Biochemical Pharmacology, Vol. 31, No. 10, pp. 1887–1892, 1982.
Printed in Great Britain.

0006-2952/82/101887-06 $03.00/0

STUDIES OF THE MODE OF ACTION OF ANTITUMOUR TRIAZENES AND TRIAZINES—IV. THE METABOLISM OF 1-(4-ACETYLPHENYL)-3,3-DIMETHYLTRIAZENE

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(*Received* 17 *November* 1981; *accepted* 13 *January* 1982)

Abstract—The metabolism of 1-(4-acetylphenyl)-3,3-dimethyltriazene has been studied *in vivo* and *in vitro* in mice. This dimethyltriazene was extensively metabolised *in vivo* and HPLC analysis of the plasma revealed the presence of two metabolites, the monomethyltriazene, 1-(4-acetylphenyl)-3-methyltriazene, and the arylamine, 4-aminoacetophenone. The dimethyltriazene was also biotransformed *in vitro* by a 9000 *g* fraction of mouse liver homogenate to products which were selectively toxic to TLX5 lymphoma cells. HPLC analysis of the products of *in vitro* metabolism under these conditions showed the presence of the monomethyltriazene but in an amount insufficient to account for the observed cytotoxicity. The monomethyltriazene was itself rapidly biotransformed by a 9000 *g* fraction of mouse liver homogenate, and by isolated mouse hepatocytes.

Considerable evidence has accumulated to corroborate the theory that 1-aryl-3,3-dimethyltriazenes are prodrugs—that is, they only develop antitumour efficacy after biochemical transformation *in vivo* [1, 2]. The events leading to the emergence of antitumour activity are still not completely understood but the balance of published evidence points to the active moeities being 1-aryl-3-methyltriazenes which are known to methylate biologically significant nucleophiles [3]. Thus, in the case of the dimethyltriazene used in the present work this would imply an activation sequence (I) → (II) → (III) with the arylamine (IV) being the by-product of the methylation reaction (Fig. 1). However, in an earlier paper in this series [2] we showed that monomethyltriazenes were equitoxic *in vitro* to mouse TLX5 lymphoma cells which were either sensitive (TLX5S) or resistant (TLX5R) to dimethyltriazenes *in vivo*; they do not exhibit the discriminating selectivity observed with dimethyltriazenes, which, when incubated *in vitro* with TLX5 lymphoma cells together with a mouse liver homogenate (9000 *g* fraction) and an NADPH generating system, liberate cytotoxic species which are more toxic to the sensitive lymphoma than the resistant variant [2]. We argued that, under these specific bioassay conditions, cytotoxic species other than monomethyltriazenes were generated and that these species were possibly responsible for the selective antitumour effects of dimethyltriazenes observed *in vivo*.

In the present study we have examined the metabolism of 1-(4-acetylphenyl)-3,3-dimethyltriazene (I) both *in vitro* under the conditions of the bioassay used previously [2] or in the presence of isolated mouse hepatocytes, and *in vivo* in the mouse. Using a novel HPLC system to separate and quantitatively estimate the dimethyltriazene substrate (I) and its metabolites we have been able to positively identify, for the first time, a monomethyltriazene (III) amongst the products.

MATERIALS AND METHODS

Chemicals. Triazenes were synthesised by literature methods [3]. 4-Aminoacetophenone was purchased from BDH Chemicals Ltd. (Poole, U.K.).

Tumours. The TLX5S lymphoma sensitive to dimethyltriazenes *in vivo* and TLX5R lymphoma resistant to dimethyltriazenes *in vivo* were those used in a previous study [2].

In vitro–in vivo *bioassay.* This has been described previously [2]. It is worthwhile noting that biotransformation experiments performed with a 9000 *g* fraction of mouse liver homogenate and an NADPH generating system were conducted in *closed* 25 ml universal tubes (Flow Laboratories Ltd, Irvine, U.K.) and not under the forced oxygenation conditions employed by other workers [1, 4, 5].

