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THE METABOLISM OF THE ANTI-TUMOUR AGENT 1(1-AZIRIDINYL)-2,4-DINITROBENZENE (CB 1837)

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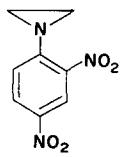
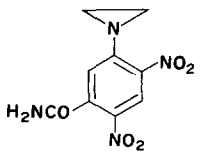
Abstract—1-(1-Aziridinyl)-2,4-dinitrobenzene (CB 1837) is the parent member of a series of nitrophenylaziridines having a highly specific action against the Walker tumour in the rat. CB 1837 was much less cytotoxic *in vitro* than was predicted on the basis of its antitumour activity *in vivo*, but its activity was enhanced in the presence of liver 9000-*g* supernatant and cofactors, which also converted it into a metabolite, 2-amino-1-(1-aziridinyl)-4-nitrobenzene. This metabolite was more active in the *in vitro* test than was CB 1837.

CB 1837 is extensively metabolised *in vivo* by the rat, and the following urinary metabolites have been identified by mass spectrometry and quantitatively determined after administration of the tritium-labelled drug: 1-(2-chloroethyl)amino-2,4-dinitrobenzene and its 2-hydroxyethylamino analogue; 2,4-dinitroaniline; 2-amino-1-(1-aziridinyl)-4-nitrobenzene, 2-amino-1-(2-chloroethyl)amino-4-nitrobenzene and their respective *N*(2)-acetyl derivatives; and *S*-[*N*-(2,4-dinitrophenyl)-2-aminoethyl] mercapturic acid.

1-(1-Aziridinyl)-2,4-dinitrobenzene (CB 1837, I) was the parent member of a series of nitrophenyl aziridines evaluated for antitumour activity by Khan and Ross [1]. Interest in these compounds was prompted by the finding that CB 1837 had a therapeutic index† against the Walker tumour in rats which was as high as that for alkylating agents in clinical use. Subsequently, the related 5-carboxamide (5-aziridinyl-2,4-dinitrobenzamide, CB 1954, Ia) proved to have the highest therapeutic index of any compound so far tested against this tumour, and studies on the mechanism of action of the nitrophenyl aziridines have centred on this agent [2–4].



(I)



(Ia)

A noteworthy feature of the biological activity of CB 1954 is its very narrow spectrum of antitumour activity, it being active only against the Walker tumour [2]. A second unusual feature is that both CB 1954 and CB 1837 are mono-functional alkylating agents, and this class of compound does not usually show antitumour activity.

The finding, reported here, that the activity *in vitro* of CB 1837 was dependent on a metabolising system suggested that a knowledge of the metabolic fate of the nitrophenyl aziridines might help to explain their unusual pharmacology. CB 1837 was initially chosen for this study because it was the simplest such compound possessing significant antitumour activity, and because its toxicity to rats was lower than that of CB 1954, thus allowing larger doses of the drug to be administered, facilitating the identification of less abundant metabolites.

As in other recent investigations in this laboratory into the metabolism of antitumour agents, mass spectrometry has afforded sufficient information for the structures of the metabolites to be deduced, and thereafter confirmed by synthesis [5, 6].

METHODS AND RESULTS

Animals. Female Wistar rats, 6–8 weeks old, weighing about 200 g were used except for tumour passaging for which male rats were used. They were fed *ad lib.* on rat cake and water.

Liver supernatant. For the preparation of rat liver 9000-*g* supernatant, rats were pretreated with phenobarbitone sodium (80 mg/kg) for 3 days immediately prior to use. Livers were homogenised with a Potter type homogeniser in 4 vol ice-cold 0·1 M Tris buffer, pH 7·4 (Tris buffer) and centrifuged at 9000 *g* for 20 min.

Walker antitumour assay in vivo. The method for determining the ID_{90} for the Walker tumour and the LD_{50} in rats was that of Rosenoer *et al.* [7]. Results for appropriate compounds follow the descriptions of their synthesis.

