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MECHANISM OF THE MICROSOMAL DEMETHYLATION OF 1-ARYL-3,3-DIMETHYLTRIAZENES

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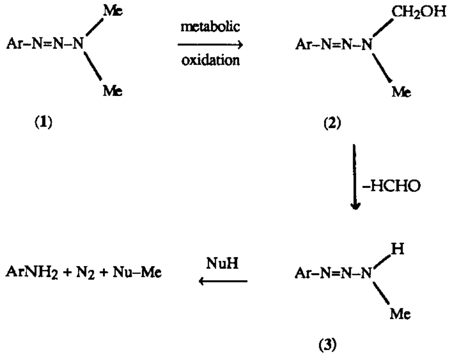
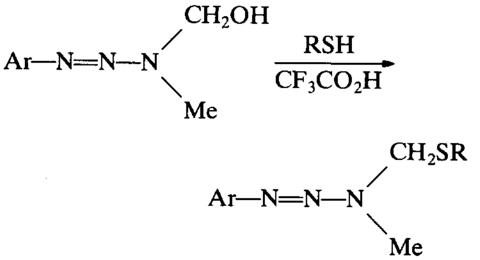
Abstract—The metabolism of 1-aryl-3,3-dimethyltriazenes by phenobarbital-induced rat liver microsomes results in the formation of 1-aryl-3-methyltriazenes and formaldehyde. Intermolecular kinetic deuterium isotope effects for the reaction are found to be 1.0 (±0.03) in both $V_{\max}$ and $V_{\max}/K_m$, respectively. The intramolecular kinetic deuterium isotope effects in $V_{\max}$ and $V_{\max}/K_m$ are found to be 4.8 (±0.05). There is no correlation of $V_{\max}$ or $V_{\max}/K_m$ with calculated ionization potentials of the triazenes. For 3-ethyl-3-methyltriazene comparison of values of $V_{\max}$ and $V_{\max}/K_m$ for ethyl vs methyl loss give rise to values of 3.68 in $V_{\max}$ and 2.02 in $V_{\max}/K_m$. Thus, loss of an ethyl group is favoured. The results are discussed in terms of a mechanism in which the triazene undergoes direct hydrogen atom abstraction to form a carbon centred radical. This radical subsequently forms a hydroxymethyltriazene that collapses to formaldehyde and a monomethyltriazene.

Dialkyltriazenes (**1**) display considerable cytotoxicity [1] but require metabolic activation to exert their cytotoxic action [2]. Preussman *et al.* originally proposed that dialkyltriazenes underwent microsomal oxidation to form an hydroxyalkyl derivative (**2**) that could lose an aldehyde spontaneously to generate the corresponding monoalkyltriazene (**3**) (Scheme 1) [3]. This monoalkyltriazene was considereed to be the ultimate cytotoxic species. Certainly, it is an alkylating agent [4] capable of alkylating DNA [5]. Gradually, evidence has accrued that alkyltriazenes are indeed metabolized by the pathway illustrated in Scheme 1. Thus, monoalkyltriazenes (**3**) and their amine decomposition products have been identified from both *in vivo* and *in vitro* metabolic studies [6–8]. Further metabolic and conjugation products of the amines have also been found [1, 6, 9]. Formaldehyde *in vitro* [3], and its *in vivo* metabolite carbon dioxide [5], are known products from metabolism of dimethyltriazenes (**1**). Even the potentially unstable hydroxymethyltriazene (**2**) has been detected in blood and also isolated from urine [10, 11]. Furthermore, a glucuronic acid conjugate of (**2**) is a metabolite excreted in the urine of rats [12]. However, it is clear from the work of Gescher *et al.* that the monomethyltriazene (**3**) cannot be solely responsible for the cytotoxicity of the dimethyltriazenes [13]. Both selective and non-selective cytotoxic agents are formed. The hydroxymethyltriazene is a possible candidate for the selective cytotoxic agent. Independently, it has been suggested that hydroxymethyltriazenes exert their effect via an interaction of the hydroxymethyl group and the nucleophilic sites of DNA/RNA/protein [14]. Indeed, work in our laboratories has shown that in protic, non-aqueous media hydroxymethyltriazenes react with cysteine containing amino acid derivatives and peptides to form just such conjugates (equation 1) [15].



