methyl derivatives requires 4-methyl-5-carbamoyl-imidazoles which are not readily available.

The most generally used synthesis for substituted purines is the Traube synthesis,[12-14] because of its flexibility and the good yields obtained. By this method all the possible $N_{(1)}$, $N_{(3)}$ and $N_{(7)}$ methylated xanthines are available by use of suitably methylated pyrimidine precursors, but these require laborious synthesis. The attractive alternative approach is by direct methylation of intermediates and this was adopted for the present work. While the direct methylation approach was expected to give various monomethyl xanthines as by-products this was not regarded as a disadvantage, since all the possible xanthines with methyl groups at $N_{(1)}$, $N_{(3)}$ and $N_{(7)}$ were of interest as potential metabolites.

4,5-diaminouracil (I) and 3-methyl-4,5-diaminouracil

are both possible common starting points for theophylline, theobromine and caffeine, but the former was preferred because higher yields were obtained in trial syntheses.

Imidazole-ring Labelling

The easiest synthesis for labelling of the imidazole ring was by reaction of a labelled cyclizing agent in the Traube synthesis to produce [8-^{14}C]xanthines (Fig.1).

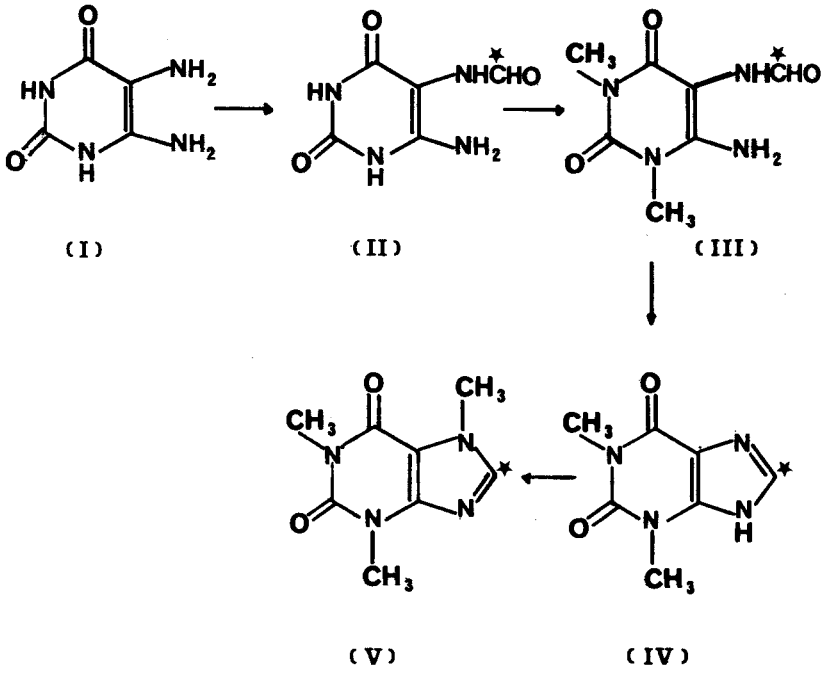

FIG. I SYNTHESIS OF [8-^{14}C] THEOPHYLLINE AND [8-^{14}C] CAFFEINE

The most efficient cyclizing agent is formamide since this gives high yields in a one-stage synthesis,[15] but this is excluded for a radiochemical synthesis because it is used as the solvent. Recourse was made to Traube's original cyclizing agent, formic acid.

With formic acid cyclization of the 4,5-diaminouracil proceeds in two stages, the first involving exclusive formylation of the amino group attached to $C_{(5)}$[16] to give 4-amino-5-formamidouracil (II). In the original method a large excess of formic acid was used but trial experiments showed that use of the stoichometric amount of formic acid did not significantly affect the yield. The second stage involves cyclization of the isolated 4-amino-5-formamidouracil to the xanthine by fusion,[12] by heating in aqueous base,[17] or by heating in a high boiling solvent, preferably formamide.[15]

Formamide could not be used in the current work because of the likelihood of losing the isotopic label through the known formyl exchange mechanism,[18] and cyclization was effected by heating with aqueous base.

However, before the cyclization reaction, methylation of (II) with dimethyl sulphate was carried out under controlled conditions of pH and temperature to give 1,3-dimethyl-4-amino-5-formamidouracil (III).[17] Cyclization to [8-^{14}C]theophylline then followed. In trial syntheses yields were low initially (10-35%) but were improved to 60% by modifications to the method. [8-^{14}C]caffeine was then obtained in 95% yield from [8-^{14}C]theophylline by methylation with dimethyl sulphate. Analysis of the various reaction mixtures and products was achieved by some new thin-layer chromatography techniques.[19]

