Biochemical Pharmacology, Vol. 18, pp. 1–13. Pergamon Press. 1969. Printed in Great Britain

MECHANISM OF CARCINOGENESIS WITH 1-ARYL-3,3-DIALKYLTRIAZENES. ENZYMATIC DEALKYLATION BY RAT LIVER MICROSOMAL FRACTION *IN VITRO*

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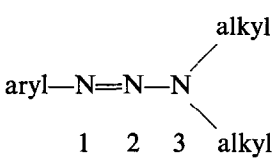
(*Received* 22 *April* 1968; *accepted* 5 *July* 1968)

Abstract—16 Aryl-dialkyltriazenes have been investigated as substrates for *N*-dealkylation by microsomal fraction from rat liver *in vitro*. The potent carcinogens 1-phenyl-3,3-dimethyltriazene (I), 1-(pyridyl-3)-3,3-dimethyltriazene (II) and several other triazenes were oxydatively dealkylated to form the corresponding aldehyde. In the case of (I) aniline was also shown to be formed as metabolite. The results suggest that carcinogenic aryldialkyltriazenes are dealkylated to form aryl-monoalkyltriazenes as proximate carcinogens. Arylmonoalkyltriazenes are known alkylating agents and carcinogenic activity of triazenes can best be explained by alkylation of biopolymers. A mechanism of action is proposed.

1-PHENYL-3,3-dimethyltriazene (I) is a potent carcinogen in BD-rats, producing after oral as well as subcutaneous application mainly malignant tumours of the brain and nervous tissue (neurotropic action).[1] Certain other 1-aryl-3,3-dialkyltriazenes of the general formula



are also carcinogenic in rats, as has been demonstrated in extensive work on structure–activity relationships in this group of compounds (unpublished).[2] Many triazenes are carcinogenic even after application of a single dose of carcinogen. In rats (I) is a powerful teratogen.[3, 4]

Certain triazenes have technical importance as intermediates in the so-called "Rapidogen" dyeing process.[5] Other triazenes have been evaluated as carcinostatic agents on transplanted animal tumours.[6–9] Aryldialkyltriazenes in patents have been claimed as rodent repellents and herbicides.[10]

In acid medium aryldialkyltriazenes are easily hydrolysed to form aryldiazonium salts and secondary amine. For this reason triazenes have been tested primarily by us for carcinogenic activity of diazotating and/or arylating agents. Methylphenylnitrosamine[11] and phenylnitrosourea,[12] forming probably phenyldiazonium ion as reactive intermediate, are carcinogenic. However, oral application of (I) did not

produce "local" tumours in the stomach, where the acid medium should easily hydrolyse the triazene; subcutaneous injection induced only very few sarcomas at the site of application. (I) therefore is a typical resorptive carcinogen, and activation to form the proximate carcinogen[13] is not effected by a simple chemical scission of the triazene, but very likely by an enzymatic metabolic process.

To investigate this possibility, (I) and other triazenes were incubated with isolated microsome fractions of rat liver and some other organs in the presence of a NADPH-generating system. It could be demonstrated that certain aryldialkyltriazenes are dealkylated by drug metabolizing enzymes[14] of this system.

MATERIALS AND METHODS

Substrates. The aryldialkyltriazenes used as substrates were synthesized by known methods: The respective diazotized aromatic amine was coupled with the corresponding secondary amine in the presence of sodium carbonate.[6] (III) (Table 3) was formed by oxidation of (II) (Table 3) with perbenzoic acid. Isolated triazenes were purified by vacuum distillation or by recrystallization. Boiling resp. melting points corresponded to literature values. All used triazenes were pure as shown by TLC. Triazenes show characteristic u.v. spectra with maxima at 225, 285 and 310 nm. In Table 1 those aryldialkyltriazenes are summarized which have not been described in literature to our knowledge.

