Biochemical Pharmacology, Vol. 17, pp. 1349–1363. Pergamon Press. 1968. Printed in Great Britain

METABOLISM OF TRICYCLOQUINAZOLINE AND ITS DERIVATIVES IN MICE AND RATS

R. W. BALDWIN, J. ANNA NIKOLIC, H. C. PALMER and M. W. PARTRIDGE

British Empire Cancer Campaign Research Laboratory and Department of Pharmacy, University of Nottingham, England

(*Received* 4 *December* 1967; *accepted* 31 *January* 1968)

Abstract—^{14}C-Tricycloquinazoline (TCQ) was slowly eliminated following i.p. injection into mice and this may be related to its rapid uptake by adipose tissue. Metabolites were principally excreted in faeces and only trace amounts of radioactivity were detected in urine. 1-Hydroxy-TCQ and 3-hydroxy-TCQ were identified, but these accounted for less than 1 per cent of the faecal radioactivity, which was mainly associated with polar substances resulting from extensive degradation of TCQ. 1-Hydroxy-TCQ and 3-hydroxy-TCQ were also identified as biliary metabolites and were formed following *in vitro* incubation of 14C-TCQ with mouse liver homogenates. The major metabolites in each case, however, were unidentified polar compounds.

STUDIES with the epidermal carcinogen tricycloquinazoline, TCQ have established the importance of several structural features for carcinogenicity, including the overall shape and size of the molecule and the number and distribution of its nitrogen atoms.[1,2] Whilst such observations suggest that the parent hydrocarbon is the proximate carcinogen, the possible involvement of metabolites cannot be excluded. The overall metabolism of TCQ in rats and mice has therefore been investigated as a preliminary to studies on the metabolic fate of topically applied carcinogen.

MATERIALS AND METHODS

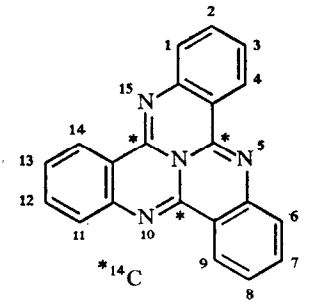
Animals

Young, adult, male albino mice of a random strain (Schofield) and male albino rats of an inbred Wistar strain were used. These were maintained on a standard cubed diet (MRC 41B) and water *ad libitum*.

Compounds and mode of administration

Tricycloquinazoline (TCQ) 2-methyl-TCQ and 3-methyl-TCQ were dissolved in arachis oil (1 mg/ml) for i.p. administration. Radioactive tricycloquinazoline (^{14}C-TCQ; sp. act. 14·7 mc/m-mole)[3] was dissolved in arachis oil (9 μg/ml). For i.v. injection, serum suspensions of ^{14}C-TCQ were prepared by adding an acetone solution of the compound (0·9 ml) to mouse serum (0·2 ml) and removing the acetone in a stream of nitrogen.

The preparation and properties of the monohydroxylated TCQ derivatives used as reference compounds are reported elsewhere.[3]



TCQ

Measurement of radioactivity

Radioactivity was estimated in a Panax scintillation counter, type SC-LP (Panax Equipment Ltd., Redhill, Surrey) at 4° to a statistical accuracy of ± 2 per cent. The counting efficiency as determined by internal standardization with *n*-hexadecane-1-^{14}C (0·97 mc/g; Radiochemical Centre, Amersham, Bucks.) was 20–40 per cent for aqueous samples and 60–70 per cent for organic samples. All counts were corrected to 100 per cent efficiency on this basis.

Organic samples were estimated in NE213 liquid scintillator (Nuclear Enterprises Ltd., Edinburgh, Scotland). Tissue, urine and faeces samples were digested in 4N-KOH and aliquots (0·1 ml) were assayed for radioactivity according to the technique of Brown and Badman.[4]

Spectra

Absorption spectra were measured on a Unicam S.P.500 spectrophotometer and fluorescence spectra on an Aminco–Bowman spectrophotofluorimeter. Absorption characteristics of TCQ and derivatives have been reported previously.[3,5]

Thin-layer chromatography (TLC)

Thin-layer chromatograms, prepared from silica gel G, (E. Merck A.-G. Darmstadt, West Germany) were of 0·25 mm thickness and were developed for 10 cm with one of the following solvent systems.

(a) chloroform;
(b) benzene-acetic acid (9:1, v/v);
(c) light petroleum (b.p. 60°–80°)—pyridine (4:1, v/v);
(d) benzene-acetic acid-acetone (17:2:1, by volume)
(e) chloroform: formic acid (9:1, v/v);
(f) butan-l-ol-acetic acid-water (63:10:27, by volume):
(g) benzene-pyridine (9:1, v/v).

Chromatograms were examined, whilst still wet, in ultraviolet light and fluorescent zones marked. Radioactive zones were located by exposure to X-ray film (Ilford Industrial G). Limits of detection were calculated on the basis of $|10^6|$ counts/cm^2 for darkening of the X-ray film. In some tests, zones of silica gel containing adsorbed metabolites were removed from the thin layer plates, packed into micro-columns (0·5 × 5·0 cm) and metabolites eluted with benzene-acetone (9:1 v/v) for radioactive assay.

The properties on TLC's of TCQ derivatives are listed in Table 1. Metabolites were

always identified by comparison of their mobilities with authentic reference compounds run at the same time. Radioactive metabolites were further characterised by chromatography in admixture with reference compounds.

TABLE 1. PROPERTIES OF TRICYCLOQUINAZOLINE AND MONOHYDROXYLATED DERIVATIVES ON TLC'S

Compound	R_f Values in solvent						
	a	b	c	d	e	f	g
TCQ	0·72	0·48	0·45	0·62	0·23	0·82	0·83
1-Hydroxy-TCQ	0·66	0·45	0·29	—	0·25	0·82	0·75
2-Hydroxy-TCQ	0·39	0·20	0·22	0·48	0·03	0·82	0·60
3-Hydroxy-TCQ	0·50	0·28	0·22	0·53	0·04	0·84	0·62
4-Hydroxy-TCQ	—	0·59	0·44	—	0·63	—	0·82

One dimensional chromatograms were developed as described in the text. Some variation in R_f values was observed between different chromatograms; the values quoted are typical.