Pyrimidine-ring Labelled Xanthines

To avoid laborious synthesis of all the necessary methylated precursors direct methylation of xanthine was used to obtained theophylline, theobromine and caffeine. As discussed above $C_{(6)}$ was considered an unsatisfactory position for the carbon-14 label, but fortunately the two most attractive approaches to xanthine lend themselves to labelling at $C_{(2)}$. These are:-

1) Cyclization of 4-amino-5-carbamoylimidazole[20-22] with [^{14}C]urea

2) Cyclization of 4,5-diaminouracil (Traube synthesis as above) itself prepared from ethyl cyanoacetate and [^{14}C]urea (see Fig.II).[23]

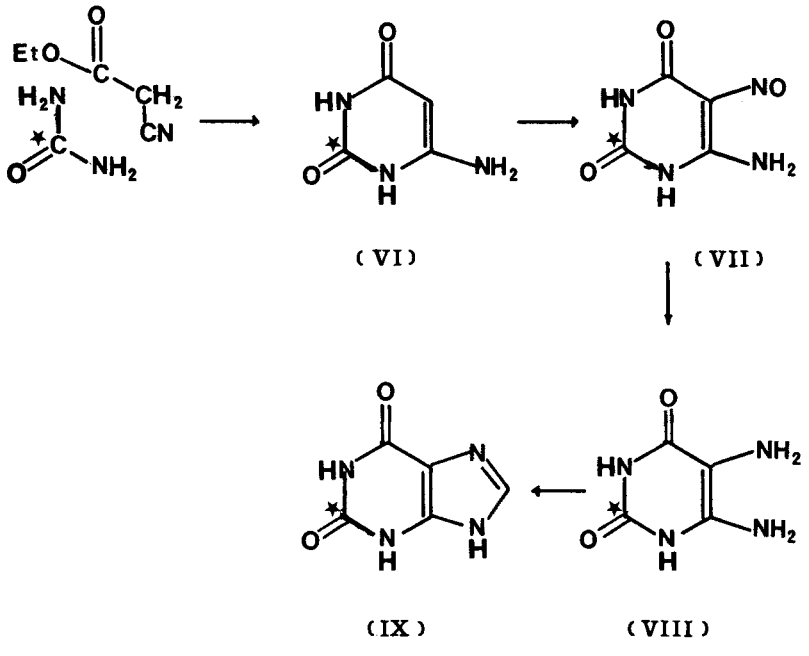

FIG. II SYNTHESIS OF [2-^{14}C] XANTHINE

Method 1 is the most direct approach, with a reported yield of 75% calculated on the imidazole in the fusion reaction with urea, but a large excess of urea is required and the method is thus less suitable for synthesis with [^{14}C]urea. Method 2 was used in the current work and 4,5-diamino[2-^{14}C]uracil was cyclized with formamide in 90% yield to give [2-^{14}C]xanthine and an overall yield based on [^{14}C]urea of 50%.

Methylated xanthines were obtained by reaction of [2-^{14}C]xanthine with dimethyl sulphate (Fig.III) under basic conditions. This reaction was investigated with

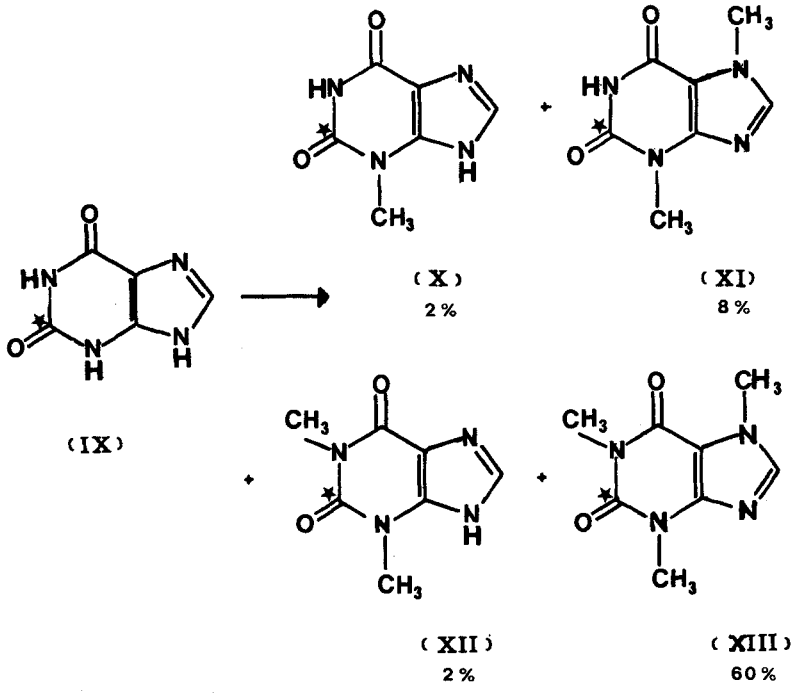

FIG. III PRODUCTS FROM METHYLATION OF [2-^{14}C] XANTHINE

[2-^{14}C]xanthine at tracer level to determine the most favourable conditions for formation of caffeine, theophylline and theobromine. The influence of molar ratio of dimethyl sulphate to xanthine on the yield of each methylated product was determined by addition of the dimethyl sulphate in portions and analysis of samples of the reaction mixture by thin-layer chromatography and liquid scintillation counting at each stage. As expected caffeine was the major product, except when the molar ratio of dimethyl sulphate to xanthine was low (see Fig.IV).