Scheme 1. Proposed pathway for the metabolism of dimethyltriazenes.



$$\text{Ar—N=N—N}\begin{matrix}\text{CH}_2\text{OH}\\ \text{Me}\end{matrix} \xrightarrow[\text{CF}_3\text{CO}_2\text{H}]{\text{RSH}} \text{Ar—N=N—N}\begin{matrix}\text{CH}_2\text{SR}\\ \text{Me}\end{matrix} \qquad (1)$$

RSH = cysteine, *N*-acetylcysteine, glutathione.

Whatever the precise nature of the cytotoxic agent(s) involved, it still remains that the mechanism by which dimethyltriazenes (**1**) are transformed to hydroxymethyltriazenes (**2**) is unknown. As dimethyltriazenes have useful anticancer activity, an understanding of this process could prove central to the design of newer triazene pro-drugs since it is known that dimethyltriazene pro-drugs are poorly

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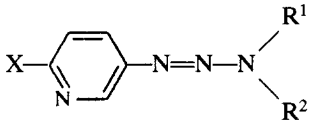
metabolized by humans [16]. We have therefore undertaken a study, involving kinetic deuterium isotope and structural effects, complementing the earlier study of Godin *et al.* [17], that attempts to identify the molecular processes involved in the conversion of (**1**) to (**2**). The compounds employed for the present study were (**4**; **a–e**)



(**4**)

	X	R^1	R^2
(**a**)	H	CH_3	CH_3
(**b**)	H	CD_3	CD_3
(**c**)	H	CH_3	CD_3
(**d**)	MeO	CH_3	CH_3
(**e**)	H	CH_3	C_2H_5

MATERIALS AND METHODS

Substrates. 1-(Pyridin-3-yl)-3,3-dimethyltriazene (**4a**), 1-(pyridin-3-yl)-3,3-di(deuteriomethyl)triazene (**4b**), 1-(pyridin-3-yl)-3-ethyl-3-methyltriazene (**4e**) and 1-(2-methoxypyridin-5-yl)-3,3-dimethyltriazene (**4d**) were synthesized from the parent dimethylamines and pyridine-3-diazonium ion in the usual manner [18] except that the compounds were purified by column chromatography. 1-(Pyridin-3-yl)-3-deuteriomethyl-3-methyltriazene (**4c**) was synthesized from the corresponding monomethyltriazene in the following way. The monomethyltriazene (100 mg) was dissolved in DMF (10 mL) under N_2 at room temperature. Sodium hydride (1.2 mol equiv.) was added and when the evolution of H_2 had subsided iododeuteriomethane (1.2 mol equiv.) was added and the solution stirred for 45 min. Water (5 mL) was then added and the reaction mixture extracted with chloroform (2 × 10 mL). The extracts were dried ($MgSO_4$), evaporated and subjected to chromatography on silica using ether-hexane (1:1) as eluant to remove the contaminating 1-methyl-3-deuteriomethyl-3-(pyridin-3-yl)triazene. The product, which was an oil, had $\delta(CDCl_3)$ 3.35(3*H*,s), 7.16(1*H*,dd,*J* = 8.5, 5 Hz), 7.62(1*H*,dt,*J* = 8.5, 2 Hz), 8.30(1*H*,dd,*J* = 5, 2 Hz), 8.64(1*H*,d,*J* = 2 Hz) and *m/z* 153, 106, 78. The intensity of the *m/z* 153 and 150 peaks, 82 and 0.1%, respectively, indicated that the compound had >99.88% incorporation of a monodeuteriomethyl group.

