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METABOLISM OF TRICYCLOQUINAZOLINE IN MOUSE SKIN

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Abstract—^{14}C-Tricycloquinazoline rapidly penetrated mouse skin when doses of the order of 0·025 μM were topically applied to a standard area of skin. Larger doses than this showed prolonged retention presumably due to the low solubility of the carcinogen in skin lipids.

Metabolism of ^{14}C-TCQ in mouse skin was demonstrated but the metabolites accounted for only a small amount of the radioactivity present in skin which was mainly unchanged carcinogen. All four monohydroxylated derivatives of TCQ were detected together with trace amounts of material not identifiable as TCQ-like compounds. Small amounts of radioactivity were also present in skin as strongly bound conjugates with protein. This radioactivity was only liberated by drastic hydrolytic conditions which precluded its identification, but it was established that metabolites, not unchanged TCQ were present.

THE epidermal carcinogen tricycloquinazoline (TCQ) has been shown to undergo metabolism in the mouse, 1-hydroxy- and 3-hydroxy-TCQ, but not 2-hydroxy- and 4-hydroxy-TCQ, being detected.[1] These hydroxylated derivatives accounted for only a small percentage of the total metabolites, however, the major products being acidic polar substances indicative of extensive degradation of the carcinogen.

Metabolic intermediates of carcinogenic hydrocarbons have frequently been shown to possess carcinogenic activity, though not generally to the same degree as the parent compounds.[2–4] Studies were therefore undertaken on the penetration, organ distribution and metabolism of TCQ following topical application in the mouse. The present paper describes the identification of some of the metabolites of TCQ found to occur freely in skin and the nature of unidentified metabolites which are firmly associated with skin proteins.

METHODS

Young, adult, male albino mice of a random strain (Schofield) maintained on a standard cubed diet (MRC 41B) with water *ad libitum* were used for all tests. Dorsal hair was removed using electric clippers at least 36 hr prior to application of ^{14}C-tricycloquinazoline (sp. act. 14·7 mc/m-mole)[5] in 0·03 ml of benzene : mineral oil (95:5 v/v) over a standard area of skin (6 cm^2) outlined by gentian violet. Mice were prevented from licking the painted area of skin either by fitting rigid polythene collars (external diameter 4 cm) or in tests of short duration, they were tranquillized with an i.p. dose (0·025 mg) of perphenazine (Fentazin, Allen & Hanbury Ltd., London) and immobilized dorsal surface uppermost.

Determination of radioactivity

Radioactivity was estimated as previously described by liquid scintillation counting.[1]

Thin layer chromatography (TLC)

Thin layer chromatograms on silica gel G (E. Merck, A-G Darmstadt, West Germany) were developed using the following solvent systems:

(a) Benzene-ethyl acetate (4:1, v/v);
(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-1-ol-acetic acid-water (63:10:27, by volume);
(g) butan-1-ol-ethanol-ammonia (0·88)—water (6:3:1:2, by volume).

The chromatographic properties of TCQ and the monohydroxylated derivatives in solvents (b) to (f) have been published previously[1]; those in solvents (a) and (g) are given in the text.

Radioactive zones were located by exposure to X-ray film.[1]

EXPERIMENTAL

Penetration of ^{14}C-tricycloquinazoline into mouse skin following topical application

At intervals after skin application of ^{14}C-TCQ (1·35 μg; 1·5 $\times$ 10^5 cpm), pairs of mice were killed by cervical dislocation and an area of skin greater than that treated was removed. Skin samples and the remaining carcass were digested in 4N KOH (4 ml and 25 ml respectively) under reflux for one hour. The digests were then made up to a standard volume and aliquots estimated for radioactivity. In some experiments, the treated area of skin was initially extracted with acetone containing non-radioactive TCQ (10 μg) under reflux for 3 hr. Acetone extracts were reduced by distillation to a volume of 5 ml before assay for radioactivity.

To examine the effect of dosage of TCQ on rate of penetration into mouse skin, doses of TCQ (1·72, 8·13 and 37·2 μg) containing known amounts of ^{14}C-TCQ (1·5–2·0 $\times$ 10^5 cpm) were applied to a standard area of skin and pairs of mice were killed after 6 and 24 hr. Skin samples were digested in 4N KOH and aliquots of the digests assayed for radioactivity.