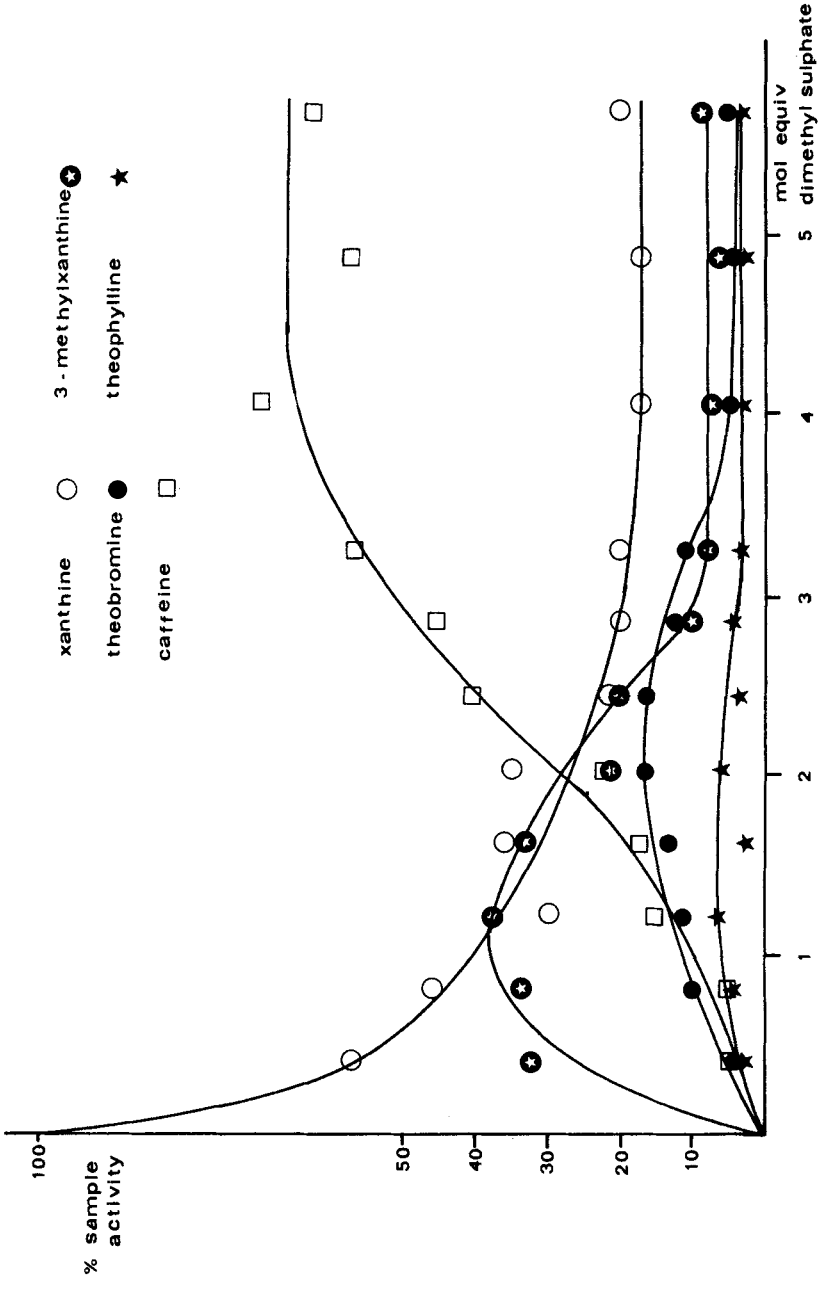

FIG. IV PRODUCTS OF METHYLATION OF [2-^{14}C] XANTHINE WITH DIMETHYL SULPHATE (AT 65°, pH 10-13)

In this experiment the pH was maintained in the range 10-13 by addition of further base as required. Formation of theobromine by control of pH [15] is widely quoted in textbooks. Unfortunately our results do not support this contention and we were unable to repeat the earlier work. It was necessary however to maintain the pH well on the basic side since it has been more recently shown that at low pH both imidazole-ring nitrogen atoms are involved in methylation to give a 7,9-dimethylxanthinium betaine.[23]

Variation of temperature over a limited range did not appear to affect the reaction significantly. Similar results were obtained under reflux (70°) and at 50° and 40°. Below 40° the reactants precipitated from solution.