Bioassay. The bioassay procedure was carried out aseptically as follows. Walker ascites cells were taken from a donor rat 6–7 days after tumour transplantation, counted on a Coulter counter and suspended at a concentration of 10^6 cells/ml in a mixture

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† Therapeutic index, $TI = LD_{50}/ID_{90}$. LD_{50} = dose lethal to 50% of animals in group, ID_{90} = dose producing 90% tumour inhibition.

Table 1. Bioassay of CB 1837 (I) against Walker cells with and without liver 9000-*g* supernatant

Conc of CB 1837 (I) (μg/ml)	Median survival time (days)	
	CB 1837 alone	CB 1837 + supernatant
400	9	—
200	10	
100	9	17
50	8	12
25	8	9
Control	6	6

— signifies no tumours.

of TC 199 and horse serum No. 2 (6:4), (both from Wellcome Reagents Ltd., Beckenham, Kent). To 10-ml aliquots of the incubation mixture were added solutions of the test drugs in dimethyl sulphoxide (0·1 ml) or solvent alone as control, and cofactors—glucose-6-phosphate (2·3 mg), NADP (3·0 mg unless otherwise stated) and magnesium chloride (1·2 mg). After incubation for 2 hr at 37°, 1-ml aliquots of each incubate were injected into groups of five rats, and their survival times determined (Tables 1–3).

Metabolism and synthesis

Silicic acid for column chromatography was Merck Kieselgel, and for thin-layer chromatography (t.l.c.) on glass plates (20 × 20 cm or 20 × 5 cm) Merck Kieselgel GF_{254} was used. Rotary evaporations were performed at 30° and 10 mm.

(i) *Isolation of metabolites of CB 1837 (I) from rat urine.* Three rats were injected i.p. with CB 1837 (150 mg/kg) in dimethyl sulphoxide and the urine was collected frozen for 24 hr. It was then extracted with ethyl acetate (3 × 50 ml) and the extract was dried ($MgSO_4$). The two phases, the ethyl acetate extract and the remaining aqueous phase, were then chromatographed as detailed below.

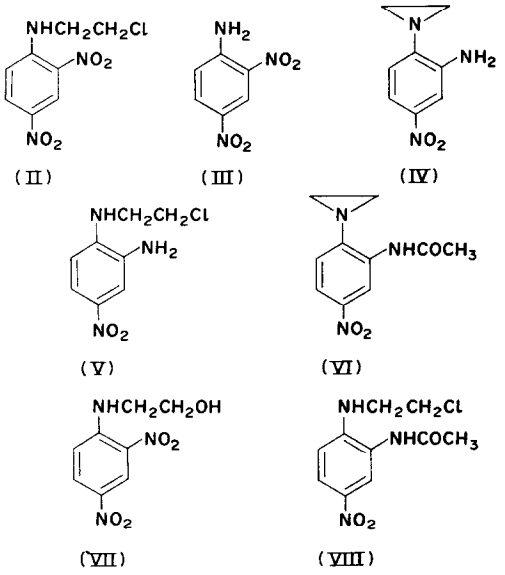
(a) Ethyl acetate extract. Aliquots (20% of the total extract) were concentrated before t.l.c. with benzene as developing solvent. Multiple developments were sometimes needed for complete separation of the mobile components. These were subsequently identified as I and II–VI by mass spectrometry (see (iv) below and Discussion; for R_f values after a single development, see Table 4). Further t.l.c. in chloroform–methanol (19:1) revealed no additional mobile components, although a hydrolysis product (VII) of CB 1837 in admixture with another component (VIII) were detected by this procedure during quantitative determination of the metabolites (see (iv) below).

Table 2. Bioassay of 2-amino-1-(1-aziridinyl)-4-nitrobenzene (IV) against Walker cells

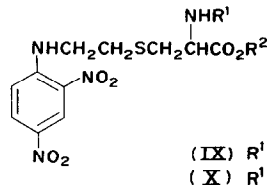
Conc of (IV) (μg/ml)	Median survival time (days)
1000	—
100	
10	8
1	7
Control	6

—signifies no tumours.

