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INVESTIGATION ON THE OXIDATIVE *N*-DEMETHYLATION OF ARYL TRIAZENES *IN VITRO*

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Abstract—The oxidative *N*-demethylation of several *p*-substituted phenyl triazenes has been determined *in vitro* under carefully controlled conditions. Three of these compounds had been reported not to be appreciably demethylated, one of them still possessing antitumour activity. The per cent demethylation figures obtained for the tested compounds ranged from about 20 to 60 per cent and was not dependent on the nature of the solvent used to keep the drug in solution. The enzymatic reaction proved to be strongly inhibited by diazonium cations either generated by spontaneous hydrolysis of the drug or added to the incubation mixture. The reaction is also inhibited at various degrees by the monomethyl derivatives produced by demethylation of the dimethyl triazenes. The inhibition by diazonium and monomethyl derivatives can not account for the observed time-course of demethylation, which occurs for all the tested compounds only in the first minutes of incubation. The possible occurrence of other reaction(s), competing with the demethylation process, the impossibility of correctly expressing kinetic parameters for demethylation, and the possible relevance of these observations for the activity of triazenes *in vivo*, are pointed out.

Dialkyl triazenes are a class of compounds endowed with interesting biological activities. 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (DIC) has been reported to have marked inhibitory activity against L 1210 leukemia and other rodent neoplasms [1, 2]. It also proved to be clinically effective, and is currently employed in the therapy of malignant melanoma [3]. Its mechanism of action is essentially unknown [4]. DIC undergoes hydrolysis to 5-diazo-imidazole-4-carboxamide and dimethylamine [5], the diazo derivative having much smaller antitumor activity than DIC itself [2]. This makes unlikely the possibility that the drug exerts its effects only by acting as an antimetabolite [4], i.e. via the diazo derivative, which has been shown to inhibit the purine biosynthesis *in vitro* [6]. Another modification of DIC concerns its oxidative *N*-demethylation by liver [7] and tumour [8] microsomes to the corresponding monomethyl derivative, which decomposes to 5-amino-imidazole-4-carboxamide, nitrogen, and methyl cations [9]. The formation of methyl cations suggests that the activity mechanism of DIC involves an alkylation of cellular components.

This possibility is supported by the reports on the biological activity of 3,3-dimethyl-1-phenyl triazene and its derivatives, for which a selective inhibitory activity on purine biosynthesis is unlikely, due to the presence of an aromatic ring instead of the imidazole moiety in their molecule. These compounds have been shown to possess carcinogenic [10] and mutagenic effects [11], and exhibit an inhibitory activity against rodent tumours [12, 13]. Also, these molecules undergo hydrolysis to the corresponding aromatic diazonium compounds [14] and microsomal oxidative *N*-demethylation, as described for DIC [13,15]. At least for the effects at systemic level, the latter pathway has been given the greatest importance [16].

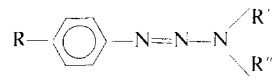
In contrast with this conclusion, some molecules with minute differences in the substituents in the aromatic ring, have been reported not to be demethylated [13, 15], although having biological activity [13]. This discrepancy has prompted a reinvestigation of the *N*-demethylation reaction, performed under carefully controlled conditions. The results we have obtained suggest that either the diazo derivatives formed by hydrolysis of the parent triazenes, or the monomethyl derivatives produced enzymatically, might inhibit the *N*-demethylation process *in vitro*. They further indicate that side reactions occurring, catalyzed by microsomal enzymes, might chemically modify the substrate, thereby affecting the main process of oxidative *N*-demethylation.

MATERIALS AND METHODS

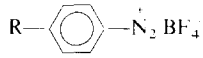
The abbreviations and the references for the synthesis of the used triazenes are reported in Table 1.