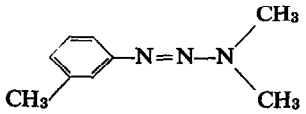
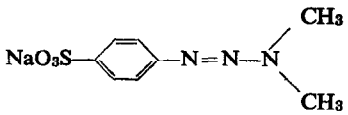
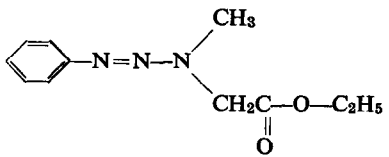
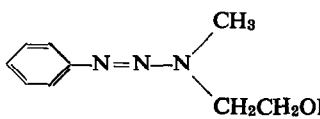
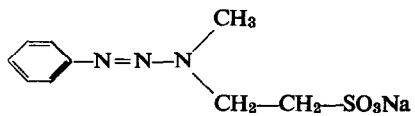
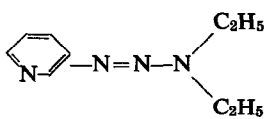
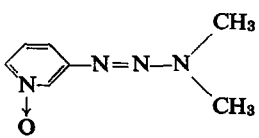
Preparation of rat liver microsomes. Male BD VI or BD IX rats[15] weighing 250–300 g were killed by cervical dislocation, livers were removed at once and rinsed with ice-cold isotonic KCl-solution to remove blood. After blotting and mincing the 1·5-fold of liver weight of 1/15 m phosphate buffer, pH 7·4, was added and homogenized at 0° in a glass homogenisator for 5 min. Oxygen was passed shortly through the homogenate to ensure oxygen saturation, which then was immediately centrifuged at 4° and 10,000 *g* for 30 min. The supernatant was again centrifuged at 4° and 100,000 *g* in a Spinco-ultra-centrifuge, model L. Sedimented microsomes were washed with cold phosphate buffer and finally resuspended in cold buffer using a homogenisator. The protein content of this microsome homogenate was assayed by the microbiuret method described by Itzhaki and Gill[16] using crystalline bovine serum albumin as reference standard.

Microsome fraction from lungs, kidney and brain. Microsomes from kidney and brain were prepared essentially in the same way as liver microsomes. Lungs were intensively rinsed with cold KCl-solution, disintegrated in a knife-homogenisator (starmix) and then homogenized and worked up as described for liver.

Incubation mixture. A quantity of microsomes corresponding to 8 mg microsomal protein was added with: 2 μmole glucose-6-phosphate (Boehringer), 0·4 μmole NADP (Boehringer), 120 B.E. glucose-6-phosphate dehydrogenase (Boehringer) as NADPH-generating system, 50 μmole nicotinamide, 25 μmole $MgCl_2$ and 100 μmole semicarbazide for trapping of aldehydes. Substrate concentration usually was 0·2 μmole triazene in phosphate buffer, pH 7·4. In certain cases substrate concentration was increased to 0·5–2·0 μmole. Total volume of the incubation mixture was 8 ml. Pure oxygen was passed through the mixture for 1 min and it was then incubated in an atmosphere of pure oxygen at 37° for 1 hr with shaking.

Assay procedures. (a) *Formaldehyde.* After incubation and cooling the mixture 4 ml 10% aqueous sulfuric acid were added and the volume was filled up to 25 ml with

TABLE 1. NEW 1-ARYL-3,3-DIALKYLTRIAZENES

Name	Formula	Mole wt.	b.p./mmHg m.p.	Calc. found	Analysis C %	H%	N%
1-(3-methylphenyl)-3,3-dimethyltriazene (XI)		163·21	115/4·2	calc.: found:	66·22 66·41	8·03 8·27	25·74 26·01
1-(4-sulphophenyl)-3,3-dimethyltriazene Na-salt (XVI)		251·25	m.p. 351°		38·24 38·27	4·01 4·12	16·72 16·43
1-phenyl-3-methyl-3-carbethoxymethyltriazene (VIII)		221·25	120/0·4		59·71 59·64	6·83 6·38	18·99 18·93
1-phenyl-3-methyl-3-(2-hydroxyethyl)-triazene (IX)		179·21	128/0·6		60·31 60·71	7·31 7·44	23·95 23·64
1-phenyl-3-methyl-3-(ethylsulphonic-acid)-triazene Na-salt (VI)		265·27	m.p. 230–240 (decomp.)		40·75 40·40	4·56 4·12	15·84 15·82
1-(pyridyl-3)-3,3-diethyltriazene (IV)		178·23	124/0·3		60·66 61·13	7·92 7·54	31·44 31·39
1-(pyridyl-3-N-oxid)-3,3-dimethyltriazene (III)		166·18	m.p. 156–7°		50·58 50·77	6·06 6·18	33·71 34·50

water and distilled. 15 ml of distillate were collected in a cooled volumetric vessel. Colorimetric formaldehyde determination was performed according to MacFadyen[17] with chromotropic acid and measured against a control without substrate. Lower detection limit: 0·01 μmole.