Microsomes. Rat liver microsomes were obtained from 10-week-old male Wistar rats that had been fed drinking water containing phenobarbital (0.05%) for 2 weeks prior to killing. Protein determination was carried out using the method of Hartree [19]. Cytochrome P450 analysis was performed by determining the absorbance at 450 nm after passing carbon monoxide through the sample of the microsomes. Cytochrome P450 levels varied between 1.32 and 2.3 nmol/mg protein.

Products. Products of the microsomal oxidation were determined in one of two ways.

(1) The triazenes were analysed, after quenching 0.5 mL aliquots of the reaction mixture by the addition of ethanol (1 mL), using an HPLC system that employed a C8 reversed phase column eluting with acetonitrile/methanol/water (2:3:5) buffered to pH 6.5 with 0.025 mol/dm³ ammonium acetate.

(2) Formaldehyde, its deuteriated isotopomers, and acetaldehyde were identified by conversion to their 2,4-dinitrophenylhydrazones. To an aliquot (1.5 mL) of the reaction mixture was added 0.5 mol/dm³ HCl (0.5 mL) followed by 0.022 mol/dm³ 2,4-dinitrophenylhydrazine (2 mol equiv. based on total triazene present). The samples were allowed to stand for 1 hr, after which time they were extracted with diethyl ether (2 × 2 mL). The ether was evaporated, the residue taken up in acetonitrile (250 μL) and analysed by HPLC using a C8 reversed phase column with an acetonitrile/water (48:52) eluant.

Reaction kinetics. Microsomes (protein content *ca.* 13 mg) and substrate (0.1–2 mmol/dm³) were incubated at 37° in pH 7.4, 0.05 mol/dm³ phosphate buffer (2 mL). Reactions were initiated by the addition of an NADPH generating system (1.25 mg NADP, 9 mg glucose-6-phosphate, 5 mg $MgCl_2$ in 0.5 mL, 0.05 mol/dm³, pH 7.4, phosphate buffer to which 3.5 μL of a glucose-6-phosphate dehydrogenase solution containing 500 units/dm³ had been added). For the dimethyltriazenes, aliquots (0.5 mL) were withdrawn at timed intervals and quenched by the sequential addition of 5% $ZnSO_4$ (1 mL) and 5% $Ba(OH)_2$ (1 mL). After centrifugation, the supernatant was analysed for formaldehyde by the Nash procedure. Alternatively, for the 3-ethyl-3-methyltriazene (**4c**), aliquots were quenched as described earlier (i.e. using HCl) and analysed for formaldehyde and acetaldehyde via their 2,4-dinitrophenylhydrazone derivatives. Such analysis requires the use of an internal standard, and the 2,4-DNP derivatives of propanal or butanal were employed. These were added (25 μL, 10 mmol/dm³) to the incubate after the addition of 2,4-dinitrophenylhydrazine. Quantitiation of the formaldehyde and acetaldehyde 2,4-DNP derivatives was carried out using the HPLC system described above, monitoring at λ 350 nm, and adjusting peak areas for the differing extinction coefficients (HCHO 17290; CH_3CHO 18457; C_2H_5CHO 19620 and C_3H_7CHO 19680 dm³/mol/cm). Standard calibration curves indicated that the system accurately assays the formaldehyde and acetaldehyde 2,4-DNP derivatives.

Mass spectral assay for HCHO *and* DCDO. 1-(3-Pyridinyl)-3-deuteriomethyl-3-methyltriazene (**5c**) was subjected to microsomal oxidation as described above. Aliquots (1 mL) were taken and quenched by the addition of 0.5 M hydrochloric acid (0.5 mL). After centrifugation at 2000 rpm for 30 min, a twofold excess of 2,4-dinitrophenylhydrazine (based on the original concentration of triazene) in acidic methanol was added to the supernatant and left to stand for 1 hr. The solution was extracted with diethylether (2 × 2 mL), and the ethereal extract concentrated and subjected to micro-column chromatography on silica using first hexane followed by hexane-diethylether (1:1). The pooled fractions containing for-