The purity of these substances was checked by elemental analysis and thin layer chromatography, using Kieselgel GF 254 (Merck) and methanol–ethyl acetate–ligroin (3:2:1) or occasionally aluminium oxide GF 254 Type E (Merck) and ethyl acetate–hexane (1:4). In particular, the purity of the samples of DM-CH_3 and DM-OCH_3 used was confirmed also by their n.m.r. spectra: δ TMS in $CDCl_3$, 2·30 (s, 3H, $ArCH_3$), 3·22 (s, 6H, NCH_3), 7·22 (sym. m., 4H, ArH) for DM-CH_3, and δ TMS, 3·07 (s, 6H, NCH_3), 3·57 (s, 3H, OCH_3), 7·20 (sym. m., 4H, ArH) for DM-OCH_3. For DM-OCH_3, the half-life at pH 7·4 and 37° was determined. Since hydrolysis of this substance gives a very stable diazonium cation, a direct spectrophotometrical determination has been preferred to the usual procedures for determining the rate constant [14]. Thus, 0·1 ml of an approximately 4×10^{-3} M solution of this substance in absolute ethanol was diluted to 10 ml with a prewarmed buffer phosphate 0·1 M solution (pH 7·4), and the absorbances were timely determined at 317 nm. Two isosbestic points, at 293 and 327 nm, were observed. Linear

Table 1. Triazene derivatives and diazonium salts used, abbreviations of their chemical names and references for their synthesis



Abbreviation	R	R′	R″	Chemical names	References
DM-H	H	CH_3	CH_3	1-phenyl-3,3-dimethyltriazene	21
DM-OCH_3	OCH_3	CH_3	CH_3	1-*p*-methoxyphenyl-3,3-dimethyltriazene	22
DM-CH_3	CH_3	CH_3	CH_3	1-*p*-tolyl-3,3-dimethyltriazene	23
DM-COOH	COOH	CH_3	CH_3	1-*p*-carboxyphenyl-3,3-dimethyltriazene	24
DM-COOEt	$COOC_2H_5$	CH_3	CH_3	1-*p*-carbethoxyphenyl-3,3-dimethyltriazene	13
DM-$CONH_2$	$CONH_2$	CH_3	CH_3	1-*p*-carboxamidophenyl-3,3-dimethyltriazene	25
$MMCH_3$	CH_3	H	CH_3	1-*p*-tolyl-3-methyltriazene	26
MMCOOEt	$COOC_2H_5$	H	CH_3	1-*p*-carbethoxyphenyl-3-methyltriazene	*



†R			Calc. C	Calc. H	Analyses N	Found C	Found H	Found N
OCH_3	u.v.$^{max}_{water}$ 311 nm	m.p. 142 dec.	37·88	3·18	12·62	38·02	3·40	12·88
$CONH_2$	u.v.$^{max}_{methanol}$ 257 nm	m.p. 114 dec.	35·78	2·57	17·88	35·73	2·56	17·78

* Obtained from *p*-carbethoxyphenyldiazonium tetrafluoborate and acqueous methylamine; recrystal. from hexane, m.p. 83 dec., u.v.$^{max}_{ethanol}$ 311 nm. Analysis— calculated: C, 57·96; H, 6·32; N, 20·28; found: C, 57·85; H, 6·23; N, 20·00.

† Prepared according to a reported procedure [24] and recrystal. from methanol and washed with ether.

regression analysis applied to 29 spectrophotometrical data, $\log_{10}(E_\infty - E_t)$, relevant to a range of 60 min, gave the equation $\log_{10}(E_\infty - E_t) = -0{\cdot}3457 - 0{\cdot}01756\,t$. The root mean square deviation of the observed points from the interpolating line was $\pm 0{\cdot}01414$.

Oxidative *N*-demethylation *in vitro* was determined essentially as described by Preussmann *et al.* [15] and Audette *et al.* [13]. Sprague–Dawley rats weighing about 250 g were given drinking water containing phenobarbitone 0·5 g/l. for 3 days. Following sacrifice by cervical dislocation, the washed livers were homogenized by a Potter–Elvehjem homogenizer at 4° in 4 vol of K phosphate buffer 0·1 M, pH 7·4. Both the supernatant obtained by a 20-min centrifugation at 10,000 *g*, or a microsomal preparation were used. Microsomes were prepared by centrifuging the 10,000-*g* supernatant for 1 hr at 100,000 *g* and resuspending the pellets in a glass homogenizer to the original volume with the phosphate buffer.