Methylation of [2-^{14}C]xanthine was used to obtain highly purified samples of [2-^{14}C]caffeine (45%), [2-^{14}C]theobromine (2.6%), [2-^{14}C]theophylline (1%) and 3-methyl-[2-^{14}C]xanthine (1.4%) with 26% of unreacted [2-^{14}C]xanthine.

EXPERIMENTAL

Materials

All chemicals and solvents were of Analar grade unless otherwise specified. Ethyl cyanoacetate, formic acid, formamide and dimethyl sulphate were purified by fractional distillation under nitrogen. Theophylline was recrystallised twice from methanol. Caffeine was recrystallised from ethanol and methanol. Theobromine and xanthine were twice reprecipitated with acetic acid from alkaline solution. Samples of monomethylxanthines were gifts of Mr. M.S. Moss of Racecourse Security Services' Laboratories. 4,5-Diaminouracil, 1,3-dimethyl-4,5-diaminouracil, [^{14}C]formic acid and [^{14}C]urea were commercial samples.

Instruments

Radiochemical assay was carried out using a Packard Tricarb Model 3003 liquid scintillation counter or a Phillips Liquid Scintillation Analyser. Assays were reproducible to better than ± 2%. Paper chromatograms were scanned for radioactivity with a Packard Model 7201 Radiochromatogram scanner, and thin-layer chromatograms were scanned with a Panax RTLS-1A Radiochromatogram Scanner. Radiochemical purities using this instrument were estimated by comparison of peak areas, and errors were estimated to be approximately ± 3%.

In some cases paper chromatogram samples were combusted using a Packard Tricarb Sample Oxidiser prior to liquid scintillation counting.

Thin-layer Chromatography

All the information relating to TLC of uracil and xanthine derivatives is summarised here, except for R_F values of uracil derivatives, which appear elsewhere.[19]

Thin-layer plates were all pre-cast, silica gel F_{254} (Merck, 5 x 20 and 20 x 20 cm, 0.25 mm layer) Kieselguhr F_{254} (Merck, 20 x 20 cm, 0.25 mm layer) and cellulose LS_{254} (Schleicher and Schull, 10 x 20 cm, 0.1 mm layer).

Samples were 1-10µl of 1 mg/ml solutions in 0.25M sodium hydroxide, except for caffeine which was applied in chloroform. Solvent systems were:

A. n-propanol-3.5% ammonium hydroxide (3:1) [19]

B. acetonitrile-3.5% ammonium hydroxide (3:1) [19]

C. Chloroform-methanol (4:1) [25]

D. ethyl acetate-methanol-0.880 ammonia (8:2:1) [25]

The spots were detected by observation of the plate in UV light of wavelength 254nm. All the xanthine derivatives

Table 1. R_F values of methylxanthines in TLC

Compound	Silica gel				Cellulose
	System A	System B	System C	System D	System B
xanthine	0.31	0.33	0.00	0.08	0.15
1-methylxanthine	0.38	0.37	0.39	0.16	0.30
3-methylxanthine	0.45	0.38	0.33	0.24	0.37
7-methylxanthine	0.34	0.34	0.34	0.13	0.30
1,3-dimethylxanthine	0.47	0.41	0.56	0.36	0.58
1,7-dimethylxanthine	0.43	0.42	0.50	0.27	0.47
3,7-dimethylxanthine	0.42	0.47	0.51	0.51	0.50
1,3,7-trimethylxanthine	0.53	0.60	0.58	0.69	0.92

absorb light of this wavelength. In addition, theobromine, theophylline and caffeine were detected by spraying the plate with a mixture of ferric chloride (5g) and iodine (2g) dissolved in a mixture of acetone (50 ml) and 20% aqueous tartaric acid solution (50 ml). R_F values for the methylxanthines are summarised in Table (1).

Paper Chromatography

Paper chromatography was used for analysis of xanthine since R_F values in TLC were low.

The paper used was Whatman No.1 chromatography paper and solvent systems were:

E. n-butanol-pyridine-water (1:1:1)

F. glacial acetic acid-water-n-butanol (1:1:2)

R_F values for xanthine were 0.64 and 0.42 in solvent systems E and F respectively. Location was by observation in UV light.

Synthesis of [8-^{14}C]theophylline

(i) 4-amino[5-^{14}C]formamidouracil (II)

4,5-Diaminouracil (1g; 7.04 mmol) was weighed into a specially designed reaction vessel (Fig.V), a Teflon coated magnetic stirrer was inserted, and the vessel connected to a high-vacuum line.