Isolation of mouse liver cells. Female C57 Black mice (20–25 g) were anaesthetised with pentobarbital (100 mg/kg i.p.) and liver cells were obtained as described by Renton *et al.* [6] including the perfusion of the liver in a retrograde fashion. Cells were counted in a Bürki chamber after staining them with a solution of 0.1% trypan blue in physiological salt solution. The total yield fluctuated between 4 and 6×10^7 cells per mouse. Viability, as measured by the ability of the cells to exclude trypan blue, was 65–80%. Cells were suspended in Krebs–Henseleit buffer [7] containing 1% bovine serum albumin satu-

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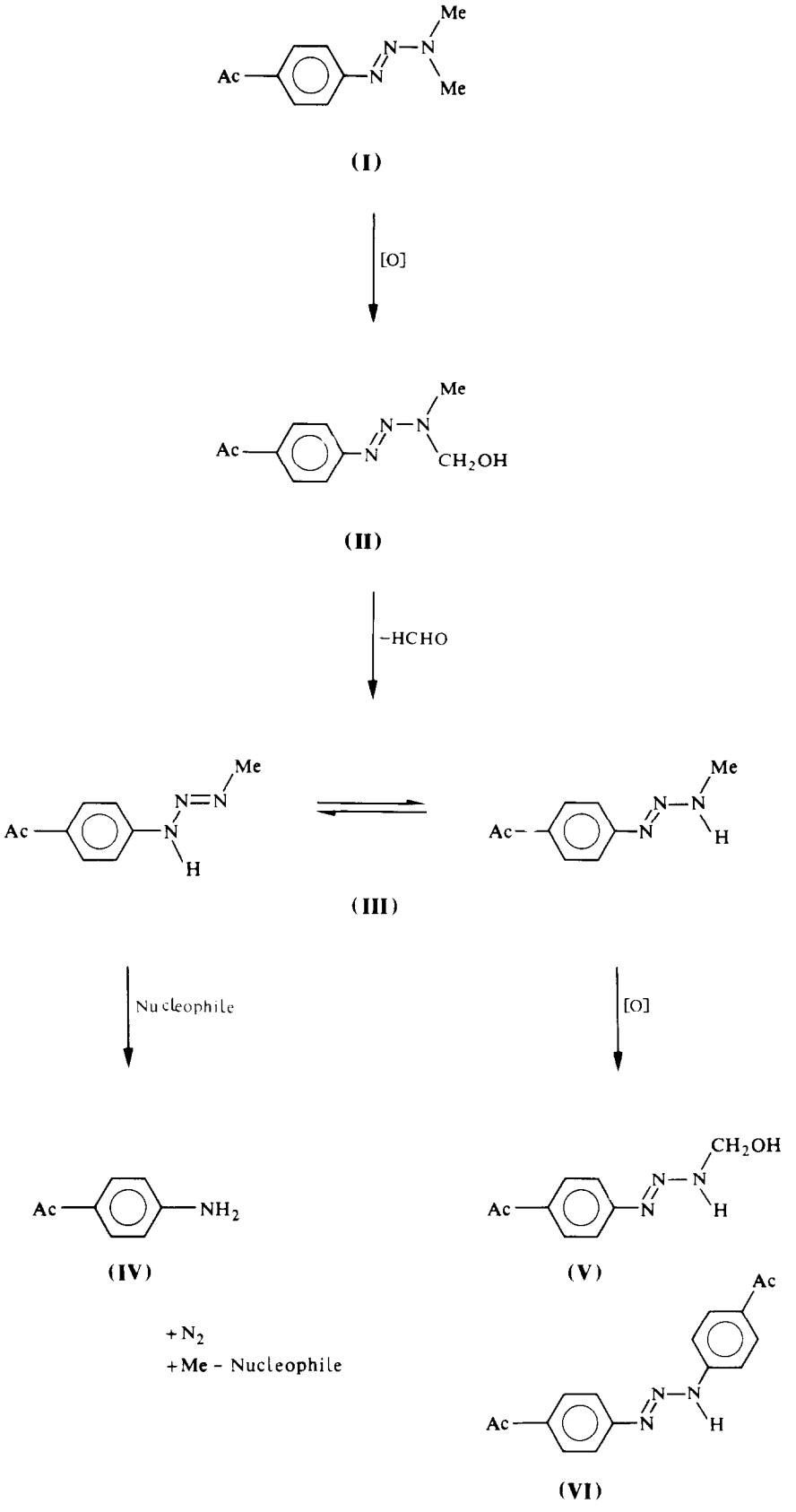