EXPERIMENTAL

Excretion studies

Mice receiving ^{14}C-TCQ (0·52 mg; 5×10^6 cpm) by i.p. injection were kept in metabolism cages designed to separate faeces and urine and in which food was provided from non-spill containers. Collections of excreta, which were cooled in ice during the period of the tests to minimize degradation of metabolites, were made after 6 hr and then daily for 6 days. Aliquots of urine, and faeces which were dried *in vacuo*, were then estimated for radioactivity. Organ distribution of radioactivity was measured in four mice, 24 hr after each had received ^{14}C-TCQ (100 μg; 10^6 cpm) i.p. Each of these mice was kept in individual glass metabolism cages to facilitate recovery of excreta.

For body burden studies, pairs of mice injected i.p. with TCQ or the monomethyl derivatives (1 mg) were killed at daily intervals over a period of 9 days and each mouse was digested under reflux for 2 hr in 4N-potassium hydroxide (100 ml), ethanol (50 ml) and toluene (100 ml). The organic phase was separated and the aqueous residue re-extracted twice with toluene (100 ml) and ethanol (20 ml). The combined organic extracts were then washed successively with water, 1% HCl and water and finally concentrated to a 50-ml vol. by distillation. Extraction of the toluene solution with 90% formic acid (v/v), furnished a fraction containing TCQ derivatives which were characterised and estimated spectroscopically in benzene.[6] In control studies, TCQ (1 mg) was injected i.p. into mice; these when immediately killed and processed as above gave recoveries of not less than 95 per cent of the injected TCQ.

To determine the rate of elimination from blood, two mice received ^{14}C-TCQ (1·8 μg; 2×10^5 cpm) in mouse serum (0·2 ml) into a dorsal tail vein and 10 μl aliquots of blood were removed at intervals by tail bleeding. These were mixed with M potassium hydroxide (0·1 ml) and M-Hyamine chloride (0·1 ml) and estimated for radioactivity. Calculation of the total blood content was based on a blood volume of $9 \pm 0{\cdot}4$ per cent of the body weight.[7] A further two mice receiving ^{14}C-TCQ (1·8 μg: 2×10^5 cpm) by i.v. injection were killed after 2 hr for measurement of the organ distribution of radioactivity.

Faecal Metabolites

Aliquots of dried faeces (2·5 g; $7·6 \times 10^5$ cpm) collected over a period of 5 days from 4 mice injected i.p. with ^{14}C-TCQ (0·52 mg; 5×10^6 cpm) were extracted continuously with 100 ml vol. of various boiling solvents for 24 hr and the extracts assayed for radioactivity and subjected to TLC.

In non-radioactive studies, dried faeces collected over a period of 10 days from 20 mice each of which received TCQ (1 mg) by i.p. injection were continuously extracted with boiling benzene (200 ml) for 24 hr. The benzene extract was shaken with 90% formic acid (3×60 ml) to yield an extract which after dilution with water (600 ml) was re-extracted with benzene (2×100 ml). This benzene extract was concentrated to 50 ml, and chromatographed on a silica gel (Mallinckrodt, New York, U.S.A., 100 mesh) column (6×4 cm) with benzene to obtain two yellow fluorescent fractions (I and II). A further yellow fluorescent fraction (III) was then eluted with benzene-acetone (9:1 v/v). Fraction I was further chromatographed on an alumina (Type H, 100–200′/s mesh; P. Spence, Widnes) column (2×10 cm) with benzene to obtain a yellow fluorescent band (IA). A second yellow fluorescent fraction was then eluted with acetone-formic acid (9:1, v/v). All five of the foregoing fractions were re-purified by formic acid extraction and re-extraction into benzene[6] for spectroscopy and TLC.

Biliary metabolism

Biliary fistulae were established in rats under sodium pentobarbitol anaesthesia[8]. When bile was flowing at a steady rate, ^{14}C-TCQ (9 μg; 10^6 cpm) in serum was injected i.v., and further subsequent samples collected at hourly intervals over a 6-hr period were assayed for radioactivity. Direct TLC of an aliquot (0·1 ml) of the pooled sample of bile (2·55 ml; $3·8 \times 10^5$ cpm) indicated that no free metabolites were present. Control studies with normal bile indicated that this procedure was capable of resolving TCQ and hydroxylated derivatives whilst the limit of radiochemical detection was 2·5% of the total radioactivity in the sample.

The bulk of the bile sample was diluted with 0·1 M sodium acetate buffer, pH 5·0 (35 ml) and treated with β-glucuronidase (Sigma Type B-3; 4000 units) at 37° for 24 hr. The incubate was extracted with benzene (20 ml) containing carrier 3-hydroxy-TCQ (40 μg.), followed by three successive extractions with benzene (20 ml). The combined organic extracts were concentrated to 5 ml, estimated for radioactivity and submitted to TLC.

The aqueous residue from the enzymic hydrolysis was concentrated to 20 ml, conc. hydrochloric acid (20 ml) was added and the mixture refluxed for 2 hr. The solution was then adjusted to pH 5·5 with 50 per cent sodium hydroxide solution and extracted as before with benzene containing carrier 3-hydroxy-TCQ. The benzene extracts were concentrated to 5 ml and examined for radioactive metabolites.

Metabolism of tricycloquinazoline and monohydroxylated derivatives by mouse liver homogenate

For each test, the livers from three mice were minced and homogenised in 0·25 M sucrose (4–5 ml/g wet wt. tissue) in a Potter–Elvehjem homogenizer with a Perspex pestle [9] Mitochondrial supernatant fractions were obtained by centrifugation (9000 g for 10 min) in a Spinco Model L ultracentrifuge. Microsomes were separated from this

fraction by centrifugation (105,000 *g* for 60 min) and resuspended in the original volume of medium.

Liver fractions, equivalent to 350 mg wet wt. liver (1·8 ml) were added to flasks containing potassium phosphate buffer, pH 7·4 (100 μ mole), potassium chloride (400 μ mole,) nicotinamide (240 μ mole) (British Drug Houses, Poole, Dorset) glucose-6-phosphate (12 μ mole), adenosine triphosphate (4·0 μ mole), $NADP^+$ (0·54 μ mole) and NAD^+ (0·48 μ mole) (all obtained as sodium salts from Sigma Chemical Co., St. Louis, Mo., U.S.A.) in a total volume of 4·2 ml. When microsome fractions were used, NAD^+ and $NADP^+$ were replaced by the reduced forms (0·54 μ mole).