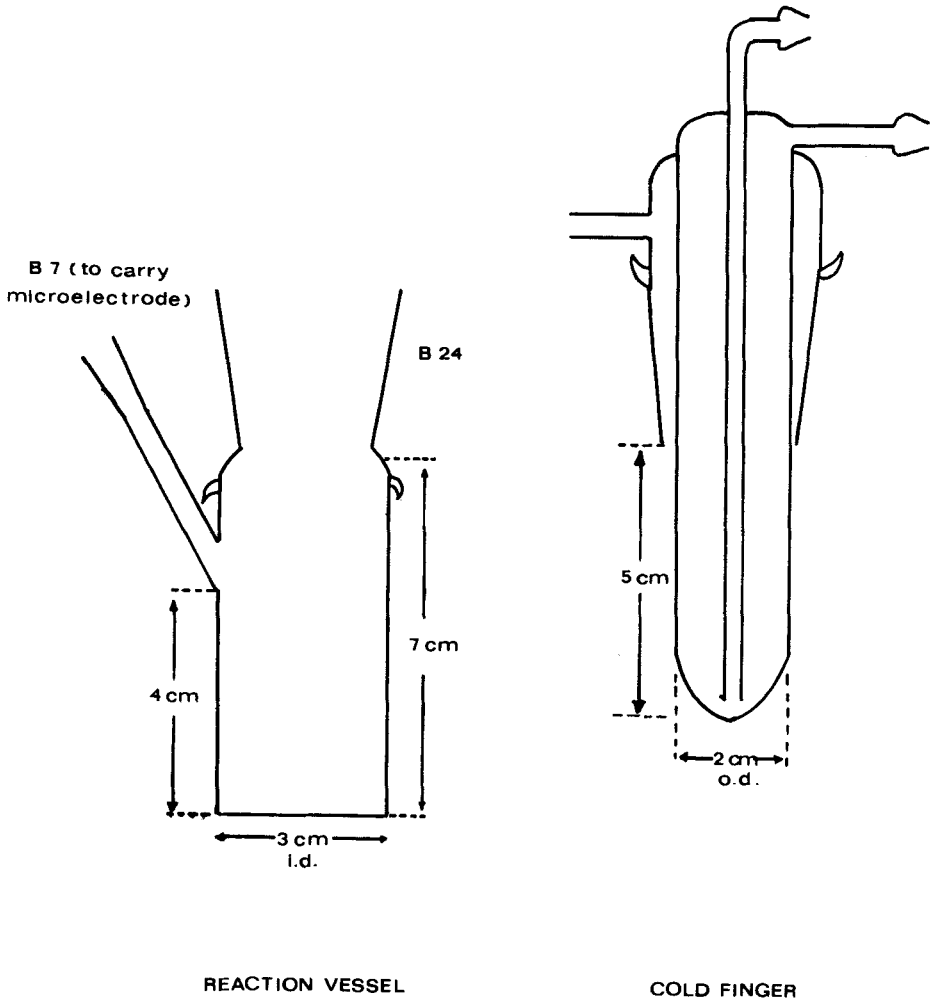

FIG. V APPARATUS FOR SYNTHESIS OF [8-^{14}C] THEOPHYLLINE AND [8-^{14}C] CAFFEINE

[^{14}C]Formic acid (6 mCi; 0.0982 mmol) was supplied in 6 break-seal ampoules, each stated to contain 1 mCi. The ampoules were attached to a high-vacuum line in turn and their contents distilled into the reaction vessel using high-vacuum technique. Each ampoule was subsequently flushed out with inactive formic acid solution (total 5.04 ml of 1.38M solution; 6.94 mmol). After the distillations were complete the reaction vessel was removed from the vacuum line, water (5 ml) was added and the mixture heated under gentle reflux for 1 hour.

(ii) 1,3-dimethyl-4-amino[5-^{14}C]formamidouracil (III)

6M sodium hydroxide solution (0.8 ml) was added to adjust the pH to 8.5, and dimethyl sulphate (1.65 ml; 17.7 mmol) was added in one portion. 6M sodium hydroxide solution (total 2.55 ml) was added dropwise at a rate sufficient to maintain the pH between 8.5-9. Temperature was maintained in the range 25-35°. The addition took about 1 hour, and the mixture was then stirred for a further hour.

(iii) [8-^{14}C]theophylline (IV)

Further 6M sodium hydroxide solution (2 ml) was added, giving a clear solution which was heated to 100° and gently boiled for 15 min to effect cyclization. The cold reaction mixture was extracted with ether (6 x 10 ml) acidified to pH4 by addition of 5M hydrochloric acid, then transferred to a continuous extraction apparatus and extracted with chloroform for 5 hours. The chloroform was removed by distillation and the product (0.775g) dried under vacuum.

The chemical yield of [8-^{14}C]theophylline calculated on the weight (0.775g) was 61%. The radiochemical yield, however, was only 20%, calculated from the assumed starting

activity of 6 mCi. This anomaly remained unexplained even after a careful activity balance on all the reactants and products and also after reference to the radiochemical suppliers.