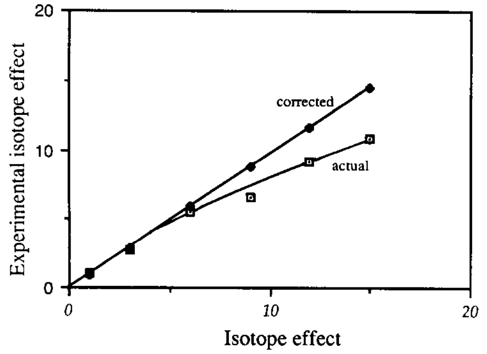


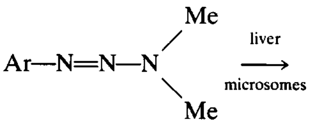
Fig. 1. Mass spectral calibration curve for the 2,4-DNP derivatives of HCHO and DCDO.

maldehyde 2,4-DNP were assayed for purity by HPLC (using the conditions described above) and also GC [BPS (25 m), start temperature 150° then 8°/min up to 220°]. Isotopic composition in the purified formaldehyde 2,4-DNP derivatives was determined using direct probe EIMS by comparison of the two molecular ion intensities at *m/z* 212 (DCDO) and 210 (HCHO). Mass spectra of the separate HCHO-2,4-DNP and DCDO-2,4-DNP derivatives (synthesized independently as standards) displays a small $(M+2)^+$ peak and no $(M-2)^+$ peaks. Thus, in mixtures of the two isotopomeric 2,4-DNPs, the $(M+2)^+$ peak of HCHO-2,4-DNP will interfere with the molecular ion peak of DCDO-2,4-DNP. That this is of importance can be seen in Fig. 1, where gravimetrically prepared mixtures of HCHO and DCDO 2,4-DNPs (HCHO-DNP always in excess as expected for an isotope effect > 1) were analysed by the mass spectral method. Without correction for this $(M+2)^+$ peak of HCHO-2,4-DNP, the isotopic composition of the mixture is underestimated by mass spectrometry. With correction (relative intensities of *m/z* 210 and 212 peaks for HCHO-2,4-DNP are 69.1 and 1.8%) a calibration graph of unit slope is obtained.

Molecular orbital calculations. Ionization potentials for 1-aryl-3,3-dimethyltriazenes were calculated using the semi-empirical AM1 SCF MO program within the MOPAC 4 package [20]. All structures were geometry optimized using the Broyden–Fletcher–Goldfarb–Shanno procedure. Computations were performed on a VAX cluster.

RESULTS AND DISCUSSION

Microsomal metabolism of dimethyltriazenes (**4**; **a–d**) results in the formation of the corresponding monomethyltriazene and formaldehyde (equation 2).



$$\mathrm{Ar{-}N{=}N{-}N(Me)_2} \xrightarrow[\text{microsomes}]{\text{liver}} \mathrm{Ar{-}N{=}N{-}NHMe + HCHO}. \qquad (2)$$

Triazene (**4e**) gives rise to both acetaldehyde and formaldehyde. In no case were we able to detect the hydroxymethyltriazene (**2**) as an intermediate (even though for the 3-pyridinyl system this compound can be assayed by HPLC [21]). Reactions were monitored by following both the release of HCHO and the formation of the monomethyltriazene (**3**). The rate of formation of formaldehyde was found to be linear up to *ca.* 10 min. Therefore, we chose to measure the rate of demethylation using the initial 10 min period. Other workers have generally used longer periods, even though non-linearity of formaldehyde production after *ca.* 10 min has been previously described [22]. A plot of the initial rate, V_i, vs [substrate] for (**4a**) is shown for both HCHO and monomethyltriazene formation in Fig. 2. It is clear that the amount of monomethyltriazene assayed is *ca.* 75% that of the formaldehyde. This is not surprising, since 1-(3-pyridinyl)-3-methyltriazene is unstable in 0.05 mol/dm^3, pH 7.4, phosphate buffer, decomposing with $T_{1/2}$ *ca.* 41.5 min [23]. In general, therefore, we consider the formaldehyde assay to be more reliable, and all data described below refer to this assay. In contrast to the curvature of the V_i vs [substrate] plot in Fig. 2, the initial rate varies linearly with the quantity of liver microsomes used in each incubation (Fig. 3) as expected for an enzyme-catalysed reaction operating under