Fig. 1. Proposed metabolic activation of 1-(4-acetylphenyl)-3,3-dimethyltriazene.

rated with oxygen and were used within 1 hr after isolation.

Hepatocyte incubations. The metabolic incubation mixtures consisted of 9 ml of Krebs–Henseleit buffer with 1% bovine serum albumin and 1 ml of foetal calf serum and contained 2×10^7 cells. The substrates were added in 0.1 ml acetone and the final concentration of triazenes was 50 μM. The presence of the drugs at this concentration did not affect the viability of the hepatocytes. Control incubations contained substrate, buffer, serum and cells which had been inactivated by boiling for 30 sec. Incubations were performed in a shaking water bath in an atmosphere of oxygen containing 5% CO_2. Under these conditions the hepatocytes maintained unchanged viability for at least 120 min. Aliquots of 1 ml of the incubation mixtures were removed at different time intervals (t = 0, 10, 20, 40 and 80 min.) and were deproteinized by addition of 1 ml of ice-cold acetonitrile followed by centrifugation. Samples were then injected into the HPLC column.

In vivo *metabolism experiments.* 1-(4-Acetylphenyl)-3,3-dimethyltriazene was dissolved in arachis oil and injected i.p. into female C57 Black mice and the animals were killed after 15, 30, 60 and 120 min. At each time interval the blood of four mice was collected separately into heparinised containers. After centrifugation the plasma was diluted with an equivalent volume of ice-cold acetonitrile. Prior to analytical determination the samples were centrifuged to precipitate proteins.

HPLC analysis. 1-(4-Carboxamidophenyl)-3,3-diethyltriazene was added as a marker to extracts of the hepatocyte supensions or plasma samples. A Waters Chromatograph Model 440 fitted with a 254 nm absorbance detector and a Lichrosorb RP18 column (Merck, Darmstadt, West Germany) was used. Separation of triazenes and 4-aminoacetophenone was achieved with an isocratic solvent system of 35% aqueous acetonitrile containing diethylamine (0.05%). The flow rate was 1.5 ml/min. The limits of detection were 0.2 μg/ml for the triazenes and 0.3 μg/ml for 4-aminoacetophenone. The reproducibility of the separation was tested by determining the mean peak area ratios of four concentrations (0.5, 1.0, 2.5 and 5 μg/ml) for the dimethyltriazene (I), the monomethyltriazene (III) and arylamine (IV). The S.D. obtained with four separate determinations was less than 5% of the mean for the triazenes, and less than 7% for the arylamine.

RESULTS

Antitumour tests. In standard antitumour tests using the mouse TLX5 lymphoma *in vivo* [2] the dimethyltriazene (I, Fig. 1) showed optimal antitumour activity against the sensitive strain (TLX5S) at 40 mg/kg to give a 56% increase in survival time (IST). In an exactly parallel experiment, but using the resistant line (TLX5R), only a 6% IST was observed.

In vitro–in vivo *bioassays.* The results of bioassays are recorded in Table 1. Experiments 1 and 2 with the dimethyltriazene (I) confirm previous observations [2] that dimethyltriazenes have no cytotoxicity to the TLX5S and TLX5R lymphoma, but are activated by incubation with a 9000 *g* fraction of mouse liver homogenate and cofactors to afford product(s) which are more toxic to the TLX5S lymphoma (experiment 3) than to the TLX5R (experiment 4). In contrast, the monomethyltriazene (III) is directly toxic towards the TLX5R tumour (experiments 5–7) and extrapolation from these results implies that the 23% ILS registered against the TLX5R tumour when the dimethyltriazene is activated (experiment 4) would be equivalent to the generation, by metabolism, of approximately 60 μg/ml of the monoethyltriazene. (The assumption is made that the monomethyltriazene is the sole species responsible for cytotoxicity.)