The product was dissolved in chloroform (80 ml) and a small amount of insoluble material filtered off. The solution was then made up to 100 ml and the total activity determined. Several samples of the solution were analysed by thin-layer chromatography using solvent systems C and D followed by radioscanning. The product contained [8-^{14}C]theophylline (∿85%), [8-^{14}C]caffeine (∿5%) and a third radioactive component of unknown identity (∿10%).

(iv) Dilution and purification of [8-^{14}C]theophylline

Pure inactive theophylline (1.858g) and crude product (0.235g) were dissolved in boiling methanol (100 ml) and after equilibration the product was crystallised, filtered off, washed with cold ether, and then recrystallised again from methanol to give sample 1 (1.600g).

The filtrates were combined and solvent evaporated to leave a residue (0.476g) to which was added further pure inactive theophylline (0.558g). This mixture was recrystallised three times from methanol to give sample 2 (0.552g). The specific activities of samples 1 and 2 determined by liquid scintillation counting were 33.3 μCi/mmol and 13.4 μCi/mmol respectively. TLC analysis on silica gel with systems A, C and D followed by radiochromatogram scanning indicated that they contained only one compound, which was radioactive. Chemical and radiochemical purities were thus approximately 100%. Reverse isotope dilution analysis indicated radiochemical purities of 98% and 102% respectively.

Synthesis of [8-^{14}C]caffeine (V)

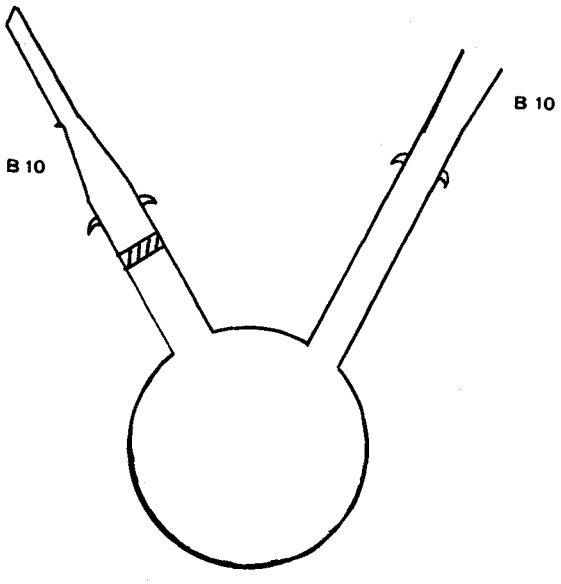

FIG. VI REACTION VESSEL FOR SYNTHESIS OF 4,5-DIAMINO-[2-^{14}C] URACIL AND [2-^{14}C] XANTHINE

Crude [8-^{14}C]theophylline (0.370g; 2.1 mmol), water (15 ml) and 6M sodium hydroxide solution (0.5 ml) were placed in the reaction vessel (fig.V) and dimethyl sulphate (0.27 ml; 2.9 mmol) was added over 30 min from an 'Agla' syringe. After stirring for a further 45 min the mixture was heated at 80° for 10 min. 5M hydrochloric acid (2 drops) was added to adjust the pH to 4, then the mixture was continuously extracted with chloroform for 5 hours. The solvent was evaporated to give crude [8-^{14}C]caffeine (0.330g; 658 μCi; 95% chemical and radiochemical yield).

Dilution and purification of [8-^{14}C]caffeine

To the crude [8-^{14}C]caffeine (0.330g) was added pure inactive caffeine (1.492g) and the mixture recrystallised twice from ethanol to give sample 3 (1.420g).

The filtrates were combined and solvent evaporated to leave a residue (0.397g) to which was added further pure

inactive caffeine (0.814g) and the mixture recrystallised three times from ethanol to give sample 4 (0.442g). The specific activities of samples 3 and 4 determined by liquid scintillation counting were 73.8 μCi/mmol and 19.1 μCi/mmol respectively. TLC analysis on silica gel with systems A, C and D followed by radioscanning showed the chemical and radiochemical purities to be ∿ 100%. Reverse isotope dilution analysis gave radiochemical purities of 101 and 99% respectively.

Synthesis of [2-^{14}C]xanthine (IX)

(i) 4-amino[2-^{14}C]uracil (VI)

[^{14}C]Urea (4 mCi; 58.1 mCi/mmol) in methanol solution was transferred to a reaction vessel (Fig.V1) containing inactive urea (295 mg; 4.49 mmol) and the methanol evaporated at 40° in a nitrogen stream. 'Superdry' alcohol (6 ml), ethyl cyanoacetate (0.532 ml; 5 mmol) and sodium ethoxide solution (4.5 ml of 2.65M solution; 11.9 mmol) were added and the mixture stirred. The vessel was fitted with a condenser carrying a calcium chloride drying tube and heated in an oil-bath at 100° for 3 hours. Water (8 ml) was added and the mixture adjusted to pH 4 by addition of glacial acetic acid to precipitate 4-amino[2-^{14}C]uracil.