Components were visualised as yellow or orange bands, or as dark bands under ultraviolet radiation. The silicic acid in the appropriate areas was removed and each component was eluted with methanol prior to mass spectrometry.



(b) Remaining aqueous phase. Thin-layer chromatography (butan-1-ol–water, 86:14 or chloroform–methanol, 1:1) revealed four components (for R_f values, see Table 4). The total aqueous phase was applied to a column (30 × 2 cm) of Amberlite XAD-2 resin which was eluted first with water (1 litre) then with methanol (100 ml). Of the four components, only one (subsequently identified as IX, see (iv) below) was abundant in the methanol eluate. This eluate was concentrated to dryness and a solution of the residue in 1N hydrochloric acid (5 ml) was extracted with ethyl acetate (3 × 5 ml). The dried ($MgSO_4$) extracts were applied to a column (22 × 1 cm) of silicic acid, which was eluted with chloroform–methanol (1:1) (10-ml fractions). Fractions 6–10 contained the chromatographically homogeneous metabolite.



(IX) $R^1 = CH_3CO$, $R^2 = H$
(X) $R^1 = CH_3CO$, $R^2 = CH_3$
(XI) $R^1 = H$, $R^2 = H$

Table 3. Effect of different NADP concentrations on the activity of CB 1837 (200 μg/ml) with liver 9000 g supernatant against Walker cells in the bioassay

Composition of incubation mixture	Median survial time (days)
CB 1837	10
CB 1837 + S + NADP (0·3 mg)	12
CB 1837 + S + NADP (3·0 mg)	—
Control	6

S = 9000-*g* supernatant + glucose-6-phosphate + $MgCl_2$.
—signifies less than 40% tumour takes.

(ii) *Quantitative determination of CB* 1837 *and its metabolites in rat urine.* The urine (frozen) was collected during 20 hr from a rat injected with ^{3}H-labelled CB 1837 (specific activity 4 mCi/m-mole, for preparation see (v) below) (160 mg/kg) in dimethyl sulphoxide. Recovery of radioactivity was 7% of the administered dose. A portion of the urine (10 ml) was extracted with chloroform (10 × 50 ml) and the contents of this phase and of the remaining aqueous phase were treated as follows:

(a) Chloroform extract. Thin layer chromatography of an aliquot (5% of the total chloroform extract after concentration to 10 ml) was carried out in two dimensions, firstly in chloroform–methanol (19:1) then in benzene (twice). The separated components, detected by the methods described above (i(a)) were transferred to counting vials containing 0·1 N hydrochloric acid (0·5 ml), diluted with scintillation fluid (napthalene, POP and POPOP in ethanol, dioxan and toluene) (10 ml) and assayed for radioactivity using a Packard Tricarb 3375 scintillation counter. Results were corrected for counts present in the silicic acid removed from appropriate 'blank' areas of the plate. The results (mean of duplicate determinations) are shown in Table 4.

(b) Remaining aqueous phase. A procedure analogous to that used for the chloroform extracts was adopted, except that 1% (0·1 ml) of the total aqueous phase was used for t.l.c. in two dimensions, firstly in chloroform–methanol (1:1) then in butan-1-ol-water (86:14). The results are shown in Table 4, where unidentified components are designated A–C.

iii) *Metabolism of CB* 1837 *in vitro.* Liver 9000 *g* supernatant was prepared from phenobarbitone-pretreated male rats. The livers were homogenised in 4 vols ice-cold Tris buffer pH 7·4, centrifuged at 9000 *g* and the pad discarded. The supernatant was used in the following incubation mixture: liver 9000-*g* supernatant, 4 ml (equivalent to 1 g liver); Tris buffer pH 7·4, 1 ml; NADP, 12 mg; glucose-6-phosphate, 9·3 mg; magnesium chloride, 5 mg. To this mixture was added a solution of CB 1837 (1 mg) in dimethyl sulphoxide (to give a concentration in the incubation mixture of 200 μg/ml) and the mixture was incubated for 2 hr at 37° in a stoppered flask. Four vols of ethanol was then added to precipitate protein and the mixture was centrifuged at 3000 *g* for 10 min at 2°. The methods for extraction with ethyl acetate and t.l.c. of the products were those described under (i) above. The products were identified by mass spectrometry (see (iv) below). Only unchanged CB 1837 and the half-reduced derivative (IV) were positively identified.