Metabolism experiments. Under conditions when activation of the dimethyltriazene to cytotoxic products by a 9000 *g* fraction of mouse liver homogenate can be achieved (Table 1, experiments 3 and 4), HPLC analysis (Fig. 2) of the metabolism mixture at various time intervals confirmed the presence of the monomethyltriazene. However, when it is considered that these conditions engender cytotoxicity to the TLX5R tumour equivalent to the effects elicited by 60 μg/ml of monomethyltriazene (calculated from experiments 5–7) the actual quantity of monomethyltriazene formed (< 2.0 μg/ml) is puz-

Table 1. Bioassay of triazenes against TLX5 lymphoma*

Experiment number	Compound (special conditions)	Concentration (μg/ml)	Tumour type†	Per cent ILS
1	I	500	TLX5S	0
2	I	500	TLX5R	0
3	I (9000 *g* + cofactors)	500	TLX5S	38
4	I (9000 *g* + cofactors)	500	TLX5R	23
5	III	25	TLX5R	6
6	III	50	TLX5R	16
7	III	100	TLX5R	47

* 2×10^6 TLX5 cells/ml were incubated with the stated concentrations of drug for 2 hr at 37°. 2×10^5 cells were then injected into animals and the increase in life span (per cent ILS) compared with animals which received untreated cells.

† For definitions see Materials and Methods.

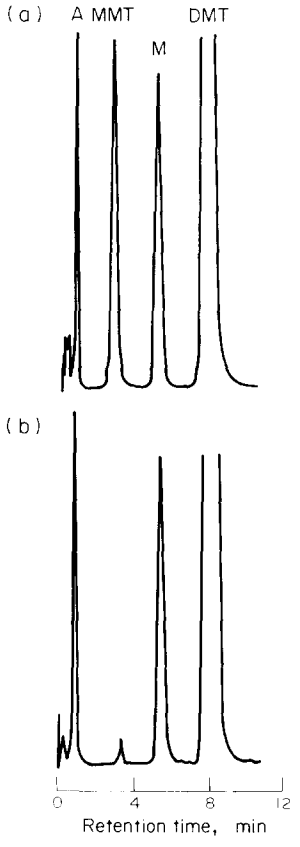

Fig. 2. High pressure liquid chromatogram of (a) a solution of 4-aminoacetophenone (A), 1-(4-acetylphenyl)-3-methyltriazene (MMT), marker 1-(4-carboxamidophenyl)-3,3-diethyltriazene (M), and 1-(4-acetylphenyl)-3,3-dimethyltriazene (DMT), and (b) an extract of a 9000 *g* mouse liver preparation incubated with 2.5 mM 1-(4-acetylphenyl)-3,3-dimethyltriazene under the conditions of the bioassay [2] after 60 min incubation. The concentration of MMT in the incubation mixture was less than 1 μg/ml.

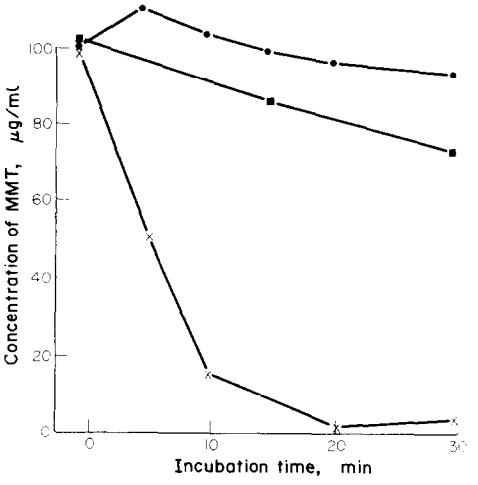

Fig. 3. Metabolism of 1-(4-acetylphenyl)-3-methyltriazene by a mouse liver 9000 *g* fraction. 0.5 mM methyltriazene was incubated with liver homogenate equivalent to 0.2 *g* of liver in Earl's buffer with cofactors generating 1 mM NADPH and with 3 mM $MgCl_2$ under access of air. The methyltriazene was determined in mixtures of the liver fraction with (×) and without (●) cofactors and in buffered solutions of the cofactors without the 9000 *g* supernatant (■).