To measure demethylation, the reaction mixture contained 1·9 ml 0·1 M K phosphate buffer (pH 7·4), 1·5 ml 10,000-*g* supernatant (about 60 mg protein) or microsomal suspension (about 15 mg protein), 0·1 ml dimethylsulphoxide (DMSO) containing 0·5 μmoles of the drug (unless otherwise stated), 100 μmoles semicarbazide, 25 μmoles $MgCl_2$, 2 μmoles NADP, 25 μmoles glucose-6-phosphate, 2·5 units glucose-6-phosphate dehydrogenase (EC 1.1.1.49) type XV from Sigma (final vol 4 ml).

At the end of the incubation, carried out at 37° in air for 60 min (unless otherwise indicated), the reaction was stopped by adding 1 ml 20% (w/v) $ZnSO_4$ and 1 ml satured $Ba(OH)_2$ solution, and, after centrifugation, the Nash colorimetric assay for formaldehyde was performed on the supernatant [17]. Occasionally, the assay was carried out on formaldehyde separated by distillation. For each determination, a blank containing the appropriate solvent and no drug, and standards containing known amounts of formaldehyde, were run.

The demethylating capacity of the system used was checked by using aminopyrine as substrate, and proved to be constant for at least 1 hr.

Protein was measured according to the biuret method, using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Data reported in Table 2 show that all the triazenes tested were demethylated, although at different degrees, by both liver postmitocondrial supernatant and microsomes. MM-CH_3 and dimethylamine did not react, thus excluding the possibility that the formaldehyde assayed originated from the demethylated triazene or from the demethylamine formed by hydrolysis of the drug.

The results concerning DM-COOH, DM-CH_3 and DM-COOEt are in disagreement with those reported by Audette *et al.* [13] and Preussmann *et al.* [15], who were unable to show any demethylation for these triazenes. Even by following their procedure exactly, i.e. using ethanol instead of DMSO, and performing the colorimetric reaction on distilled formaldehyde, we found a percentage of demethylation (see Tables 2 and 3) ranging from about 20 to 60 per cent.

Some hint to explain the observed discrepancy derives from the experiments described in Table 3. When the triazenes were preincubated with either 10,000-*g* supernatant or microsome before adding the NADPH-generating system, there was a decrease in the per cent of demethylation. This effect is less evident with the 10,000-*g* supernatant than with microsomes, which provides a 4-fold purified *N*-demethylation system. Since the inhibitory effect of preincubations is greater the shorter the half-life of the drugs

Table 2. Demethylation of substituted aryl triazenes by rat liver 10,000-*g* supernatant or microsomes

Drug	10,000-*g* supernatant	Microsomes
DM-H	46·8 ± 4·05 (3)	34·6 ± 5·05 (3) 44·2*
DM-OCH_3	55·7 ± 7·17 (3)	32·2 ± 5·35 (3)
DM-$CONH_2$	48·6 ± 5·15 (4)	42·5 (1)
DM-CH_3	58·5 ± 4·74 (6)	60·0 ± 4·81 (6) 85·4*
DM-COOH	22·6 (1)	18·9 (1)
DM-COOEt	26·7 ± 5·1 (4)	20·1 (1)
$HN(CH_3)_2$	1·9 (2)	—
MM-CH_3	4·2 (2)	0·7 (2)

Each value is the mean ± S.E., expressed as per cent of the drug demethylated in 1 hr (number of determinations in parentheses).

* The formaldehyde assay was performed after distillation as described by Audette *et al.*[13], on half scale.