(iv) *Identification of metabolites by mass spectrometry.* The procedures for obtaining mass spectra from metabolites isolated by t.l.c. were those previously described [5]. Mass spectra were determined by the direct insertion technique, using an AEI-MS 12 spectrometer, operating at a source temperature of 100–200° and an ionising voltage of 70 eV. Spectra are shown in Figs. 1–3, and correspond to the compounds detailed in Table 4, except that the polar metabolite isolated after chromatography on XAD-2 resin as described in (i(b)) was first converted into a derivative as follows.

A methanolic solution (0·5 ml) of this metabolite was treated with a solution of diazomethane in ether (0·5 ml). After 15 min, the solution was concentrated, prior to t.l.c. in chloroform–methanol (19:1). The principal product (R_f 0·55) was eluted with methanol and the mass spectrum was obtained. This was identical with the mass spectrum (Fig. 3) of the methyl ester (X) of the mercapturic acid adduct (IX) of CB 1837, and the t.l.c. mobilities of the metabolite and its methylation product corresponded with those of synthetic (IX) and (X) respectively.

(v) *Synthesis.* ^{3}H-labelled CB 1837. This was prepared by Dr. A. H. Khan. 1-Chloro-2,4-dinitrobenzene, which had been catalytically tritiated in the aromatic nucleus (Procedure TR 1, Radiochemical

Table 4. Quantitative determination of urinary metabolites of CB 1837 (I) in the rat: (a) Non-polar metabolites; (b) Polar metabolites

Component	R_f value (solvent in brackets)	% of total urinary radioactivity
(a)		
I	0·22 (benzene)	0·9
II	0·30 (benzene)	1·3
III	0·13 (benzene)	0·2
IV	0·10 (benzene)	2·2
V	0·07 (benzene)	1·9
VI	0·10* (2 developments in benzene)	not determined
VII + VIII	0·00* (benzene)	1·9
A + IX	0·00 (benzene)	3·8
(b)		
A (unidentified)	0·50 (n-BuOH-H_2O, 86:14)	6
IX	0·35* (n-BuOH-H_2O, 86:14)	25
B (unidentified)	0·35* (n-BuOH-H_2O, 86:14)	18
C (unidentified)	0·20 (n-BuOH-H_2O, 86:14)	27

* Other R_f values: In chloroform–methanol (19:1), VI, 0·43; VII, 0·27. In chloroform–methanol (1:1); IX, 0·05; (B), 0·35.

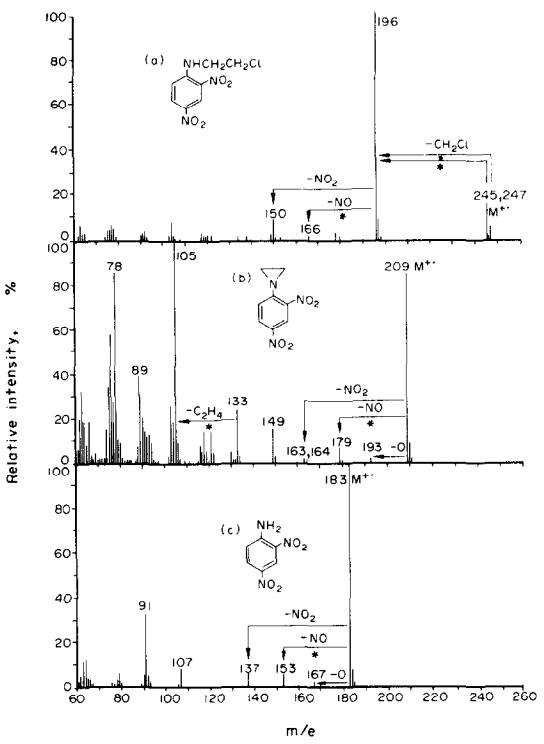


Fig. 1. (a) 1-(2-chloroethyl)amino-2,4-dinitrobenzene (II); (b) 1-(1-aziridinyl)-2,4-dinitrobenzene (CB 1837, I); (c) 2,4-dinitroaniline.