zlingly low. One can assume that cytotoxicity towards the TLX5R cells is due to the monomethyltriazene, since these cells are resistant to the, as yet, unidentified active species formed by metabolism of the dimethyltriazene *in vivo*. This is reflected in the enhanced cytotoxicity of the activated dimethyltriazene to the TLX5S cells (Table 1, experiment 3) compared to the TLX5R cells (experiment 4). The monomethyltriazene (III) can itself potentially give rise to a series of chemical degradation products, for example 4-acetylbenzenediazonium ion, 4-hydroxyacetophenone, 4-aminoacetophenone (IV) and 1,3-di-(4-acetylphenyl)triazene(VI). The latter compound, the product of a 'diazomigration' reaction [3], was readily formed when the monomethyltriazene was chromatographed on alumina or silica gel thin layer chromatography plates, but not when a solution of the monomethyltriazene in phosphate buffer (pH 7.4) was allowed to decompose at 37°. In the latter case 4-aminoacetophenone was the sole u.v.-absorbing product as adjudged by the identity of the U.V. spectrum of the totally degraded monomethyltriazene solution with that of spectrum of authentic amine.

However, although these chemical degradation products have not been examined for activity against the TLX5S and TLX5R tumours in the present study, structurally related compounds have been shown to have no significant cytotoxicity when tested under comparable conditions [2]. One attractive explanation for the discrepancy between observed cytotoxicity of the activated dimethyltriazene to the TLX5R cells and the low amounts of monomethyltriazene found in the bioassay incubation mixture is that the monomethyltriazene is, in fact, generated in substantial amounts but is then further metabolised, perhaps to another cytotoxic species. This possibility has been advanced by ourselves [2] and by Pool [8] and is supported here by the observation that the monomethyltriazene is indeed a substrate for further metabolism by a 9000 *g* fraction of mouse liver homogenate (Fig. 3) Alternatively, levels of free monomethyltriazene may be depleted by interaction with cellular nucleophiles thus leading to the formation of the arylamine metabonate (IV) which was also detected (HPLC) in the products of metabolism under the conditions of the bioassay.

Metabolism of the dimethyltriazene to monomethyltriazene and arylamine was also observed in suspensions of isolated mouse hepatocytes (Fig. 4); this system was also capable of further metabolising the monomethyltriazene (Fig. 5). In the latter case control experiments indicated that disappearance of monomethyltriazene was a truly metabolic process since nucleophile promoted chemical degradation of the substrate in the manner illustrated in Fig. 1 was comparatively slow. For example, the half-life of the monomethyltriazene in Krebs buffer at 37° was 107 min and in the presence of hepatocytes inactivated by heating 61 min (Fig. 5).

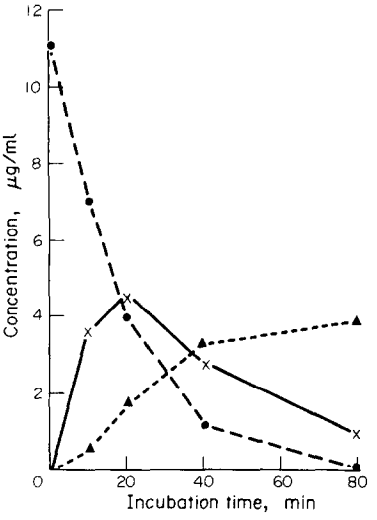

Fig. 4. Metabolism of 1-(4-acetylphenyl)-3,3-dimethyltriazene by isolated mouse hepatocytes. The initial concentration of the triazene was 0.05 mM, the hepatocyte concentration was 2×10^6 cells/ml. Symbols indicate the disappearance of the dimethyltriazene (●), and the appearance of the metabolites 1-(4-acetylphenyl)-3-methyltriazene (×) and 4-aminoacetophenone (▲). Values are the mean of two experiments.