For organ distribution studies, two mice painted with ^{14}C-TCQ (19·5 μg; 2·2 $\times$ 10^6 cpm) were killed after 6 hr and various organs as well as treated and untreated samples of skin were removed, digested in 4N KOH and aliquots assayed for radioactivity.

Metabolism of ^{14}C-tricycloquinazoline in mouse skin

Groups of four mice were killed 6 hr after topical application of ^{14}C-TCQ (1·72 μg; 1·9 $\times$ 10^5 cpm), the treated areas of skin were removed, and freed of muscle and fatty tissue.[6] In controls, an equivalent amount of ^{14}C-TCQ was added to freshly prepared mouse skin from four untreated mice. Minced skin fractions were equilibrated with acetone (10 ml) containing 20 μg each of TCQ and the four monohydroxy-TCQ derivatives, and hot extracted with fresh acetone solution (2 $\times$ 10 ml). Thereafter, the skins were extracted for 24 hr successively with 3 $\times$ 50 ml vol. of boiling acetone, benzene and finally water.

The combined organic extracts were reduced in volume (5 ml) and aliquots estimated for radioactivity and subjected to TLC. For quantitative estimation of ^{14}C-TCQ and ^{14}C-hydroxy-TCQ derivatives, 0·3 ml aliquots of each extract were chromatographed as a band on TLC's (20 × 15 cm). 2-Hydroxy-TCQ and 3-hydroxy-TCQ were separated on silica gel plates with solvent b and 1-hydroxy-TCQ with solvent c. 4-Hydroxy-TCQ was isolated on silica gel plates prepared in 0·5 M oxalic acid and developed with solvent a. In this system, the R_f of 4-hydroxy-TCQ (0·48) was markedly different from that of TCQ and 1-hydroxy-TCQ (0·15) and 2-hydroxy- and 3-hydroxy-TCQ (0·02). Silica gel zones containing fluorescent material were removed from the plate, packed into micro columns (5·0 × 0·5 cm) and eluted with 2 ml of benzene-acetone (9:1 v/v).

Since a trace impurity in the ^{14}C-TCQ, accounting for approximately 1 per cent of the total radioactivity, interfered with estimations of 4-hydroxy-TCQ, a sample (250 μg) was further purified by TLC using solvent e. The silica gel containing the major radioactive component was removed from the plate and the compound eluted with benzene-acetone, (9:1 v/v). This purified sample of ^{14}C-TCQ which migrated as a single spot (R_f 0·21 in solvent e) was then applied to the skins of four mice (dose, 1·33 μg; 1·5 × 10^5 cpm). Six hr later, the mice were killed and the treated skins processed as described above.

Tissue-bound metabolites

Enzymatic hydrolysis. TCQ-treated mouse skin residues remaining after exhaustive extraction with organic solvents and water were incubated with 0·5% pepsin (Armour Laboratories, London) in 0·2 N HCl.[7] The incubates were centrifuged (1500 *g* for 20 min) and the supernatants divided into three equivalent aliquots and adjusted to acid, neutral, and alkaline pH respectively. Each portion was then extracted with benzene (3 × 5 ml) and ethanol: ether (1:3 v/v) (3 × 5 ml). Extracts were reduced to small volume, assayed for radioactivity, and subjected to TLC. The insoluble residues were washed with ethanol, dried and assayed for radioactivity.

Acid and alkaline hydrolysis. Skin residues were digested with 6N HCl in vacuum-sealed tubes at 100° for 24 hr or 4N KOH in 85% ethanol under reflux for 6 hr. Hydrolysates were successively extracted at acid, neutral, and alkaline pH with benzene, ethanol-ether (1:3 v/v) and butan-1-ol until no further radioactivity could be recovered. The residual aqueous phase was concentrated to small volume by distillation and assayed for radioactivity. Organic extracts were similarly concentrated, dried over magnesium sulphate, assayed for radioactivity and examined by TLC.