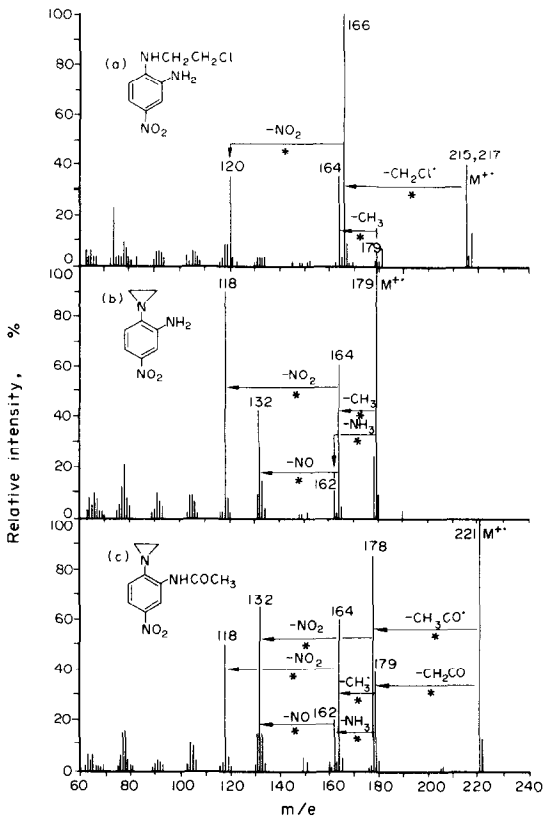


Fig. 2. (a) 2-amino-1-(2-chloroethyl)amino-4-nitrobenzene (V); (b) 2-amino-1-(1-aziridinyl)-4-nitrobenzene (IV); (c) 2-acetylamino-1-(1-aziridinyl)-4-nitrobenzene (VI).

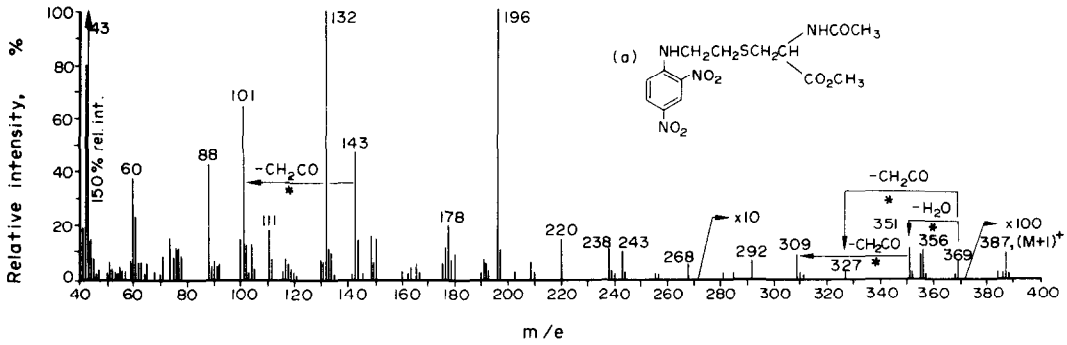


Fig. 3. Mass spectrum of synthetic *S*-[*N*-(2,4-dinitrophenyl)-2-aminoethyl]mercapturic acid methyl ester (X) corresponding to a methylated polar metabolite (IX) of CB 1837.

Figs. 1–3. Mass spectra of synthetic compounds corresponding to those isolated from rat urine after administration of CB 1837 (I) (in order of decreasing R_f value (see Table 4a)).

Centre, Amersham, Bucks.) was treated with ethyleneimine, as described previously [1] to give ^{3}H-labelled CB 1837 of sp. act. 19 mCi/m-mole.