TCQ or 1-hydroxy- or 3-hydroxy-TCQ (20 μg) in methylcellosolve (0·1 ml) was added to each flask and incubated at 37° with agitation for 3 hr. Metabolism was then stopped by immersing the flasks in boiling water for 5 min. Controls included flasks in which TCQ derivatives were omitted until after the incubation period, or, in some cases, tissue was immediately inactivated after the compounds were added. Following incubation, the contents of each flask were extracted twice with benzene-ethanol (5:2 v/v; 35 ml). Tissue residues were further extracted with boiling ethanol (2 × 5 ml) and the combined organic extracts concentrated to a volume of 2–3 ml. Because of difficulties in recovering 1-hydroxy-TCQ from tissue in controls, liver residues were hydrolysed by boiling for 90 min in 6N hydrochloric acid. After neutralisation, digests were extracted with benzene (3 × 10 ml).

Aliquots of each organic extract were then taken for TLC and the remainder chromatographed on silica gel columns (6 × 1 cm) with benzene-acetone (99:1 v/v). Fluorescent bands which separated were purified by extraction into 90% formic acid[6]. These formic acid extracts were then diluted with water and fluorescent compounds re-extracted into benzene for TLC and spectroscopic analysis. The quantities of TCQ, and 1-hydroxy-TCQ and 3-hydroxy-TCQ recovered were determined from their absorption maxima at 402, 401 and 389 mμ respectively.

In radioactive experiments, ^{14}C-TCQ (18 μg; 2× 10^6 cpm) was incubated as described above with mouse liver mitochondrial supernatant fractions at 37° for 3 hr. The contents of each flask were then extracted with chloroform (3 × 15 ml) and the residual liver protein fractions were extracted successively with ethanol (3 × 10 ml), and methanol-aqueous ammonia (19:1, v/v; 3 × 10 ml). Aliquots of these extracts as well as the residual aqueous fractions were assayed for radioactivity and subjected to TLC.

RESULTS

Elimination studies

Following i.p. injection of ^{14}C-TCQ (0·52 mg; 5 × 10^6 cpm) into mice, the compound was eliminated at such a rate that 80 per cent of the administered dose was excreted by 6 days (Fig. 1.) Radioactivity was excreted almost exclusively in faeces (77%) and only a trace amount (2·6%) was present in urine. Hot extraction of dried faeces with TCQ solvents such as benzene and chloroform removed less than half of the faecal radioactivity and none of this material was identifiable as TCQ by radioautography of TLC's. These observations demonstrate that the radioactive materials excreted in faeces were metabolites of TCQ.

The chemical stability of TCQ and the development of sensitive spectroscopic methods of assay[6] also allowed the determination of the body burden of carcinogen

in mice at intervals following i.p. injection (1 mg). These results indicated that the loss of TCQ was slow; 6 per cent of the administered dose being still present after 9 days (Fig. 2). This suggests that following i.p. administration, TCQ is sequestered in the

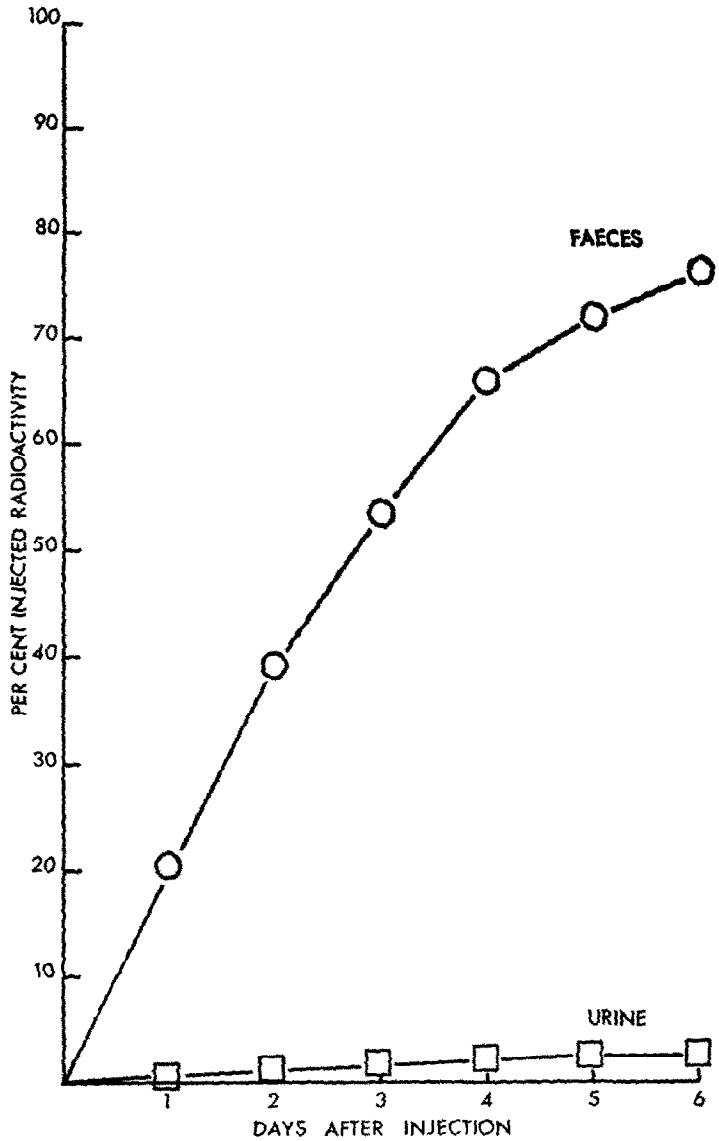


FIG. 1. Excretion of radioactivity following i.p. administration of ^{14}C-TCQ (0·52 μg; 5 × 10^6 cpm) to mice. Points represent the mean values from two mice.

body, possibly in tissue fat and under these conditions, the rate of metabolism is controlled by the rate of release of carcinogen from tissue depots. In support of this, direct observation of TCQ distribution by means of its characteristic yellow fluorescence under u.v. light in mice killed at intervals after i.p. injection in arachis oil, indicated that the compound was gradually transferred from the vehicle to fatty depots. During

the first four days after TCQ injection, oily droplets containing carcinogen wer present in the peritoneal cavity. Once these droplets had disappeared, the TCQ-fluores cence of the fatty tissues was observed to decrease as the compound was eliminated