RESULTS

Penetration of ^{14}C-tricycloquinazoline into mouse skin

Following topical application of ^{14}C-TCQ to mouse skin, radioactivity was rapidly eliminated so that only about half the applied dose remained in the treated area after 6 hr and by 24 hr, the radioactive content of the skin was down to less than 20 per cent of the applied dose (Table 1). In control studies, up to 100 per cent of the radioactivity could be recovered when the same dose of ^{14}C-TCQ was applied to the skin of freshly killed mice. Comparable penetration rates were observed in immobilised mice after 5 min and 6 hr whilst the radioactivity lost from skin within 16 hr could be accounted for almost entirely by that in the carcass (Table 1), so that physical

loss from skin could be excluded. Whilst the bulk of the radioactivity remaining in the untreated area during the 24-hr period following treatment was extractable into acetone, a limited amount, representing up to 10 per cent of the applied TCQ radioactivity, could not be readily recovered. This radioactivity probably represented TCQ metabolites or material strongly bound to tissue.

To examine the effect of dosage on skin penetration, increasing doses of TCQ containing approximately equivalent amounts of ^{14}C-TCQ were applied to a standard area of skin and the radioactivity retained in treated skin was determined after 6 and 24 hr. The results in Table 2 demonstrate that with the lowest dose examined (1·7 μg), 78 per cent of the applied radioactivity was still present in the skin after 6 hr. As the applied dose was increased approximately 5- and 20-fold the amount of TCQ penetrating the skin increased proportionately although this represented a smaller percentage of the applied dose.

TABLE 1. PENETRATION OF ^{14}C-TRICYCLOQUINAZOLINE INTO MOUSE SKIN FOLLOWING TOPICAL APPLICATION

Time after skin treatment	Percentage of radioactivity recovered from treated skin			carcass		Total recovery	
0	98,	102		—		98,	102
5 min	71,	69,	67*	19,	13	90,	82
6 hr	48,	50,	48*	43,	41	91,	91
12 hr	50,	40		—		—	
16 hr	33,	19		51,	65	84,	84
24 hr	10,	18		58,	62	68,	80

Mice were killed at intervals after topical application of ^{14}C-TCQ (1·35 μg; 1·5 × 10^5 cpm) in 0·03 ml benzene: mineral oil (95:5 v/v) to a 6 cm^2 area of skin. Treated skin and the residual carcass were digested in 4N KOH (4 ml and 25 ml respectively) and aliquots of the digests assayed for radioactivity. Figures represent values from individual mice.

* Mice tranquillized with perphenazine and immobilized.

TABLE 2. EFFECT OF DOSAGE ON THE PENETRATION OF ^{14}C-TRICYCLOQUINAZOLINE THROUGH MOUSE SKIN

Dose of TCQ μg	Dose of TCQ cpm × 10^5	Percentage recovery of radioactivity from treated skin after: 6 hr		24 hr	
1·7	1·9	77,	78	8	
8.1	1·7	85,	85	62,	48
37·1	1·5	87,	83	69,	51

Doses of ^{14}C-TCQ were applied in 0·03 ml benzene: mineral oil (95:5, v/v) to a standard area of skin (6 cm^2). Skin samples from pairs of mice killed at appropriate intervals, were digested in 4N.KOH for radiochemical assay. (All mice examined at 6 hr were tranquillized with perphenazine and immobilized).

At 24 hr, only 8 per cent of the radioactivity contained in the lowest dose of TCQ (1·7 μg) was still present in the treated area. Larger amounts of radioactivity had penetrated the skin with the higher doses of TCQ but in both cases these represented smaller percentages of the applied dose so that there was an accumulation of TCQ or metabolites in the treated skin.

Tissue distribution studies 6 hr following skin painting with ^{14}C-TCQ (19·5 μg; 2·2 $\times$ 10^6 cpm) showed that the greatest amount of radioactivity, other than in the treated area of skin, was located in the intestinal tract. Thus at the high dose level necessary for the detection of organ radioactivity, 95 per cent of the applied radioactivity was still located in the treated skin and only 1–2 per cent was detected in the intestinal tract (Table 3). Only traces of radioactivity could be demonstrated in other organs whilst a further 1 per cent of the applied radioactivity could be accounted for in the residual carcass. This pattern of organ distribution is similar to that found following i.p. administration of TCQ and shows that the compound is rapidly metabolised following absorption. This is further emphasised by the observation that only 0·8 per cent of a dose of ^{14}C-TCQ (1·7 μg; 1·9 $\times$ 10^5 cpm) was detectable after 6 hr in the adipose tissue immediately below the painted area of skin whereas 43 per cent was localised in the dermis and epidermis.