2-Amino-1-(1-aziridinyl)-4-nitrobenzene (IV). Sodium borohydride (1 g) was added to a stirred solution of CB 1837 (I, 1 g) and $CoCl_2.6H_2O$ (1 g) in methanol (100 ml). After stirring for 0·5 hr at 20° the mixture was filtered through a short column of silicic acid then concentrated to dryness. The residue was further chromatographed on silicic acid, with chloroform as eluant, to give a pale yellow eluate followed by an orange eluate. The latter was collected and chromatographed on a column of activated alumina (Spence type II) with chloroform as eluant. Addition of cyclohexane to the concentrated orange eluate yielded (IV) as small orange plates, m.p. 144–146° (Found: C, 53·3; H, 5·2; N, 23·1%. $C_8H_9N_3O_2$ requires C, 53·6; H, 5·1; N, 23·4%). In the test against the Walker 256 carcinoma in rats the product gave: LD_{50}, 179 mg/kg; ID_{90}, 100 mg/kg; TI 1·8.

2-Acetylamino-1-(1-aziridinyl)-4-nitrobenzene (VI). A solution of 2-amino-1-(1-aziridinyl)-4-nitrobenzene (IV, 0·35 g) in ethyl acetate (5 ml) was heated at 40° with acetic anhydride (0·5 g) for 6 hr. The solution was concentrated. Trituration with ethyl acetate yielded (VI) as yellow prisms (0·32 g, 74%) m.p. 178–179° (Found: C, 54·2; H, 5·2; N, 18·9%. $C_{10}H_{11}N_3O_3$ requires C, 54·3; H, 5·0; N, 19·0%).

2-Acetylamino-1-(2-chloroethyl)amino-4-nitrobenzene (VIII). (a) To a solution of 2-amino-1-(2-chloroethyl)amino-4-nitrobenzene [8] (V, 54 mg) in glacial acetic acid (1 ml) was added acetic anhydride (0·03 ml) in diethyl ether (1 ml). The mixture was stirred at room temperature until solution was complete, then concentrated. Slow addition of petroleum ether (b.p. 40–60°) to a solution of the residue in acetone yielded (VIII) as yellow needles, m.p. 181–182° (Found: C, 46·3; H, 4·65; Cl, 14·2; N, 16·0%. $C_{10}H_{12}ClN_3O_3$ requires C, 46·6; H, 4·7; Cl, 13·8; N, 16·3%). (b) [unambiguous route which could not afford the alternative product 2-amino-1-(2-chloroethyl)acetylamino-4-nitrobenzene which is a possible product from method (a)]. Acetic anhydride (0·2 ml) was added to a solution of 2-amino-1-(1-aziridinyl)-4-nitrobenzene (IV, 50 mg) in dry pyridine (0·5 ml). After stirring for 30 min at 30° the solution was concentrated to dryness and a solution in chloroform of the residue was applied to a column of silicic acid, which was eluted with chloroform. The first eluates contained unchanged (IV) and later eluates contained material presumed to be the acetamide (VI). Dry HCl–diethyl-ether was added to a solution of the latter material in acetone. After 15 min, the solvents were removed *in vacuo* and the residue was treated with a few drops of saturated aq. NaOAc and the solution was extracted with chloroform. The dried ($MgSO_4$) extract was concentrated to dryness and the residue was crystallized from acetone-petroleum ether (b.p. 40–60°) to give needles, m.p. 182–183°, undepressed by admixture with the product obtained by method (a).

S-[*N*-(2,4-Dinitrophenyl)-2-aminoethyl]mercapturic acid (IX). A mixture of 0·25 g each of CB 1837 (I), mercapturic acid and sodium hydroxide was stirred in methanol (25 ml) for 2 days at room temperature. Silicic acid (5 g) was added and the mixture was concentrated. The residue was applied to a column of silicic acid (20 × 2 cm) which was eluted with chloroform–methanol, 1:1 (10-ml fractions). Fractions 5–30 were concentrated, and a solution of the residue in 1 N hydrochloric acid (10 ml) was extracted with ethyl acetate (3 × 10 ml). The dried extracts ($MgSO_4$) were concentrated (to *ca* 10 ml) when the product separated as a yellow powder (0·18 g, 41%). Recrystallization from ethanol afforded (IX) as colourless needles, m.p. 176–178° (Found: C, 42·15; H, 4·5; N, 14·6%. $C_{13}H_{16}N_4O_7S$ requires C, 41·9; H, 4·35; N, 15·05%).