(b) *Acetaldehyde.* 2 ml 10% aqueous sulfuric acid were added to the cooled mixture and filled to a volume of 15 ml and distilled. Colorimetric acetaldehyde determination was performed according to Stotz[18] with 4-hydroxybiphenyl. Lower detection limit: 0·01 μmole.

(c) *Aromatic amine (aniline).* Aniline as metabolite of (I) was estimated by extracting with ether a fourfold incubation mixture after treatment with 2 n NaOH. The combined ether layers were re-extracted with 0·1 m hydrochloric acid and the acid aqueous solution was divided in two parts. The first half was coupled directly with *N*-ethyl-1-naphthylamine for determination of intact triazene.[19] For determination of aniline the second half of the acid extract was diazotized with $NaNO_2$, surplus nitrite destroyed by addition of urea and coupled with *N*-ethyl-1-naphthylamine. The difference of both colorimetric determinations was a measure of aromatic amine present. Appropriate controls were used as references.

RESULTS

Results are summarized in Tables 2–5.

(1) *Demethylation of 1-phenyl-3,3-dimethyltriazene (I)*

As shown in Table 2 phenyldimethyltriazene (I) was oxidatively demethylated with formation of formaldehyde by a microsome fraction from rat liver *in vitro* in the presence of a NADPH-generating system and oxygen ("complete system"). No formaldehyde formation was observed, when the NADPH-generating system was omitted or when a nitrogen atmosphere was used instead of oxygen in the incubation vessel. Neither was formaldehyde found on incubation without microsomes. This is typical for dealkylations by the so-called drug metabolizing enzymes.[14]

One alkyl group taken as unit, the demethylation yield in the medium of 19 experiments was 50 per cent with a rather wide range from 20 to 80% yield in the different experiments. The demethylation rate within one given experiment was quite constant with an error of less than ± 10 per cent. The high differences result from animal to animal, although highly inbred rats[15] were used and care was taken to treat the animals as uniform as possible before killing. D. F. Heath,[20] for example, observed similar differences in microsomal hydroxylase activity in male rats with the carcinogen dimethylnitrosamine as substrate.

Addition of a small quantity (0·25 per cent of the total volume) of ethanol significantly reduced formaldehyde yield. This experiment was necessary, since some of the examined triazines were not enough water soluble and ethanol was used to get clear solutions.

It was possible that the observed formaldehyde production was not the result of a demethylation of intact (I), but of dimethylamine formed by hydrolysis of (I). Therefore 0·2 μmole of dimethylamine were incubated in the complete microsome system. No demethylation was observed. This shows that formaldehyde is formed by demethylation of the intact triazene (I).

As also shown in Table 2 microsome fraction from lung tissue had low activity in

TABLE 2. DEMETHYLATION OF PHENYL-DIMETHYLTRIAZENE WITH MICROSOMAL FRACTIONS OF RAT LIVER, BRAIN, LUNGS AND KIDNEY *IN VITRO*. VARIATION OF INCUBATION SYSTEM. COMPLETE SYSTEM CONTAINED NADP, GLUCOSE-6-PHOSPHATE, GLUCOSE-6-PHOSPHATE-DEHYDROGENASE, NICOTINAMID, Mg^{++} AND SEMICARBAZID AND WAS SHAKEN FOR 1 hr AT 37° IN AN ATMOSPHERE OF OXYGEN. DETAILS IN EXPERIMENTAL SECTION. % YIELD REFERS TO THE DEALKYLATION OF ONE ALKYL GROUP

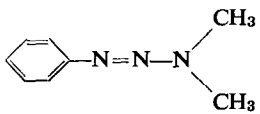
No.	Name formula	Substrate (μmole)	Organ	Incubation system	No. of experiments	μmole formaldehyde found	% yield
I	1-phenyl-3,3-dimethyl-triazene	0·2	liver	complete	19	0·1 (0·03–0·16)	50
		0·2	liver	without NADPH	2	neg.	
		0·2		without microsomes	2	neg.	
		0·2	liver	without oxygen	4	neg.	
		0·2	liver	complete + 0·25 % C_2H_5OH	4	0·04 (0·02–0·06)	20
		0·2	brain	complete	6	neg.	
		0·2	lung	complete	4	0·01 (0·005–0·015)	5
		0·2	kidney	complete	2	neg.	