TABLE 3. BODY DISTRIBUTION OF RADIOACTIVITY 6 HR AFTER TOPICAL APPLICATION OF ^{14}C-TRICYCLOQUINAZOLINE TO MOUSE SKIN

Organ	Percentage of dose*		Radioactive content cpm/g wet wt. $\times$ 10^3	
Skin (treated)	93,	97	3900,	3600
Skin (untreated)	0·11,	0·11	1·3,	1·3
Intestines	1·6,	2·2	14,	20
Stomach	0·03,	0·03	1·8,	1·4
Liver	0·04,	0·04	2·2,	2·6
Spleen	0·01,	0·02	2·0,	2·7
Fat	0·09,	0·06	2·3,	0·9
Lung	0·03,	0·02	3·3,	2·6
Carcass	1·0,	1·1	1·9,	1·7
Recovery	96,	100	—	—

* 19·5 μg (2·2 $\times$ 10^6 cpm)

Organs were digested in 4N KOH and aliquots assayed for radioactivity. Values represent individual results from 2 mice.

The nature of the ^{14}C-labelled material in subcutaneous and abdominal fat from ^{14}C-TCQ treated mice (Table 3) was examined. Benzene extraction of alkaline digests removed 37 per cent of the radioactivity and TLC of these extracts indicated the presence of unchanged ^{14}C-TCQ, thus demonstrating that TCQ penetrates skin at least partly unchanged.

Metabolism of ^{14}C-tricycloquinazoline in mouse skin

The bulk of the radioactivity remaining in mouse skin 6 hr after topical application of ^{14}C-TCQ (1·72 μg; 1·9 $\times$ 10^5 cpm) could be extracted into benzene and acetone (Table 4). Further radioactivity, accounting for between 1 and 3 per cent of the applied dose was recovered by hot aqueous extraction and the residual skin contained a trace of radioactivity (0·5%) which was resistant to extraction.

TLC of the combined organic extracts indicated that the major component recovered from the skin was unchanged TCQ, (Table 5). Four metabolites were detected also, accounting for less than 2 per cent of the radioactivity, together with trace amounts of unidentified radioactive material which could not be resolved by TLC (Table 5).

The first metabolite (R_f 0·30 in solvent c) had the chromatographic properties of 1-hydroxy-TCQ, the second (R_f 0·23 in solvent b) had the properties of 2-hydroxy-TCQ and the third (R_f 0·30 in solvent b) those of 3-hydroxy-TCQ. The fourth metabolite had the properties of 4-hydroxy-TCQ (R_f 0·63 in solvent e),

TABLE 4. RECOVERY OF RADIOACTIVITY FROM MOUSE SKIN TREATED WITH ^{14}C-TRICYCLOQUINAZOLINE

Extract	Percentage recovery of radioactivity at:			
	Zero time		6 hr	
Organic	95,	99	42,	46
Aqueous	0·2,	0·7	1·2,	2·6
Skin residue	0		0·5,	0·4

Pooled skins from groups of 4 mice treated with ^{14}C-TCQ (1·72 μg; 1·9 $\times$ 10^5 cpm) were freed of muscle and connective tissue and extracted successively (3 $\times$ 50 ml) with boiling acetone, benzene and water. The radioactivity in these extracts and in skin residues solubilized in 4N.KOH, was estimated as described in the text.

TABLE 5. METABOLITES DETECTED BY TLC OF ORGANIC EXTRACTS OF ^{14}C-TRICYCLOQUINAZOLINE-TREATED MOUSE SKIN

Compound	Percentage recovered at:			
	Zero time		6 hr	
TCQ	95·3,	97·8	46·0,	37·6
1-Hydroxy-TCQ	0·13,	0·07	0·45,	0·66
2-Hydroxy-TCQ	0·05,	0	0·15,	0·13
3-Hydroxy-TCQ	0·06,	0	0·30,	0·37
4-Hydroxy-TCQ	0·28,	0·14	0·38,	0·35
Unidentified material (remaining at origin)	0·06	—	—	0·24
Total	95·9,	98·0	47·3,	39·4

Radioactive monohydroxylated metabolites in organic extracts of pooled skins from 4 mice treated with ^{14}C-TCQ (1·72 μg; 1·9 $\times$ 10^5 cpm) were identified by direct comparison with the authentic compounds on TLC's. Quantitation of each metabolite was obtained as detailed in the text. Figures represent values obtained from 2 independent samples.