S-[*N*-(2,4-Dinitrophenyl)-2-aminoethyl]mercapturic acid methyl ester (X). A solution of the mercapturic acid (IX, 0·15 g) in methanol (10 ml) containing acetyl chloride (0·5 ml) was left at room temperature for 2 hr. The solution was twice treated with methanol (5 ml) and concentrated. Silicic acid (2 g) in methanol (5 ml) was added, the mixture was concentrated and the residue applied to a column of silicic acid (16 × 2 cm) which was eluted with chloroform–methanol, 9:1 (10-ml fractions). Fractions 6–19 contained the product (X), a yellow solid (0·11 g, 71%), which crystallized from methanol at 0°, m.p. 97–100° (Found: C, 43·5; H, 4·75; N, 14·0%. $C_{14}H_{18}N_4O_7S$ requires C, 43·5; H, 4·7; N, 14·5%).

S-[*N*-(2,4-Dinitrophenyl)-2-aminoethyl]cysteine (XI). Cysteine hydrochloride (2·4 g) was dissolved in warm dimethyl sulphoxide (30 ml) before CB 1837 (I, 3 g) and triethylamine (4·2 ml) were added. The solution was stirred at 60° for 3·5 hr before cooling. Ammonium hydroxide (d. 0·88, 5 ml) in water (50 ml) was added followed by diethyl ether (100 ml). After vigorous agitation, to complete solution, the aqueous layer was collected and saturated with sodium acetate. On addition of glacial acetic acid (6 ml) an orange precipitate slowly formed. After 1 hr, this was filtered off and washed successively with water, acetone and diethyl ether. The product (2·1 g, 44%) was recystallized by dissolution in a slight excess of dilute aqueous sodium hydroxide and then slow addition of an equivalent amount of dilute aqueous hydrochloric acid to yield the product (XI) as yellow prisms, m.p. 201° (decomp.) (Found: C, 39·8; H, 4·6; N, 16·9%. $C_{11}H_{14}N_4O_6S$ requires C, 40,0; H, 4·3; N, 17·9%). In the Walker test: LD_{50} was *ca* 1000 mg/kg; no tumour growth inhibition occurred up to 1600 mg/kg.

DISCUSSION

The toxicity of CB 1837 *in vitro* (see Table 1) was very low for a drug with an ID_{90} of 23 mg/kg, which would normally be active in the bioassay at a concentration of *ca* 23 μg/ml. This toxicity was more nearly approached when liver 9000-*g* supernatant and cofactors were included in the incubation mixture in the bioassay, a finding consistent with metabolic activation of CB 1837 by the liver supernatant fraction. Hence a study of the metabolism of CB 1837 might provide some insight into its mechanism of action, and that of other nitrophenyl aziridines.

Metabolism of CB 1837 by liver 9000-*g* supernatant and cofactors afforded the half-reduced analogue (IV). This was more active in the bioassay system (Table 2) than CB 1837, and was comparable in activity to CB 1837 in the presence of the liver supernatant (Table 1). The requirement of a relatively high concentration of NADP to produce activation of CB 1837 (Table 3) was consistent with the involvement of nitroreductase in the activation step [9] and thus with the conversion of CB 1837 into the half-reduced analogue (IV) as the activating step. The finding that the metabolite (IV) had a low TI in the *in vivo* test might appear to suggest that it was not responsible for the activity of CB 1837 *in vivo*. However, the active form of a drug may be more effective when generated by metabolism than when administered externally.