(ii) 4-amino-5-nitroso[2-^{14}C]uracil (VII)

To the 4-amino[2-^{14}C]uracil was added a solution of sodium nitrite (380 mg; 5.5 mmol) in water (4 ml). The mixture was stirred at 50° for 15 min then the solid filtered off and washed with ice-cold water (2 x 4 ml). The radioactivity of the filtrate was 2156 μCi, leaving an activity of 1725 μCi in 4-amino-5-nitroso[2-^{14}C]uracil

(44% yield).

(iii) 4,5-diamino[2-^{14}C]uracil (VIII)

To the 4-amino-5-nitroso[2-^{14}C]uracil was added water (10 ml) and the mixture warmed to 50°. Sodium dithionite (1g) was added, the mixture stirred for one hour and then filtered. The solid (313 mg) was washed with water (2 x 5 ml) and acetone (2 x 5 ml) then dried under vacuum. The filtrate contained 384 μCi of activity, leaving 1340 μCi in the product, which represented a radiochemical yield of 34% after three synthetic steps.

(iv) [2-^{14}C]xanthine (IX)

To the 4,5-diamino[2-^{14}C]uracil was added formamide (8 ml) and the mixture heated under reflux for 30 min. After cooling, TLC analysis (silica gel, systems A and B) showed that xanthine was the major constituent.

Water (10 ml) was added, the mixture filtered and the solid washed with water (2 x 5 ml) and acetone (2 x 5 ml) then dried under vacuum. The weight of crude xanthine was 274 mg, representing a chemical yield of 36% on urea, and its radioactivity was 1270 μCi, a radiochemical yield of 32% on [^{14}C]urea.

(v) Purification

A column (30 x 1.5 cm) of Dowex I (IX4-100, 4% crosslinked mesh 50-100) ion exchange resin was converted from acetate to chloride form by washing with 2M hydrochloric acid (400 ml) and then with distilled water (2l). The crude [2-^{14}C]xanthine was dissolved in 0.25M sodium hydroxide (40 ml) and applied to the column with a flow rate of 2 ml/min. After washing the column with deionized water elution was begun with a solution of 4M ammonium hydroxide-0.2M ammonium chloride. 50 ml

fractions were collected and the activity of a 5 µl sample of each fraction determined by liquid scintillation counting. A plot of fraction activity with fraction number showed that most of the activity was present in fractions 5-24. The presence of [2-^{14}C]xanthine was confirmed by TLC (silica gel, system B).

Fractions 5-24 were combined and the solvent volume reduced to 50 ml by rotary evaporation at 100°. The precipitate of [2-^{14}C]xanthine was filtered off, dissolved in 0.880 ammonia, reprecipitated with glacial acetic acid, washed with water and acetone and dried under vacuum. The chemical yield was 23% of xanthine monohydrate on urea and the total radioactivity of the product was 1040 µCi, representing an 82% recovery from the crude product and an overall radiochemical yield of 27% on [^{14}C]urea.

TLC analysis (silica gel, system B) and PC analysis (Whatman No.1, systems E and F) followed by radioscanning indicated chemical and radiochemical purities of 100%. Reverse isotope analysis indicated a radiochemical purity of 108%. The high value was probably due to slightly different degrees of hydration of carrier and active products.

Reaction of [2-^{14}C]xanthine (at tracer level) with dimethyl sulphate

[2-^{14}C]Xanthine (3-4 mg; 15-20 µCi) and xanthine (196 mg; 1.5 mmol) were dissolved in 2% aqueous sodium hydroxide (10 ml) in the reaction vessel (Fig.V). Methanol (10 ml) was added, the mixture stirred, heated to 65° and the pH monitored. Dimethyl sulphate was added in 0.05 ml portions from an 'Agla' syringe, and further sodium hydroxide solution added when necessary to maintain the pH in the range 10-13. After each addition of dimeth'l sulphate 5 min were allowed

for equilibration, then a sample (5 µl) of the reaction mixture taken and spotted on a 20 x 20 cm silica gel plate. The plates were twice developed in solvent system D, which resolved caffeine, theobromine, theophylline, 3-methylxanthine and xanthine. Each spot was scraped into a scintillation counting vial and assayed by a modified version of the method of Shaw, Harlan and Bennett.[26] Ethanol (4 drops) was added to soften the gel, then hydrofluoric acid (0.4 ml) was added to digest the gel surface. After 15 min ethanol (5 ml) and 1% butyl-PBD in toluene (5 ml) were added and the sample counted in the usual way. The recovery of radioactivity from the silica gel was checked by digestion of spots of known activity, and found to be 96 ± 6% (standard deviation).

The proportions of activity in each product in each sample were calculated and are plotted in Fig.IV as a function of the number of mol-equivalents of dimethyl sulphate added.