The low values obtained for 1-hydroxy-TCQ in control skin extracts where ^{14}C-TCQ was added to mouse skin immediately following excision was due to trailing of the TCQ band on TLC's. The recovery of 1-hydroxy-TCQ from treated skin was invariably greater, however, than that in controls (Table 5). Evidence for the presence of 4-hydroxy-TCQ was equivocal because of the high values in controls (Table 5) attributable to a small radiochemical impurity in the ^{14}C-TCQ. The presence of 4-hydroxy-TCQ as a skin metabolite was confirmed in experiments with a sample of ^{14}C-TCQ purified by TLC. When this material (1·33 μg; 1·5 $\times$ 10^5 cpm) was applied to mouse skin, 4-hydroxy-TCQ, representing 0·09 per cent of the applied radioactivity was detected after 6 hr by TLC (R_f 0·64 in solvent e) of organic extracts of skin, whereas none was observed in zero time controls.

In order to exclude the possibility that the hydroxylated TCQ derivatives found in skin were actually formed at another site such as liver, a group of 10 mice were treated with ^{14}C-TCQ (total dose, 5·7 μg; 5·9 $\times$ 10^5 cpm) on one flank and 6 hr later, samples of treated skin and untreated skin from the opposite flank were examined for TCQ metabolites. No radioactive material was detected in untreated dermis and epidermis whereas 37 per cent of the applied radioactivity could be recovered with organic solvents from the treated area. TLC of a sample of the combined organic extracts indicated that TCQ was the main component, accounting for 35 per cent of the applied radioactivity. ^{14}C-Labelled 1-hydroxy-TCQ (0·6%), 2-hydroxy-TCQ (0·4%), 3-hydroxy-TCQ (0·6%) and 4-hydroxy-TCQ (0·6%) were also detected thus indicating that metabolism occurs within the treated skin.

Hot aqueous extraction of ^{14}C-TCQ treated mouse skin residues after successive treatment with benzene and acetone removed a small amount of radioactive material (Table 4). Re-extraction of this fraction with benzene containing carrier TCQ and the monohydroxy derivatives removed 29 per cent of this radioactivity. TLC of this benzene extract in solvent c demonstrated ^{14}C-labelled TCQ, and 3-hydroxy-TCQ and three compounds in addition to material at the origin, not identifiable with known TCQ compounds (R_f's 0·03, 0·08 and 0·53). Further successive hydrolyses of the aqueous residues with β-glucuronidase and 6N HCl rendered more radioactivity extractable into benzene. In each case, however, traces of unchanged TCQ were detected in the extracts by TLC so that it was not possible to assess whether the components detected represented conjugated metabolites or small amounts of material which had resisted the various extraction procedures.

Tissue bound metabolites of ^{14}C-tricycloquinazoline

Skin residues after exhaustive extraction with organic solvents and water retained about 0·5 per cent of the radioactivity of the applied TCQ (Table 4). Solubilisation of these residues, which were essentially protein in nature, with pepsin rendered only 4·7 per cent of the radioactivity in the hydrolysate extractable into benzene (Table 6). Of this radioactivity, 3·2 per cent was associated with acidic substances, the remainder being associated with basic and neutral substances. It was not possible to recover any further radioactive compounds from the hydrolysate by extraction with ethanol-ether (1:3, v/v) since the bulk of the radioactivity (77·1%) was firmly associated with the soluble products of tissue hydrolysis and the remaining 17·7 per cent with the insoluble residue.

TLC and radioautography of the benzene extracts revealed that the radioactive material was clearly distinguishable from TCQ and the monohydroxy derivatives. Thus, radioactivity isolated from the acidified hydrolysate moved more slowly in solvent c than the reference compounds, as a diffuse trailing band indicative of a heterogeneous mixture of labelled substances. In addition, radioactive compounds from the alkaline phase, which were clearly unlike TCQ and the monohydroxy derivatives, remained at the origin in this solvent and were not identifiable.