Examination of the urinary metabolites of CB 1837 in the rat showed extensive metabolism of the drug (Table 4). The metabolites were separated into polar and non-polar components by extraction of the latter with an organic solvent and this extract was resolved into six mobile components by t.l.c. Mass spectrometry of each metabolite allowed its chemical structure to be predicted on the basis of certain character-

istic fragmentation pathways, in particular those associated with the aromatic nitro group [10]. The structures (II–VI) thus derived were confirmed by a comparison of the mass spectra and chromatographic properties (Figs. 1–2 and Table 4 respectively) with those of synthetic materials. One of the polar metabolites remaining in the aqueous phase after solvent extraction afforded a satisfactory mass spectrum only after methylation. The mass spectrum of the methylated product was identical with that of the synthetic methyl ester (X) (see Fig. 3) of the mercapturic adduct (IX) of CB 1837. The preponderance of the appropriate $(M + 1)^+$ ion over $M^{+\cdot}$ observed here has been noted for other *N*-acetylamino acid methyl esters [11] as have some of the signals in the lower mass range (e.g. *m/e* 88, 101, 111 and 143). Formation of the mercapturic acid conjugate (IX) would appear to represent a detoxification mechanism for CB 1837, since the related cysteine adduct (XI) was inactive against the Walker tumour.

Tritium-labelled CB 1837 was used in a quantitative evaluation of the urinary metabolites (Table 4). Only 7% of the administered radioactivity was excreted in this experiment, but a higher figure (23%) has been observed in a separate experiment designed to follow the excretion of the drug with time [12]. Identified metabolites accounted for 36%, and unidentified metabolites for 51% of excreted radioactivity, of which the latter consisted of those polar metabolites (A–C in Fig. 4) which were not readily separable from naturally occurring urinary constituents. Additionally detected during this quantitative experiment were the compounds (VII) and (VIII), which coincided on t.l.c. The mass spectrum contained *inter alia* the appropriate molecular ions (*m/e* 227 and *m/e* 257) in the intensity ratio 19:1. That synthetic (VIII) was produced both from (V) and from the product of partial reduction of CB 1837 served to establish that the structure (IV) had been correctly assigned to the latter product, since the aziridino and amino substituents must therefore bear the *ortho* relationship of the corresponding substituents in the known [8] compound (V). The half-reduced analogue (IV) of CB 1837, which had earlier been implicated as a possible activated form of the drug, was the most abundant of the potentially alkylating metabolites present in the urine, and was indeed more than twice as abundant as unchanged CB 1837. Its *N*-acetyl derivative (VI) was insufficiently abundant to be quantitatively determined at the levels of radioactivity employed.

Thus the studies *in vivo* lend additional support to the hypothesis that CB 1837 might be activated by partial reduction. In this connection, Khan and Ross [13] have considered the possibility that CB 1837 might be metabolised by partial reduction to an azoxy-derivative, 2,2'-di(1-aziridinyl)-5,5'-dinitroazoxy-benzene, which could be considered as a bifunctional alkylating agent, thus reconciling the usual requirement for such bifunctionality with the apparently anomalous antitumour activity of the monofunctional nitrophenylaziridines. Since the azoxy-derivative is mobile on t.l.c. in benzene (R_f 0·05) and in chloroform–methanol, 19:1 (R_f 0·70) it was sought among the relatively non-polar metabolites of CB 1837, but has not so far been detected. Other urinary metabolites of CB 1837 which were tested in the bioassay system were the *N*-(2-chloroethyl)amino analogue (II) and 2,4-dinitroaniline. Both were inactive at concentrations of 1 mg/ml.

The possibility that the Walker tumour itself could metabolise CB 1837 was also investigated. The urinary metabolites of CB 1837 produced by a tumour-free rat as described above were compared with those produced by a rat bearing the advanced Walker tumour. There were no discernible differences, but this finding does not entirely preclude the possibility that specific metabolites are formed by the tumour-bearing rats, since these may be excreted by an alternative route, or may be highly reactive and locally acting.

In summary, whilst the present study has afforded some insight into the mechanism of action of CB 1837, the wider questions, namely the reason for the selectivity of the nitrophenyl aziridines towards the Walker tumour, and for the absence of the usual requirement for bifunctionality in an antitumour alkylating agent remain unanswered. It is hoped that a complementary study of the metabolism of CB 1954 (Ia) for which this work provides a basis, will aid a solution to these problems.

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