Table 3. Effects of preincubation on the demethylation of aryl triazenes, dissolved either in DMSO or ethanol

Drug	Solvent	10,000-*g* supernatant (−)*	10,000-*g* supernatant (+)	Microsomes (−)	Microsomes (+)
DM-H	EtOH	68·4	56·6	41·8	31·0
	DMSO	45·1	45·0	29·6	28·9
DM-CH_3	EtOH	75·1	45·3	59·7	18·7
	DMSO	64·6	58·4	56·1	19·2
DM-OCH_3	EtOH	42·2	16·2	26·1	1·65
	DMSO	42·6	21·7	26·9	0·70
DM-COOH	EtOH	20·5			
DM-COOEt	EtOH	22·4			
DM-$CONH_2$	EtOH	62·8			
$HN(CH_3)_2$	EtOH	1·3			

0·5 μmoles of the drug in 100 μl. of DMSO or 10 μl. of ethanol (EtOH) were added to 1·4 (1·49) ml of the phosphate buffer and, when indicated, preincubated for 30 min at 37°. After completion of the reaction mixture, the per cent demethylation was determined after 60 min.

* (−): no preincubation; (+) preincubation).

is [14], it is likely that the diazonium salts, formed by spontaneous hydrolysis of the triazenes, are responsible for this impairment of the enzyme system. Evidence in favour of this hypothesis also derives from the experiments shown in Fig. 1, where the direct effect of two diazonium salts has been tested on the *N*-demethylation of DM-$CONH_2$. Both diazonium salts tested exerted an inhibitory activity, although of different degree, the inhibition being more pronounced when using microsomes than with 10,000-*g* supernatant. The results so far described would suggest that a partial hydrolysis of the drug before the experiments could seriously affect a following assay. In addition, the diazonium generated during the assay by hydrolysis of short lived molecules could affect the final results, particularly in microsomal preparations.

The formation of diazonium salt can not fully account however, for the observed inhibitory effects: in fact, the rate of demethylation of DM-COOEt and DM-CH_3, more stable than DM-OCH_3*, also levels off after a few minutes of incubation (Fig. 2). To understand this result better, we have studied the demthylation in 10-min incubations, following an exposure of the enzyme system to either the dimethyl triazenes itself or to the corresponding monomethyl triazenes. The results in Table 4 show that with a triazene with a moderate rate of demethylation, such as DM-COOEt, a previous incubation of either 10,000-*g*

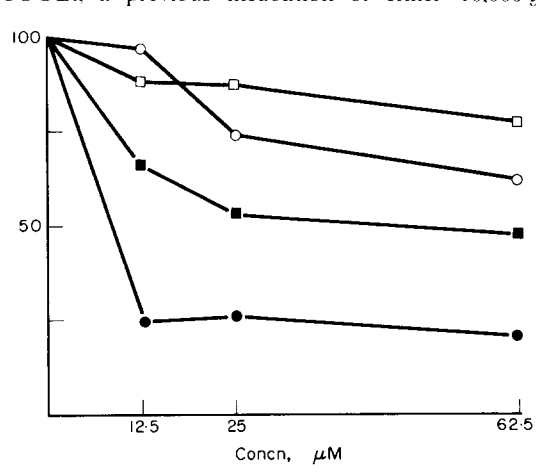


Fig. 1. Effects of two diazonium salts on the demethylation of DM-$CONH_2$. Each value represents the per cent demethylation of 125 μM DM-$CONH_2$ in 1 hr, in the presence of different concentrations of *p*-methoxyphenyldiazonium tetrafluoborate (● ○) or *p*-carboxamidophenyldiazonium tetrafluoborate (■ □), by 10,000-*g* supernatant (○ □) or microsomes (● ■).

* For this substance the half-life at pH 7·4 and 37° is 17·14 min: the reported value at pH 7 and 37° was 11·0, and for DM-CH_3 and DM-COOEt was 37·0 and 5·0 × 10^4 min respectively [14].