Liberation of significant amounts of bound radioactivity into organic solvents was achieved only under conditions which extensively degraded the tissue proteins. Thus, hydrolysis of skin residues with 6N HCl rendered 20·5 per cent of the radioactivity associated with the proteins extractable into organic solvents (Table 6), the greater proportion (16·5%) of which was recovered with butanol. However, the total

TABLE 6. ISOLATION OF PROTEIN-BOUND METABOLITES FROM MOUSE SKIN TREATED WITH ^{14}C-TRICYCLOQUINAZOLINE

Conditions of hydrolysis	Wt. of skin (mg)	Total radioactivity Before hydrolysis (cpm)	Total radioactivity After hydrolysis (cpm)	Extract	Percentage recovery of radioactivity Soluble fraction Acids	Neutrals	Bases	Total	Insoluble residue	Overall recovery
Pepsin	309	$4{\cdot}2 \times 10^4$	$4{\cdot}1 \times 10^4$	Benzene	3·2		1·5	81·8	17·7	99·5
				Aqueous		77·1				
6N HCl	63	$1{\cdot}8 \times 10^4$	$1{\cdot}1 \times 10^4$	Benzene	0·4	1·0	0·7			
				Ethanol: ether	0·4	0·8	0·7	40·8	0	40·8
				Butanol	10·1	5·7	0·7			
				Aqueous	4·1	7·2	9·0			
4N.KOH/ 85% Ethanol	74	$1{\cdot}8 \times 10^4$	$1{\cdot}8 \times 10^4$	Benzene	5·0	7·8	0·5			
				Ethanol-ether	1·9	4·6	1·5	53·4	0	53·4
				Butanol	6·3	1·9	3·1			
				Aqueous	2·5	6·7	11·7			

Pooled skin from ^{14}C-TCQ treated mice was exhaustively extracted with acetone, benzene and water and hydrolysed under various conditions. Metabolites were extracted from the digests into organic solvents for radiochemical quantitation.

recovery (40·8%) of radioactivity from the acid hydrolysate indicated that degradation of some metabolites had occurred during the digestion.

The polar nature of the radioactive compounds recovered from the acid hydrolysate by extraction with butanol was suggested by the observation that on chromatography in solvent d, all the radioactive material remained at the origin. However, when solvent f was used, in addition to material which remained at the origin, no less than four components were detected (R_f 0·28, 0·34, 0·43 and 0·49) all of which migrated more slowly than TCQ-like compounds.

Hydrolysis of skin residues with 4N KOH in 85 per cent ethanol resulted in the liberation into organic solvents of larger amounts of radioactive material (33%) than in the corresponding acid hydrolysis. Moreover, the overall recovery of radioactivity (53·4%) was greater (Table 6). Most of this liberated radioactivity (28%) comprised acidic and neutral substances, only a small proportion (5%) being recovered from the alkaline phase. The acidic and neutral substances were extracted in approximately equal proportion with benzene (12·8%) and ethanol-ether (1:3, v/v) or butanol (14·7%).

When the combined benzene extract from the acidic and neutral fractions was chromatographed in solvents b, c and e, the bulk of the labelled material moved slowly and some remained at the origin, properties which markedly distinguished it from TCQ and the monohydroxy derivatives. Improved resolution was, however, obtained using solvent d and at least three discrete radioactive zones were detected (R_f 0·20, 0·40 and 0·48), the foremost of which ran very closely to 2-hydroxy TCQ, but was distinguishable from the latter.

The ethanol-ether and butanol extracts of skin digests contained large amounts of ninhydrin–positive material which interfered with the separation of the radioactive metabolites. As with the polar metabolites recovered after acid hydrolysis, the radioactive components scarcely moved from the origin on chromatography in solvents b, c and e. However, at least five discrete radioactive spots (R_f 0·35, 0·41, 0·45, 0·52 and 0·60) were resolved in solvent g some of which were similar in their chromatographic behaviour to those recovered following acid hydrolysis. These components, which were acidic in nature, were quite clearly distinguishable from TCQ (R_f 0·75) and the monohydroxy derivatives which in this system migrated together (R_f 0·62). The minute amounts of material and the lack of suitable reference compounds did not permit the elucidation of their identity.