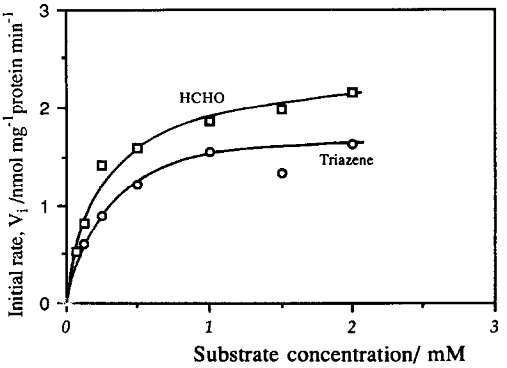


Fig. 2. Plot of initial rate, V_i, vs substrate concentration for (**4a**).

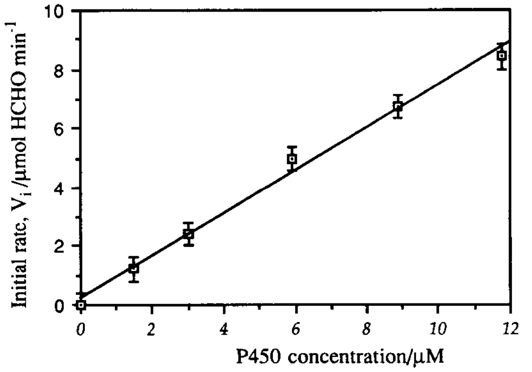


Fig. 3. Dependence of the initial rate for the demethylation of (**4a**) with the microsomal P450 content.

Table 1. V_{max} and V_{max}/K_m values for the metabolism of triazenes by rat liver microsomes at 37°, pH 7.4

Triazene	V_{max} (nmol/mg/min)	Isotope* effect	V_{max}/K_m (nmol/mg/min/mM)	Isotope* effect
4a	2.4		11.4	
4b	2.33	1.03	11.7	0.97
4c	2.37		10.2	
	1.96		8.33†	
	0.41	4.8	1.72‡	4.85
4d	3.56		9.52	
4e	2.06		9.1	
	1.62		9.7§	
	0.44	3.68	4.8†	2.02

* For **4e** the relative loss of ethyl vs methyl. † Loss of HCHO. ‡ Loss of DCDO. § Loss of MeCHO.

Michaelis–Menten conditions. Interpolation of the data in Fig. 2 by the Hanes method gives rise to the V_{max} and V_{max}/K_m values in Table 1. Corresponding values obtained from similar plots for compounds (**4**; **b–d**) [that for (**4c**) is shown in Fig. 4] are also included in Table 1. Several observations are worthy of note. First a comparison of the data for (**4a**) and (**4b**) shows there is no observable intermolecular kinetic deuterium isotope effect in V_{max} or V_{max}/K_m. Not surprisingly, compound (**4c**) has very similar global kinetic constants to compounds (**4a**) and (**4b**). Second, despite the fact that there is no kinetic deuterium isotope effect observed in the rate of demethylation, as far as product formation is concerned there are clearly isotope effects in V_{max} and V_{max}/K_m of 4.8 and 4.85, respectively. Third, an electron-donating substituent in the pyridyl ring has little effect on either V_{max} or V_{max}/K_m. Since Godin *et al.* investigated the substituent effect for the benzene series of 1-aryl-3,3-dimethyltriazenes in some detail, we did not pursue this further [17]. However, we note that we observe a similar effect; that is, aryl ring substituents have little influence on the rate of metabolism of dimethyltriazenes.