Synthesis of 3-methyl[2-^{14}C]xanthine (X), [2-^{14}C]theobromine (XI), [2-^{14}C]theophylline (XII) and [2-^{14}C]caffeine (XIII).

(i) Reaction of [2-^{14}C]xanthine with dimethyl sulphate

[2-^{14}C]Xanthine (200 mg; 820 µCi) was dissolved in 2% aqueous sodium hydroxide (10 ml), methanol (10 ml) added, the mixture stirred and warmed to 50° and the pH monitored. Dimethyl sulphate was added in 0.05 ml portions from an 'Agla' syringe. After each addition a 5 µl sample of the reaction mixture was analysed by TLC (silica gel, system 3) and radioscanning. Methylation was continued until the proportion of [2-^{14}C]xanthine had fallen to approximately 20%.

The final approximate composition of the mixture as determined by TLC and radioscanning was [2-^{14}C]caffeine

(60%; 490 μCi), [2-^{14}C]theobromine (8%; 75 μCi), [2-^{14}C]theophylline (2%; 20 μCi), 3-methyl[2-^{14}C]xanthine (2%; 20 μCi) and [2-^{14}C]xanthine (26%; 210 μCi).

After cooling the reaction mixture was transferred to a continuous extraction apparatus, acidified to pH 2 with 5M hydrochloric acid and extracted with chloroform for 3 hours to give extract A (140 mg; 530 μCi), then for a further 18 hours to give extract B (16 mg; 50 μCi). TLC analysis (silica gel, system D) of these extracts showed that A contained mainly caffeine, with some theophylline and theobromine, and B contained mainly 3-methylxanthine.

(ii) Isolation of [2-^{14}C]caffeine

Extract A was eluted from an alumina column (10 x 1.5 cm) with chloroform and 20 ml fraction were collected and analysed by TLC (silica gel, system D).

Fractions 1-3 were combined and evaporated to dryness to leave [2-^{14}C]caffeine (103 mg; 370 μCi). The solvent was then changed to acetonitrile-3.5% aqueous ammonia (3:1) and a residue (40 mg; 130 μCi) containing [2-^{14}C]theophylline, [2-^{14}C]theobromine and [2-^{14}C]caffeine eluted.

The specific activity of [2-^{14}C]caffeine was 730 μCi/mmol in good agreement with the specific activity (750 μCi/mmol) of the [2-^{14}C]xanthine monohydrate starting material. TLC analysis (silica gel, systems A, C and D) and radioscanning indicated chemical and radiochemical purities of 100%. Reverse isotope dilution analysis gave a radiochemical purity of 98%.

(iii) Separation of [2-^{14}C]theobromine and [2-^{14}C]theophylline by preparative TLC

The mixture of [2-^{14}C]theobromine, [2-^{14}C]theophylline

and [2-^{14}C]caffeine was dissolved in acetonitrile-3.5% ammonium hydroxide (1:1) and applied to four silica gel TLC plates (20 x 20 cm x 0.25 mm) which were triply developed in solvent system D. The bands containing [2-^{14}C]theobromine and [2-^{14}C]theophylline were scraped from the plates and eluted with acetonitrile-3.5% ammonium hydroxide (1:1). The recovery of these compounds was 58% of the total activity applied to the plates. The solvents were removed by rotary evaporation to leave residues of [2-^{14}C]theobromine ($\sim$15 mg) and [2-^{14}C]theophylline ($\sim$5 mg).

TLC analysis (silica gel, system D) showed that the [2-^{14}C]theophylline was radiochemically pure but that the [2-^{14}C]theobromine still contained 10% of [2-^{14}C]theophylline, and a further purification using a small silica gel column was carried out.

(iv) Column purification of [2-^{14}C]theobromine

The [2-^{14}C]theobromine was added to the top of a silcia gel column (12 x 1.5 cm) and the column eluted with solvent system D. Fractions (5 ml) were collected and analysed by scintillation counting and TLC (silica gel, system D). Theophylline was eluted before theobromine, a reversal of the order found in TLC. Fractions containing theobromine were combined and evaporated to give pure product (10 mg; 40 µCi; 74% of the activity originally applied).

(v) Isolation of 3-methyl[2-^{14}C]xanthine

Extract B (17 mg; 50 µCi) was dissolved in 4 ml of methanol-0.25M sodium hdyroxide (1:1) and applied to four silica gel TLC plates (20 x 20 cm x 0.25 mm). Double elution in system 3 gave a clean band of 3-methyl[2-^{14}C] xanthine which was scraped off the plate and eluted with 0.25M aqueous sodium hydroxide solution. To the solution

was added pure inactive 3-methylxanthine (67 mg), the mixture equilibrated and the pH adjusted to 5 with glacial acetic acid. After storage at 0° for 48 hours the pure 3-methyl[2-^{14}C]xanthine (65 mg; 11.5 μCi) was collected.

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