Organ distribution studies 24 hr after i.p. administration of ^{14}C-TCQ (100 μg. 10^6 cpm) showed that the major portion of the radioactivity was located in the carcas and the gastrointestinal tract (Table 2). Smaller amounts of radioactivity were de tected in the liver (10 per cent) and fatty tissue consisting of s.c. and genital fat bodie

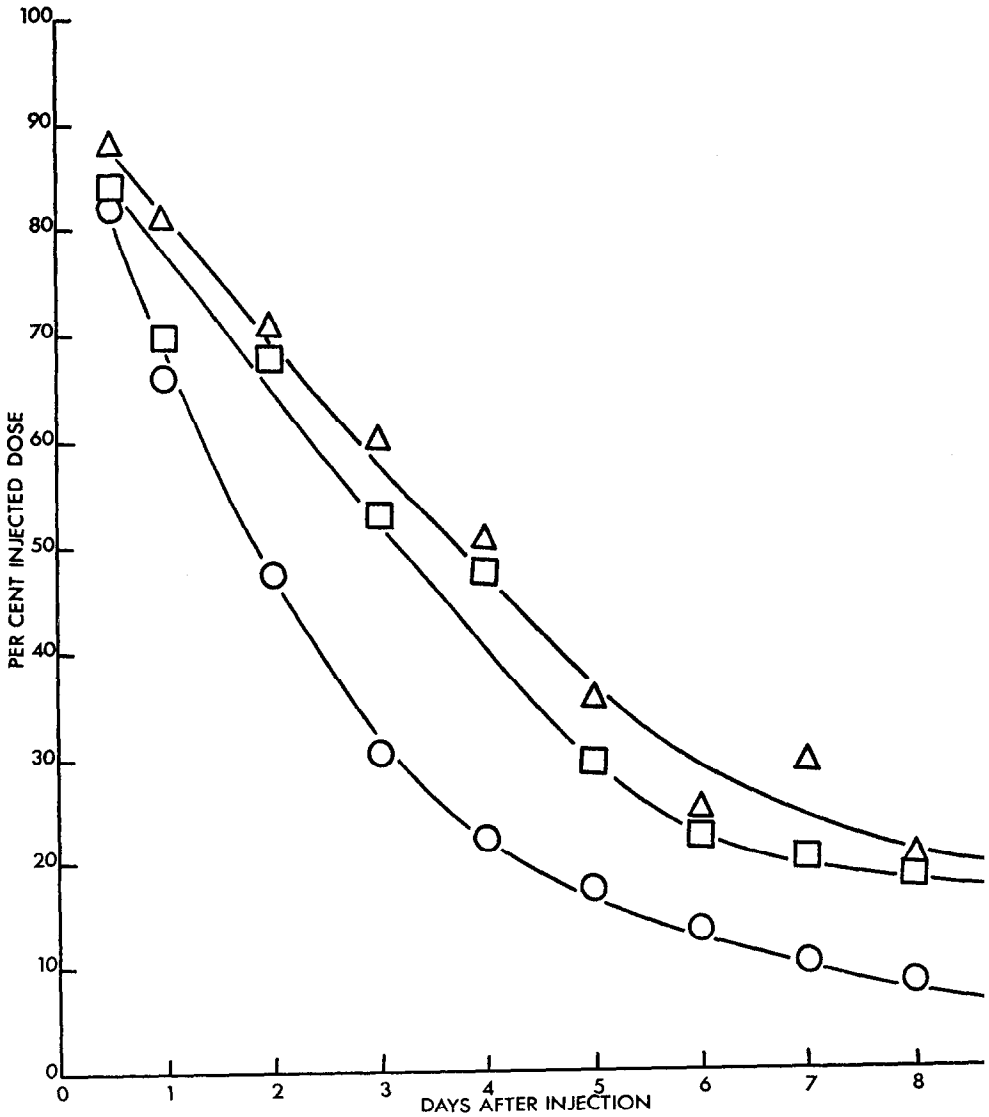


FIG. 2. Body retention following i.p. administration of 1 mg. doses of TCQ (○), 2-methyl-TCQ (△ or 3-methyl-TCQ (□) to mice. Pairs of mice treated with each compound were killed at daily intervals over a period of 9 days. Each mouse was digested under reflux for 2 hours in 4N KOH (100 ml ethanol (50 ml) and toluene (100 ml). TCQ compounds were extracted from the tissue digests described in the text and estimated spectroscopically.

as well as fat adhering to the intestines (8·9%). Direct observation of the localisation of TCQ in fatty tissue was also possible as revealed by its characteristic yellow fluorescence under ultraviolet light. Since the radioactivity detected in certain organs may have been due to surface contamination with unabsorbed TCQ, the peritoneal cavity of one mouse was washed out with acetone prior to removing the organs; only 11 per cent of the injected radioactivity was present in these washings.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY FOLLOWING ADMINISTRATION OF ^{14}C-TRICYCLOQUINAZOLINE TO MICE

Organ	Intraperitoneal injection (24 hr)		Intravenous injection (2 hr)	
	Percentage injected dose (100 μg: 10^6 cpm)		Percentage injected dose (1·8 μg: 2×10^5 cpm)	
	Mean	Range	Mean	Range
Carcass	31·0	7·3–63·0	36·0	31·0–42·0
Intestines	27·0	9·5–45·0	38·0	38·0–39·0
Stomach	1·1	0 – 1·8	3·8	1·9– 5·7
Kidney	0·9	0·5– 1·3	0·5	0·3– 0·7
Lung	0·1	0 – 0·4	0·6	0·4– 0·7
Spleen	0·5	0·4– 0·7	0·3	0 – 0·6
Liver	10·0	5·3–14·0	3·8	1·9– 5·7
Fat	8·9	2·1–14·0	3·0	2·0– 4·0
Excreta	23·0	12·0–29·0	0	—
Total recovery	106		91	

Organs removed from 4 mice and 2 mice receiving ^{14}C-TCQ by i.p. or i.v. injection respectively were digested in 4N KOH and assayed for radioactivity.