Microsomal dealkylation of compound (**4e**) yields formaldehyde and acetaldehyde. However, monitoring the reaction with time shows that whereas formaldehyde liberation continues to increase (albeit more slowly as time proceeds), acetaldehyde formation reaches a maximum within 10 min and thereafter decreases (Fig. 5). Though we did not pursue the biochemical reasons for this decrease, this observation has important ramificiations in deducing the preference for dealkylation vs demethylation. For example, Wilman *et al.* examined the extent of demethylation vs dealkylation for a series of 1-aryl-3-alkyl-3-methyltriazenes [24]. Upon incubation for 60 min it was found that demethylation contributed 47 (±6)%, and dealkylation 53 (±6)%, of the total alkyl group metabolism for alkyl groups ranging from ethyl to dodecyl. Our results indicate that the time elapsed before quantitation is crucial to the results obtained and the deductions reached. Using the first 10 min of incubation we were able to determine initial rates for both methyl and ethyl loss. Plots of V_i for both types of dealkylation exhibit saturation from which V_{max} and V_{max}/K_m values given in Table 1 were determined. From these, we conclude that loss of an ethyl group is 2–4 times more likely than loss of a methyl group, depending on the substrate concentration, rather than the almost equal probability observed by Wilman *et al.* [24]. In all probability the same is true for other alkyl groups, and it is clear that a proper kinetic study is required before

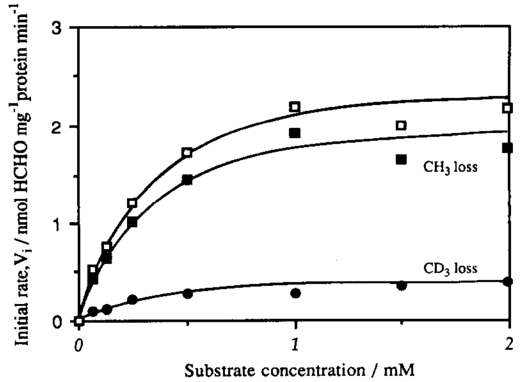


Fig. 4. Plot of initial rate, V_i, vs substrate concentration for (**4c**).

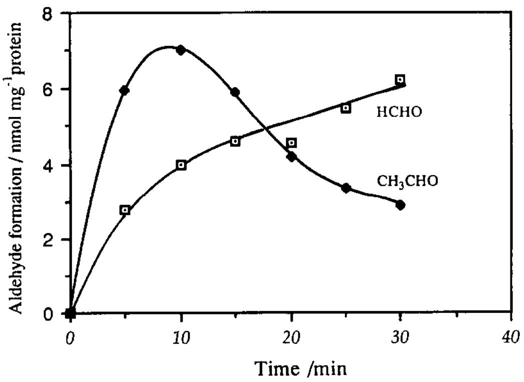
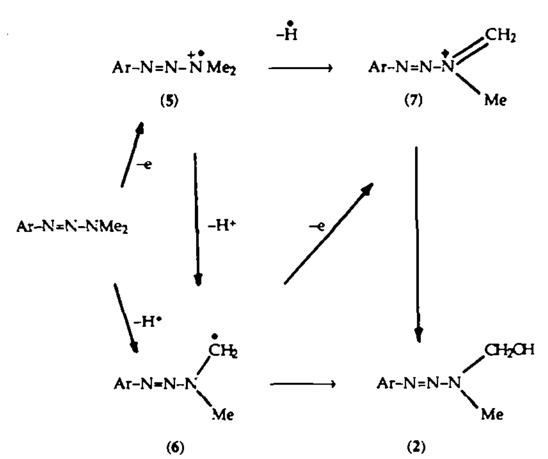


Fig. 5. Time course for the production of HCHO and CH$_3$CHO by the microsomal metabolism of (**4e**).



Scheme 2. Potential pathways for the microsomal oxidation of dimethyltriazenes.