Table 4. Effects of preincubation with triazene derivatives on the subsequent demethylation of an added dimethyl triazene

10,000-g supernatant Incubations			Microsomes Incubations		
0–10 min	10–20 min	% Demethylation	0–10 min	10–20 min	% Demethylation
DM-OOEt		17·1	DM-COOEt		16·4
	DM-COOEt	14·7		DM-COOEt	17·8
DM-COOEt	DM-COOEt	26·2	DM-COOEt	DM-COOEt	34·2
			MM-COOEt		2·0
			MM-COOEt	DM-COOEt	17·5
			MM-COOEt + DM-COOEt		17·5
$DM\text{-}CH_3$		63·5			
	$DM\text{-}CH_3$	30·6			
$DM\text{-}CH_3$	$DM\text{-}CH_3$	77·1			
$MM\text{-}CH_3$	$DM\text{-}CH_3$	17·9			
$DM\text{-}OCH_3$		50·3			
	$DM\text{-}OCH_3$	31·8			
$DM\text{-}OCH_3$	$DM\text{-}OCH_3$	70·9			

The triazenes were added to the reaction mixture, containing the cofactors and incubated at 37°, either at time 0 or 10 min; the reaction was stopped 10 min after the last addition.

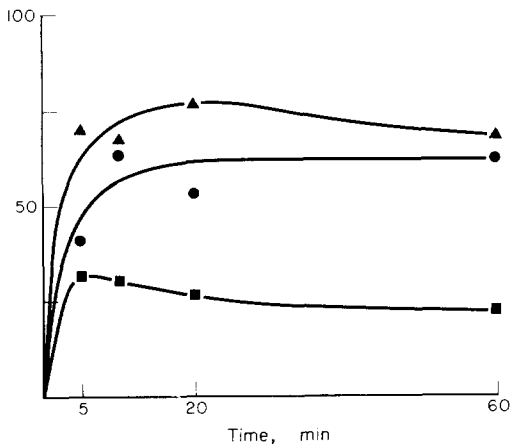


Fig. 2. Time-course of demethylation of substituted aryl triazenes by 10,000-g supernatant. Each value represents the per cent demethylation of 0·5 μmoles of DM-COOEt ■, $DM\text{-}CH_3$ ●, $DM\text{-}OCH_3$ ▲, at the time indicated.

supernatant or microsomes with the drug itself or with its monomethyl derivative is without effect. With $DM\text{-}OCH_3$, two additions of the drug to the same assay system give a demethylation per cent, which is somehow lower than the number calculated by summing up the demethylation per cent obtained with single additions. Finally, with $DM\text{-}CH_3$ the results are more complicated. First, the preincubation of the enzyme system alone produces a 50 per cent reduction of the demethylation of the drug subsequently added. Further, a preincubation of the 10,000-g supernatant with either the drug itself or its monomethyl derivative results in an inhibition of its demethylating capacity, corresponding respectively to 84 and 57 per cent of the controls.

In conclusion, the data described so far cannot explain the discrepancy between the demethylation figures for $DM\text{-}CH_3$, DM-COOH and DM-COOEt obtained by us and by other authors. The fact that three triazene derivatives, already reported to possess antitumour activity *in vivo*, have now been shown to undergo oxidative *N*-demethylation *in vitro*, supports the hypothesis put forward by several authors [16, 18–20] that this metabolic pathway is generally involved in the activity of these compounds *in vivo*.

As far as the time-course of demethylation *in vitro* is concerned, the lack of a complete block of the enzymatic system due to diazonium or monomethyl triazene derivatives, observed in these experiments, cannot explain the occurrence of demethylation only in the first minutes of incubation (Fig. 2). Other enzymatic reaction(s) are thus likely to occur on the intact drug, competing with the demethylation process, and transforming the triazene into derivatives uncapable of undergoing demethylation.

All these data indicate the impossibility of expressing kinetics figures correctly related to the capacity of triazenes to behave as substrate for microsomal demethylation. They also indicate the need for a more detailed examination of the reactions occurring *in vitro* by hepatic microsomal preparations, and their relevance with the activity of this class of drugs *in vivo*.

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