Following i.v. injection of ^{14}C-TCQ (1·8 μg; $2{\cdot}0 \times 10^5$ cpm) radioactivity was substantially eliminated from blood within 10 min (Table 3.). This is somewhat less rapid than the rate observed with benzo [a] pyrene[8] and whilst this compound was completely eliminated from blood, within 10 min, a small amount of TCQ or a metabolite persisted for at least 80 min. Body distribution studies 2 hr after i.v. administration of ^{14}C-TCQ (1·8 μg; 2×10^5 cpm) indicated that most of the radioactivity was again present in the gastrointestinal tract and the carcass with a smaller amount in the liver (3·8%) (Table 2.) Localisation of radioactivity in the subcutaneous and abdominal fat did

TABLE 3. CLEARANCE OF ^{14}C-TRICYCLOQUINAZOLINE FROM BLOOD AFTER I.V. INJECTION IN MICE

Time after injection (min)	Radioactivity in blood cpm/ml. (10^{-3})	Percentage of injected dose
5	27·0	113
10	3·3	14
15	2·4	9
25	4·1	18
40	1·6	9
83	3·5	15

Two mice received ^{14}C-TCQ (1·8 μg.; 2×10^5 cpm) in 0·2 ml mouse serum i.v. and blood samples (10 μl) were removed at intervals by tail bleeding for radioactive assay.

not occur to any great extent and the small amounts of activity in the kidneys and lungs may have been due entirely to radioactive material in the blood.

Faecal metabolites

Efforts to identify faecal metabolites from mice injected i.p. with ^{14}C-TCQ were hindered by their unfavourable solubility properties (Table 4), the only generally effective solvent being hot dimethylformamide and this also removed much contaminating material. Almost half of the faecal radioactivity could be extracted with hot water, but little of this activity could be re-extracted into organic solvents even after acid hydrolysis (4N HCl, 100°, 3 hr). Furthermore, it was not possible to characterise the metabolites in the aqueous extracts of faeces by TLC since the radioactivity remained at the origin when thin layer plates were developed with solvents a to e. These observations together with the finding that a major portion of the radioactivity in aqueous extracts of faeces was precipitated on acidification suggest that metabolites are carboxylic acids, polyhydric phenols, or phenolic acids.

TABLE 4. EXTRACTION AND CHARACTERIZATION OF MOUSE FAECAL METABOLITES OF ^{14}C-TRICYCLOQUINAZOLINE

Extraction solvent	Percentage extraction of faecal radioactivity	Metabolites detected
Dimethyl formamide	100	1- and 3-hydroxy-TCQ (trace)
Water	47	—
Chloroform-methanol (1:1, v/v)	29	1- and 3-hydroxy-TCQ (trace)
Methanol	12	3-hydroxy-TCQ (trace)

Aliquots of dried faeces (2·5 g; $7{\cdot}6 \times 10^5$ cpm) collected over 5 days from 4 mice injected i.p. with ^{14}C-TCQ (0·52 mg; 5×10^6 cpm), were extracted with 100 ml vol. of various boiling solvents for 24 hr. The extracts were assayed for radioactivity and examined by TLC as described in the text.

Smaller amounts of faecal radioactivity (29 per cent) were extractable with chloroform-methanol (1:1, v/v) and TLC of this extract yielded three metabolites together with radioactive material which remained at the origin. 3-Hydroxy-TCQ and 1-hydroxy-TCQ (R_f's 0·50 and 0·66 respectively in solvent a) were detected in trace amounts whilst a third metabolite (R_f 0·10) could not be identified as a hydroxylated TCQ derivative. 3-Hydroxy-TCQ was also detectable by TLC in trace amounts in dimethylformamide and methanol extracts of faeces. The only other metabolite in the dimethylformamide extract was 1-hydroxy-TCQ, present in trace amounts, but the bulk of the radioactivity could not be resolved.

The presence of 1-hydroxy-TCQ as a faecal metabolite was confirmed in non-radioactive studies. Hence, three yellow fluorescent bands were separated by silica gel column chromatography of benzene extracts of faeces from 20 mice each injected i.p. with TCQ (1 mg). Fraction I was further resolved into two components by chromatography on alumina with benzene. The four absorption maxima at 310, 380, 400 and 426 mμ and fluorescence maxima at 472, 495, 527 and 562 mμ of Fraction IA were identical with those of TCQ[5]. Fraction IB had absorption maxima at 332, 382, 403, and 430 mμ and fluorescence maxima at 495, 530 and 570 mμ identical with those

of authentic 1-hydroxy-TCQ. The other fluorescent band, Fraction III, had the same mobility as 3-hydroxy-TCQ on TLC (R_f 0·28 in solvent b).

Thus only 1-hydroxy-TCQ and 3-hydroxy-TCQ could be demonstrated as faecal metabolites of TCQ. Radioactivity measurements indicated that these compounds accounted for approximately 1 per cent of the total faecal metabolites, the bulk of which appeared to be acidic substances probably representing extensively degraded material.

Biliary metabolism of TCQ in rats

Following i.v. injection of ^{14}C-TCQ in rat serum (9 μg; 10^6 cpm), radioactivity was rapidly excreted in bile so that within 6 hr, 38 per cent of the administered dose had been eliminated (Fig. 3). A similar rate of biliary excretion was previously observed[8] with a comparable dose of benzo [a] pyrene. Direct examination of bile containing ^{14}C-TCQ metabolites by TLC revealed no radioactive spots corresponding to TCQ or hydroxy-TCQ derivatives. Control experiments indicated that this procedure was capable of resolving TCQ and its derivatives from bile, the limit of detection being equivalent to 2·5 per cent of the biliary radioactivity. Following hydrolysis of bile with β-glucuronidase, benzene extraction removed 12 per cent of the total biliary radioactivity (Fig. 4). TLC of this extract furnished 3-hydroxy-TCQ, (R_f 0·25 in solvent b), accounting for 5 per cent of the total biliary metabolites, together with a second compound (4%) which could not be identified as any known hydroxy-TCQ derivative (R_f 0·03 in solvent b). Treatment of this zone on the TLC with concentrated hydrochloric acid at 80° induced some degradation but did not yield a mono-hydroxy-TCQ thus excluding the possibility of a dihydrodiol derivative[10]. A trace amount of 1-hydroxy-TCQ (R_f 0·30 in solvent c) was also found but 2-hydroxy-TCQ and 4-hydroxy-TCQ were excluded above the level of detection (0·1 per cent of total biliary radioactivity).