DISCUSSION

When low doses of TCQ are applied to mouse skin, penetration is rapid and absorption is almost complete within 24 hr (Table 1). The rate of penetration is dependent, however, upon the dose of TCQ so that amounts of 0·025 μM or more applied to a standard area of skin result in considerable retention (Table 2). This must, in part, be related to the solubility of the carcinogen in tissue components, presumably lipid, involved in the initial transfer from the solvent used for application. Carcinogen which is not so transferred is precipitated upon the skin surface and little of this material is then absorbed. Direct demonstration of this was provided in a study[8] on the localization of TCQ in mouse skin by fluorescence microscopy which showed that following treatment with relatively large doses (300 μg), carcinogen was deposited on the skin

surface as a solid film. This material was still demonstrable after carcinogen penetrating into the hair follicles had disappeared. The problems of surface deposition of polycyclic hydrocarbons following evaporation of the solvent used for skin painting was previously emphasized by Bock[9] in studies on carcinogen penetration and such observations indicate that grossly excessive amounts of carcinogen are frequently administered in skin tumour-initiation tests.

The level of covalent binding of radioactivity to skin proteins following topical application of ^{14}C-TCQ was shown previously not to be increased when doses greater than 0·03 μM were applied to a standard area of skin.[10] In comparison, the present results suggest that uptake of TCQ into skin is a critical factor in limiting exposure of epithelial cells to carcinogen and thereby influences the degree of tissue interaction.

The rapid localization of TCQ in hair follicles following skin application has been demonstrated[8] and it is likely that this represents a major route of penetration into skin. That passive transfer of TCQ is involved in its absorption from skin is supported by the detection of unchanged carcinogen in s.c. and abdominal fat. Direct metabolism of TCQ in mouse skin has also been demonstrated and this provides an alternative route of elimination. The low concentration of radioactivity in all organs except the intestinal tract (Table 3) indicates, however, that once TCQ has penetrated the skin, it is rapidly absorbed and metabolised.

The bulk of the radioactivity recovered from skin after treatment with ^{14}C-TCQ was unchanged carcinogen (Table 5). Small amounts of all four monohydroxylated TCQ derivatives, together with trace amounts of unidentified metabolites were also detected and these were shown to result from direct metabolism of TCQ in skin. The pattern of hydroxylation differed from that observed in studies on the *in vitro* metabolism of TCQ by mouse liver[1] since in this case, 2-hydroxy-TCQ and 4-hydroxy-TCQ were not detected. It is unlikely, however, that the hydroxylated metabolites are involved in the carcinogenic process since both 2-hydroxy-TCQ and 3-hydroxy-TCQ are without significant activity.[11,12] The carcinogenicities of 1-hydroxy-TCQ and 4-hydroxy-TCQ have not been determined but methyl substitution at these positions results in a marked reduction in carcinogenic activity.[11]

Whereas the bulk of the metabolites formed *in vivo* following i.p. injection of TCQ into mice or *in vitro* incubation with mouse liver are polar compounds,[1] only trace amounts of metabolites with similar properties were detected in TCQ-treated skin. This suggests that either marked degradation of TCQ does not occur in skin or alternatively that these metabolites once formed are rapidly eliminated. Whichever process is operative, this suggests that the polar metabolites have little significance in carcinogenesis.

The small amount of bound radioactivity in ^{14}C-TCQ treated mouse skin was only liberated following extensive degradation of skin proteins and whilst the presence of unchanged TCQ or monohydroxylated derivatives could be excluded, none of the metabolites were identified. From their solubility properties (Table 6) and behaviour on TLC's it was evident that metabolites were polar compounds, but in view of the drastic hydrolytic conditions necessary for their liberation, the possibility that these are degradation products of the bound metabolites cannot be excluded. Since, however, TCQ is unaffected by the hydrolytic treatments, these results imply that metabolites rather than unchanged carcinogen are involved in tissue binding. It was similarly shown that metabolites of dibenz[ah]anthracene[7] and benzo[a]pyrene[13] were involved

in binding to mouse skin proteins but the importance of these metabolites in carcinogenesis has yet to be established.

These observations support the concept that TCQ is the proximate carcinogen involved in uptake by cells. Such a concept is in accord with structure-action data in this group of compounds which emphasizes the critical dependence of carcinogenicity on molecular structure.[14,15]

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