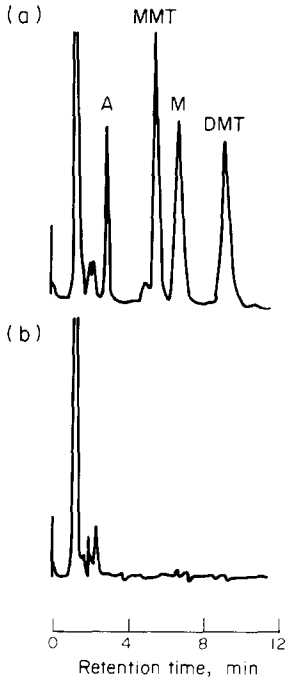

Fig. 6. High pressure liquid chromatogram of extracts of mouse plasma samples 30 min after injection of (a) 1-(4-acetylphenyl-3,3-dimethyltriazene (40 mg/kg) dissolved in arachis oil, and (b) arachis oil. For the explanation of the peak identifying letters see legend of Fig. 2. Details on the sample preparation and chromatography under Materials and Methods.

An HPLC analysis of plasma collected 30 min after the i.p. injection of dimethyltriazene into mice confirmed the presence of monomethyltriazene and arylamine (Fig. 6). Plasma concentrations measured after administration of the optimum *in vivo* antitumour dose (40 mg/ml) afforded estimates of the areas under the plasma concentrations vs time curve for the drug and its metabolites up to 2 hr after drug administration. These values, calculated by the trapezoidal rule [9], were very similar: 100 μg/min/ml for the dimethyltriazene; 98 μg/min/ml for the monomethyltriazene; and 91 μg/min/ml for the arylamine. No monomethyltriazene was detected in the plasma 2 hr after drug administration (Fig. 7).

DISCUSSION

That tumour-inhibitory and carcinogenic dimethyltriazenes undergo oxidative *N*-demethylation to monomethyltriazenes, which may be the ultimate cytotoxic or carcinogenic species, was originally proposed by Preussman in 1969 [5] and has since been supported by several studies [1, 4, 8, 10, 11]. How-

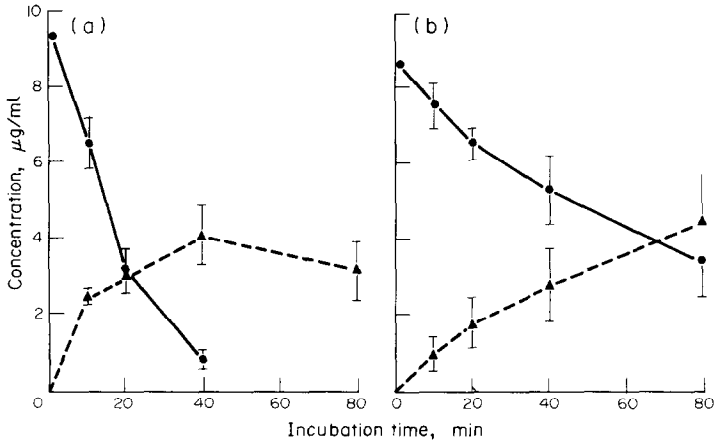

Fig. 5. Metabolism of 1-(4-acetylphenyl)-3-methyltriazene by (a) isolated mouse hepatocytes, and (b) by hepatocytes inactivated by heating. The graphs show the disappearance of the monomethyltriazene (●) and the appearance of 4-aminoacetophenone (▲). The initial concentration of the monomethyltriazene was 0.05 mM, the hepatocyte concentration of 2×10^6 cells/ml. Values are the mean ± S.E.M. of three experiments.