Acid hydrolysis of the aqueous residue remaining after treatment with β-glucuronidase rendered a further 16 per cent of the total biliary radioactivity extractable into benzene. The only compound identifiable in this extract was 3-hydroxy-TCQ (R_f 0·26 in solvent b) which accounted for trace amounts of the radioactivity, but the bulk of the metabolites could not be characterised.

Less than 30 per cent of the total biliary radioactivity was extractable into benzene after enzymic and acid hydrolysis. It appears therefore that the biliary metabolites were similar to those present in faeces.

Metabolism of tricycloquinazoline by mouse liver

Following incubation of TCQ with mouse liver mitochondrial supernatant fractions, two yellow fluorescent compounds were isolated from benzene extracts. These were identified as TCQ and 3-hydroxy-TCQ by their mobilities on TLC's (R_f's 0·50 and 0·29 in solvent b) and by their absorption spectra[5]. No 3-hydroxy-TCQ was detected in control experiments where TCQ was incubated with heat-inactivated liver fractions. Quantitative experiments indicated that, after incubation of TCQ (20 μg) with a mouse liver mitochondrial supernatant fraction for 3 hr, only 37 per cent was recovered in an unchanged form whereas complete recoveries were obtained in controls containing heat-inactivated liver (Table 5). However, 3-hydroxy-TCQ accounted for only 6 per cent of the metabolised TCQ; no other fluorescent metabolites were detected, indicating that metabolism probably involves more than ring hydroxylation.

In order to investigate further the nature of the non-fluorescent metabolites, the *in vitro* metabolism of ^{14}C-TCQ was studied. After incubation of ^{14}C-TCQ (18 μg; 2 $\times$ 10^6 cpm) with mouse liver mitochondrial supernatant fractions for 3 hr, 37 per cent of the radioactivity was extractable into chloroform. Unchanged TCQ, accounting

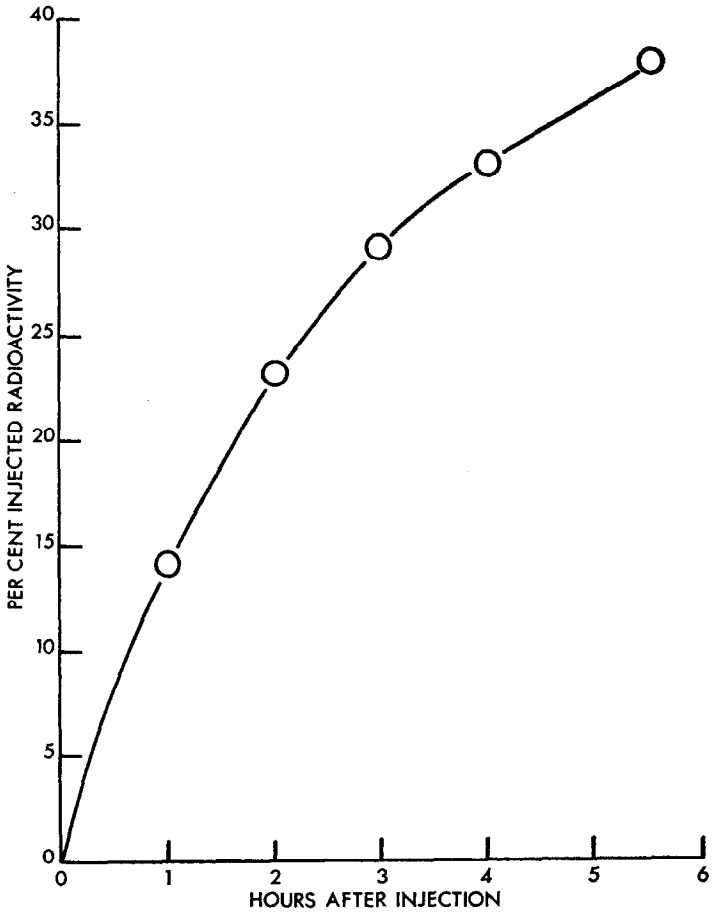


FIG. 3. Biliary excretion of radioactivity following i.v. administration of ^{14}C-TCQ (9 μg; 10^6 cpm) to a rat.

for 25% of the total radioactivity, was the major component identified in this extract, (Table 5). 1-Hydroxy-TCQ (R_f 0·29 in solvent c) and 3-hydroxy-TCQ (R_f 0·30 in solvent b) were also detected by TLC but these accounted for about 3 per cent of the total radioactivity. 2-Hydroxy-TCQ and 4-hydroxy-TCQ were excluded above their limit of radiochemical detection (0·1%). A further 8 radioactive compounds could be demonstrated by TLC but qualitative comparison of the radioautographs indicated that none was a major metabolite. None of these metabolites could be identified as a TCQ-like compound and the amounts of material on the thin layer plates were insufficient to obtain qualitative reactions with spray reagents.

A further 25 per cent of the TCQ radioactivity was extracted from liver incubates with ethanol and methanol containing 5 per cent ammonia, leaving 14 per cent in

the aqueous residue (total recovery of radioactivity, 76%). The ethanol extract was resolved into three fractions by TLC in solvent f. The first fraction containing approximately 5 per cent of the total radioactivity could be further resolved by two dimensional TLC (solvent f followed by g) into several components. TCQ, 1-hydroxy and 3-hydroxy-TCQ were identified by comparison with the thin layer chromatographic properties of the authentic compounds. No other component could not be identified as a TCQ-like compound. The second and third fractions containing

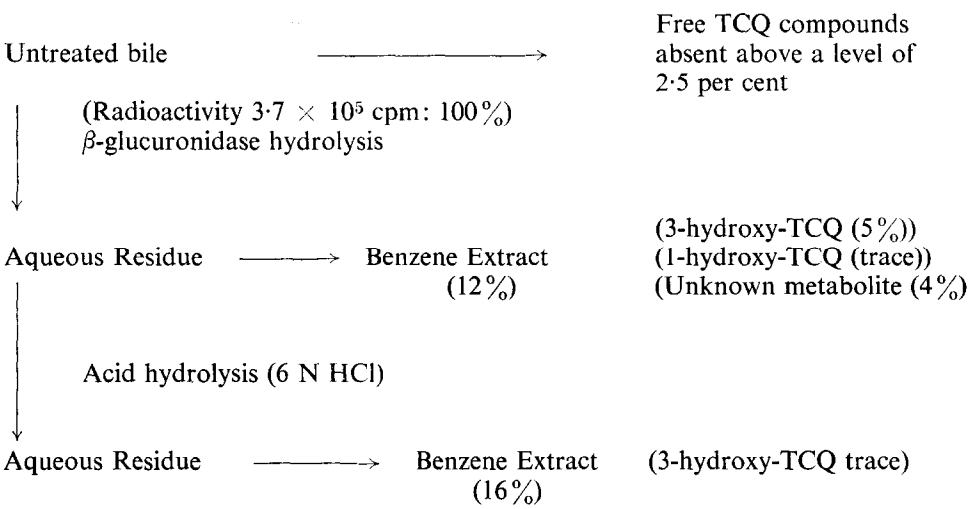


FIG. 4. Characterization of rat biliary metabolites following i.v. injection of ^{14}C-TCQ