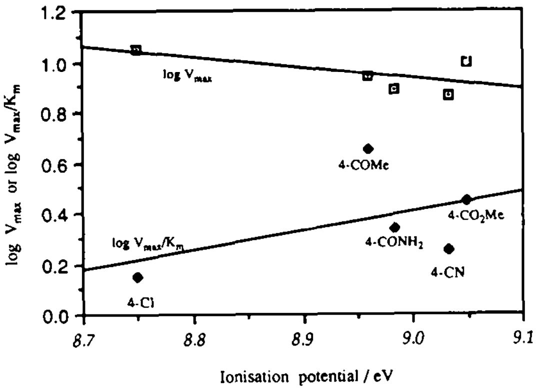
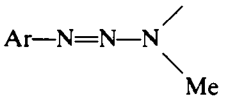


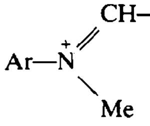
Fig. 6. Plots of log V_{max} and log V_{max}/K_m for the demethylation of dimethyltriazenes vs the ionization potential of the triazene.

any deductions may be made regarding the relative rates of metabolism of methyl vs alkyl groups.

Our results, together with those of Godin *et al.* [17], enable us to outline a mechanism for the conversion of the dimethyltriazene (**1**) to the hydroxymethyltriazene (**2**). Like the analogous dimethylamines [25], potential pathways involve electron transfer to form a radical cation (**5**) or direct hydrogen atom abstraction to form the carbon centred radical (**6**) (Scheme 2). The radical cation may lose H^+, to form the radical (**6**), or $H^•$ to form the iminium ion (**7**). Both (**6**) and (**7**) may interact with the appropriate haemhydroxyl complex to form the hydroxymethyltriazene (**2**). Miwa has identified the criteria for H^+ or $H^•$ loss; H^+ loss is associated with an intrinsic kinetic deuterium isotope effect <*ca.* 3.6 [25]. We observe an intramolecular isotope effect of 4.8 (an intramolecular isotope effect is a good approximation to, but less than, the intrinsic isotope effect for a reaction [26]) from which we deduce that the hydrogen is lost as $H^•$. Whether this $H^•$ is lost from the substrate (**2**) or the radical cation (**5**) can be ascertained from substituent effects. For the analogous dimethylanilines direct hydrogen atom abstraction from substrate is associated with a Hammett ρ value of *ca.* 0, whereas formation of a radical cation is associated with a Hammett ρ value between −0.6 and −1.0 [27–29]. The data of Godin *et al.* for the demethylation of 1-(substituted phenyl)-3,3-dimethyltriazenes reveal that there is little direct correlation, if any, between log V_{max} or log V_{max}/K_m and the Hammett σ substituent constant [17]. Furthermore, a plot of log V_{max} or log V_{max}/K_m vs the ionization potential calculated by the semi-empirical AM1 procedure (Fig. 6) clearly shows that these kinetic constants are largely independent of the ability of the triazene to lose an electron. In contrast, biomimetic demethylation of dimethyltriazenes using tetraphenylporphyrinato-manganese III chloride-iodosobenzene, which does involve the formation of a triazene radical cation, yields a Hammett ρ value of *ca.* −1.3 [30]. Thus, we exclude the radical cation (**5**) as an intermediate in the microsomal reaction and conclude that the metabolism of dimethyltriazenes involves formation of the carbon centred radical (**6**) via direct hydrogen atom transfer. Unfortunately, our results are unable to identify whether the radical (**6**) undergoes subsequent electron transfer to form the iminium ion (**7**). The preferential loss of an ethyl group is consistent with both intermediates; formation of an electron deficient carbon centred radical (**8**) or iminium ion (**9**) will be favoured over their counterparts (**6**) and (**7**) because of the electron donating effect of the methyl group.



(**8**)



(**9**)

The differential effects observed for the loss of an ethyl group, i.e. 3.68 in V_{max} but 2.02 in V_{max}/K_m, suggests that at saturation the substrate preferentially loses an ethyl group but that at low substrate concentrations the substrate binds in such a way that loss of the methyl group is somewhat more favoured (though not enough to overcome the preferential loss of ethyl). Thus, it is clear that the extent of demethylation vs deethylation, is dependent on both the time after which the assay is carried out and also the substrate concentration employed.

Work is in hand to probe this mechanism further.

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