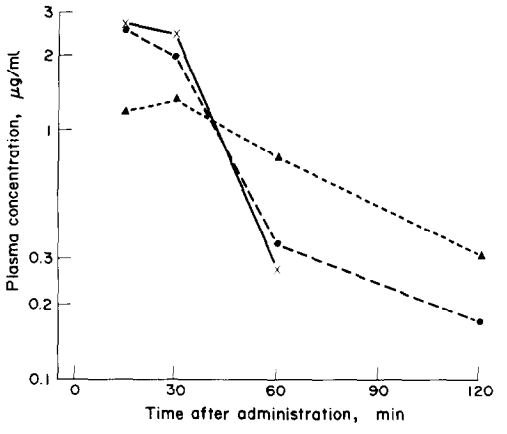

Fig. 7. Plasma concentrations of 1-(4-acetylphenyl)-3,3-dimethyltriazene (●) and its metabolites 1-(4-acetylphenyl)-3-methyltriazene (×) and 4-aminoacetophenone (▲) in mice after i.p. administration of 40 mg/kg of the dimethyltriazene. Values are the mean of four experiments.

ever, the intermedicacy of monomethyltriazenes has not been demonstrated directly but instead the measurement of formaldehyde, produced by oxidative demethylation, has been used as a presumptive measure of their production (Fig. 1). A recent study described the isolation of an *N*-acetylated derivative of a monomethyltriazene from an *in vitro* metabolism experiment, but not the monomethyltriazene itself [8].

The results presented here establish unequivocally that monomethyltriazenes are metabolites of dimethyltriazenes and are liberated both *in vitro* (Figs. 2 and 4) and *in vivo* (Fig. 6). After administration of the dimethyltriazene (I) *in vivo* the emergence of the monomethyltriazene metabolite (III) and the arylamine (IV) in the plasma was followed by the rapid disappearance from the plasma of both the dimethyltriazene and the monomethyltriazene (Fig. 7); a pattern of metabolism similar to that observed with isolated mouse hepatocytes (Fig. 4).

In view of our previous work [2] we were particularly interested in noting any biotransformations occurring under conditions *in vitro* where metabolites are formed which are more toxic towards the sensitive TLX5S tumour than the resistant TLX5R tumour (Table 1, experiments 3 and 4). Metabolism of a dimethyltriazene under these bioassay conditions, utilising a 9000 *g* fraction of mouse liver homogenate, did however result in the death of resistant cells (experiment 4), presumably due to metabolically generated monomethyltriazene. This is consistent with our earlier study [2] in which we showed that a series of monomethyltriazenes are toxic to both sensitive and resistant cell lines. However HPLC analysis of the metabolism mixture at various times showed that only a very small quantity of the monomethyltriazene was present (less than 2 μg/ml incubation medium). Comparisons of the cytotoxicities observed when the dimethyltriazene was activated (experiment 4) with the direct action of the monomethyltriazene (experiments 5–7) suggested that a titre of 60 μg/ml of the monomethyltriazene would account for the observed cell death. However, the levels of monomethyltriazene actually quantified (HPLC) in comparable metabolism mixtures were insufficient to achieve this antitumour effect.

It is extremely difficult to relate cytotoxicity under these different conditions however, and one possible explanation for this degree of discrepancy is that the addition of genuine monomethyltriazene to a bioassay is not equivalent to its cumulative production by metabolism *in situ* (complicated by its disappearance due to chemical breakdown). We believe that a possible alternative explanation is that as the monomethyltriazene is generated by metabolism it is then rapidly removed by further metabolism (Figs. 3 and 5). This is a novel observation and work is currently in progress to identify the product(s) of this intriguing metabolic transformation. It is pertinent to note that formaldehyde could hardly be detected when monomethyltriazenes were metabolised *in vitro* [1, 4]. It is possible that a stable monohydroxymethyltriazene (VI, Fig. 1) could be formed which may be a candidate for the truly ultimate, selective toxic species generated under the bioassay conditions, and also possibly *in vivo*.

Acknowledgements—We than Dr. K. Vaughan and Dr. M. D. Threadgill for the synthesis of triazenes, the Cancer Research Campaign and CNR (project CT 8100176.04) for financial support, and the British Council for an academic interchange award between the laboratories at Aston and Milan.

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