A bile sample collected over a 6-hr period from a rat receiving ^{14}C-TCQ (9 μg: 10^6 cpm) by i.v. injection was buffered at pH 5 and incubated with β-glucuronidase. Liberated metabolites were extracted as described in the text and the aqueous residue subjected to acid hydrolysis (6N HCl for 2 hr under reflux). The hydrolysate was neutralised with 50% NaOH and further extracted to isolate liberated metabolites. Each extract was examined by TLC and radiochemically estimated as described in the text.

TABLE 5. *IN VITRO* METABOLISM OF TCQ AND HYDROXYLATED DERIVATIVES BY MOUSE LIVER MITOCHONDRIAL SUPERNATANTS

Compound	Amount added to substrate (μg)	Percentage recovery of compound in: Controls	Test	Metabolites detected†
TCQ	20	102	37	3-Hydroxy-TCQ (6 per cent)
^{14}C-TCQ	18	—	25	3-Hydroxy-TCQ, 1-Hydroxy-TCQ (3 per cent); Unidentified metabolites (12 per cent)
3-Hydroxy-TCQ	20	65	8	Unidentified metabolite (7 per cent)
	20*	83	37	Unidentified metabolite (11 per cent)
1-Hydroxy-TCQ	20	49	12	—

* Microsomal fractions used in the incubation system.

† Figures in parentheses represent percentage recovery of compound as metabolites.

TCQ, 1-hydroxy-TCQ or 3-hydroxy-TCQ (20 μg) were incubated with liver fractions (equivalent to 350 mg wet wt. liver) in a fortified medium (see text for details) for 3 hr at 37°. Metabolites extracted into organic solvents were characterised by TLC and estimated either spectroscopically or radiochemically.

approximately 2 and 5 per cent respectively of the total radioactivity, could be not identified as TCQ-like compounds and it was not possible to recover these from TLC's without extensive degradation.

Metabolism of 1- *and* 3-*hydroxy-TCQ derivatives by mouse liver*

Following incubation of 3-hydroxy-TCQ (20 μg) *in vitro* with mouse liver mitochondrial supernatant or microsome fractions, extensive metabolism occurred (Table 5). Benzene-ethanol extraction yielded two yellow fluorescent fractions one of which was identified as unchanged 3-hydroxy-TCQ by its mobility on TLC's in solvent b and by its absorption spectrum. The mobility of the second fraction (R_f 0·06 in solvent b) and the absorption maxima at 384, 407, 409 440 and 446 mμ differed from those of other available TCQ derivatives although its spectroscopic properties showed that its chromophore was similar to that of TCQ. Only a single yellow fluorescent fraction was isolated from controls containing heat killed liver fractions and this was identified as 3-hydroxy-TCQ. Quantitative estimation showed that the metabolite did not account for more than 11 per cent of the 3-hydroxy-TCQ metabolised indicating that non-fluorescent polar metabolites are formed.

Recoveries of 1-hydroxy-TCQ following incubation with heat killed liver fractions were low owing to difficulties in extraction. Nevertheless, the results demonstrate that the compound is metabolised by mouse liver mitochondrial supernatants (Table 5) Only a single yellow fluorescent fraction was detected in benzene-ethanol extracts from control and test liver preparations and this was identified in each case as unchanged 1-hydroxy-TCQ by its absorption spectrum and its mobility on TLC in solvent d.

DISCUSSION

One of the most prominent features in the elimination of ^{14}C-tricycloquinazoline following either i.p. or i.v. injection into mice was the rapid uptake of the carcinogen from the vehicle (lipid or serum) into tissue fat. This is illustrated by the high radioactive content of the carcass which included the osseous system, fat and muscle compared with all other organs except the intestines (Table 2). Moreover, in non-radioactive studies where direct location of the carcinogen was possible by means of its yellow fluorescence under ultraviolet light, uptake of TCQ into abdominal fat was observed following i.p. administration. By comparing the rate of excretion of radioactivity from ^{14}C-TCQ (Fig. 1) with body retention of unchanged carcinogen (Fig. 2), it was shown that the rate of elimination from the mouse was controlled by the rate at which it is released from these tissue depots. It is likely that the carcinogen is retained in an unchanged form until its release for metabolism and excretion by the liver and biliary system. (Fig. 3).

The rapid concentration of carcinogenic polycyclic hydrocarbons in adipose tissue following enteral or parenteral administration has been demonstrated in previous studies. Thus Heidelberger and Weiss[11] showed that 90 min after i.v. administration of ^{14}C-benzo [a] pyrene to mice, 10·6 per cent of the radioactivity was localised in abdominal and carcass fat whilst the bulk of the radioactivity (72%) was retained by the carcass. In this case, however, the radioactivity was not retained for long and by 24 hr, only 2·4 and 7·6 per cent remained in these tissues. In an earlier study[12], localisation of radioactivity in body fat was observed following s.c. or i.p. injection of

^{14}C-dibenz [ah] anthracene. Whilst the material retained in s.c. tissue was only slowly eliminated over a period of weeks, that in body fat had substantially disappeared within 7 days. Extensive tissue distribution studies have been reported with 7,12-dimethylbenz [a] anthracene in connection with its carcinogenic potential in mammary tissue. Thus Flesher[13] recently found that ^{3}H-DMBA accumulated in adipose tissue of perirenal fat and mammary gland, mainly in an unchanged form, following oral administration. In an extensive study of the absorption of DMBA following oral administration, Daniel, Pratt and Prichard[14] showed that much of the absorbed carcinogen is taken up into body fat where it is often retained for a long period. The pattern of absorption and excretion of 3-methylcholanthrene and dibenz [ah] anthracene was closely similar to that of DMBA and both showed retention in tissue fats.

Body retention studies indicated that the carcinogenic derivative 3-methyl-TCQ, and the inactive 2-methyl-TCQ, were eliminated at rates comparable to that of TCQ following i.p. injection (Fig. 2) These observations suggest that the overall susceptibility to metabolism cannot account for the observed difference in carcinogenic activity following skin painting[15].

Following i.p. injection of ^{14}C-TCQ, metabolites were excreted almost exclusively in faeces (77%) and only trace amounts (2·6%) were present in urine (Fig. 1). This proportion of urinary metabolites is lower than that observed with the homocyclic hydrocarbon carcinogens, 3-methylcholanthrene[16], benzo [a] pyrene[12] and dibenz [ah] anthracene[11] and possibly reflects the lower water solubility of TCQ and its metabolites.

1-Hydroxy- and 3-hydroxy-TCQ were detected as faecal metabolites of ^{14}C-TCQ, but these accounted for less than 1 per cent of the total metabolites whilst 2-hydroxy-TCQ and 4-hydroxy-TCQ were not demonstrable. Only trace amounts of unchanged TCQ were detectable and whilst the greater part of the radioactivity excreted was unidentifiable, its solubility properties (Table 4) suggested that metabolites were polar compounds presumably resulting from extensive degradation of TCQ.

The only identifiable metabolites in bile from rats given an intravenous injection of ^{14}C-TCQ were 1- and 3-hydroxy-TCQ and an unknown compound all of which were present as glucosiduronic acid conjugates (Fig. 4). Again, however, the radioactivity was associated principally with polar compounds which could not be characterised, the implication being that metabolism of TCQ results in extensive degradation of the molecule. This is supported by the finding that whilst 1-hydroxy TCQ and 3-hydroxy-TCQ were formed following *in vitro* incubation of ^{14}C-TCQ with mouse liver homogenate, these metabolites accounted for less than 3 per cent of the total radioactivity and the bulk of the metabolites could not be identified with any TCQ-like compound (Table 5).

The products of total metabolism of TCQ are not known but since 3-hydroxy-TCQ and to a lesser extent, 1-hydroxy-TCQ have been consistently demonstrated as metabolites, it is probable that hydroxylation at these positions represents a primary metabolic change. That this can lead to further metabolism has been demonstrated since *in vitro* incubation of these compounds with mouse liver homogenates results in the formation of metabolites which no longer possess the characteristic fluorescent properties of TCQ-like compounds (Table 5). The *in vitro* metabolism studies also indicated that 1-hydroxv-TCQ and 3-hydroxy-TCQ were as resistant as TCQ to metabolism. Hence

the small amounts of these compounds detected as in vitro metabolites would argue against hydroxylation at these positions being a major route of metabolism. The absence of 2-hydroxy-TCQ and 4-hydroxy-TCQ as metabolites also suggests that epoxide formation as involved in the metabolism of benz [a] anthracene and its derivatives[17] is not a major metabolic change with TCQ.

Because of the limited amounts of material available, it has not been possible to investigate the nature of the non-TCQ-like metabolites. It is unlikely that any of these would be involved in the carcinogenic action of TCQ since the available evidence from studies on the relationship between carcinogenic action and structure amongst a wide range of TCQ derivatives and analogues has pointed to the importance of the overall molecular shape and size[1, 2, 18, 19].

The possible involvement of 3-hydroxy-TCQ in TCQ carcinogenesis can be excluded also since this compound was shown to lack tumour initiating activity (unpublished findings) and 3-methoxy-TCQ was virtually inactive in long term skin painting tests[19].

Acknowledgements—We wish to thank Mrs. M. E. Marshall for skilled technical assistance. This work was supported by a block grant from the British Empire Cancer Campaign for Research and by the award of a scholarship to one of us (J.A.N.) by the Medical Research Council.

REFERENCES

1. M. W. Partridge, J. M. Sprake and H. J. Vipond, *J. chem. Soc.* (C) 1245 (1966).
2. R. W. Baldwin and M. W. Partridge, *Cellular Control Mechanisms and Cancer* (Eds P. Emmelot and O. Muhlbock), p. 313. Elsevier, Amsterdam (1964).
3. H. G. Dean, R. J. Grout, M. W. Partridge and H. J. Vipond, *J. chem. Soc.* (C) 142 (1968).
4. W. O. Brown and H. G. Badman, *Biochem. J.* **78**, 571 (1961).
5. F. C. Cooper and M. W. Partridge, *U.V. Atlas of Organic Compounds*, vol. 2. Butterworths, London (1966).
6. R. W. Baldwin, H. C. Palmer and M. W. Partridge, *Br. J. Cancer* **16**, 740 (1962).
7. J. J. Friedman, *Am. J. Physiol.* **196**, 420, (1959).
8. P. Kotin, H. L. Falk and R. Busser, *J. natn. Cancer Inst.* **23**, 541 (1959).
9. R. W. Baldwin, *Br. J. Cancer* **18**, 285 (1964).
10. E. Boyland and P. Sims, *Biochem. J.* **91**, 493 (1964).
11. C. Heidelberger and S. M. Weiss, *Cancer Res.* **11**, 885 (1951).
12. C. Heidelberger and H. B. Jones, *Cancer, N.Y.* **1**, 252 (1948).
13. J. W. Flesher, *Biochem Pharmac.* **16**, 1821 (1967).
14. P. M. Daniel, O. E. Pratt and M. M. L. Prichard, *Nature, Lond.* **215**, 1142 (1967).
15. R. W. Baldwin, G. J. Cunningham, M. W. Partridge and H. J. Vipond, *Br. J. Cancer* **16**, 276 (1962).
16. W. G. Dauben and D. Mabee, *Cancer Res.* **11**, 216 (1951).
17. E. Boyland and P. Sims, *Biochem. J.* **97**, 7 (1965).
18. R. W. Baldwin, G. J. Cunningham, A. T. Davey, M. W. Partridge and H. J. Vipond, *Br. J. Cancer* **17**, 266 (1963).
19. R. W. Baldwin, G. J. Cunningham, H. G. Dean, M. W. Partridge, S. J. Surtees and H. J. Vipond, *Biochem. Pharmac.* **14**, 323 (1965).