TABLE 3. DEALKYLATION OF 1-(PYRIDYL-3)-DIALKYLTRIAZENES WITH MICROSOMAL FRACTION OF RAT LIVER *IN VITRO*. COMPLETE INCUBATION SYSTEM AS DESCRIBED IN TABLE 2 AND IN EXPERIMENTAL SECTION. % YIELD REFERS TO THE DEALKYLATION OF ONE ALKYL GROUP

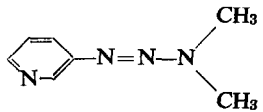
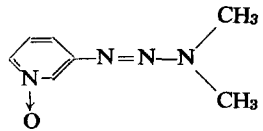
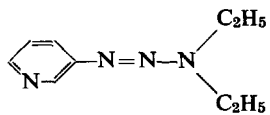
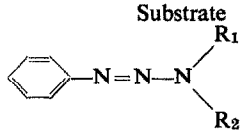
No.	Name	Formula	Substrate (μmole)	No. of experiments	Estimated aldehyde	Micromole aldehyde	% Yield
II	1-(pyridyl-3)-3,3-dimethyltriazene		0·2	6	formaldehyde	0·01 (0·005–0·02)	5
			1·0	1		0·1	10
			2·0	3		0·2 (0·17–0·23)	10
III	1-(pyridyl-3-N-oxid)-3,3-dimethyltriazene		0·2	3	formaldehyde	neg.	—
			1·0	5		0·01 (0·004–0·02)	1
			2·0	2		0·06	3
IV	1-(pyridyl-3)-3,3-diethyltriazene		0·2	6	acetaldehyde	0·09 (0·05–0·13)	45
			1·0	2		0·16	16

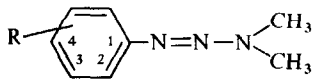
TABLE 4. DEALKYLATION OF 1-PHENYL-3,3-DIALKYLTRIAZENES WITH MICROSOMAL FRACTION OF RAT LIVER *IN VITRO*. VARIATION OF THE ALKYL RESIDUES. COMPLETE INCUBATION SYSTEM AS DESCRIBED IN TABLE 2 AND EXPERIMENTAL SECTION. % YIELD REFERS TO THE DEALKYLATION OF ONE ALKYL GROUP



No.	R_1	R_2	Substrate (μmole)	No. of experiments	Estimated aldehyde	Micromole aldehyde	% Yield
V	$-C_2H_5$	$-C_2H_5$	0·2	5	acetaldehyde	0·11 (0·09–1·13)	55
VI	$-CH_3$	$-CH_2-CH_2-SO_3Na$	0·2	2	formaldehyde	neg.	—
			0·5	3	formaldehyde	neg.	—
VII	$-CH_3$	$-CH_2-CH_2-OH$	0·2	2	formaldehyde	neg.	—
			0·5	2	formaldehyde	neg.	—
VIII	$-CH_3$	$-CH_2-\overset{O}{\overset{\|}{C}}-O-C_2H_5$	0·2	4	formaldehyde	neg.	—
			1·0	1	formaldehyde	neg.	—
IX	$-CH_3$	$-OH$	0·2	2	formaldehyde	neg.	—
			0·5	4	formaldehyde	neg.	—

TABLE 5. DEMETHYLATION OF 1-ARYL-3,3-DIMETHYLTRIAZENES WITH MICROSOMAL FRACTION OF RAT LIVER *IN VITRO*. VARIATION OF THE ARYL RESIDUE. COMPLETE INCUBATION SYSTEM AS DESCRIBED IN TABLE 2 AND IN EXPERIMENTAL SECTION. % YIELD REFERS TO THE DEALKYLATION OF ONE ALKYL GROUP

Substrate



No.	R	Position	Substrate (μmole)	No. of experiments	formaldehyde (μmole)	% Yield
X	$-CH_3$	4	0·2	4	neg.	—
XI	$-CH_3$	3	0·2	7	0·03 (0·028–0·032)	15
XII	$-CH_3$	2	0·2	5	neg.	—
XIII	$-\overset{\overset{O}{\|}}{C}-O-C_2H_5$	4	0·2	4	neg.	—
XIV	$-NO_2$	4	0·2	5	0·17 (0·145–0·195)	85
XV	$-Cl$	4	0·2	5	0·17 (0·145–0·195)	85
XVI	$-SO_3Na$	4	0·2	2	neg.	—
			1·0	3	neg.	—

demethylating (I), while kidney as well as brain did not demethylate the substrate within the detection limit of the formaldehyde assay method.

(2) *Detection of aniline as metabolite from (I)*

Using the method described in the experimental part, aniline was formed after incubation of (I) with liver microsomes. A half-quantitative estimation of aniline showed that approximately equimolar quantities of aniline and formaldehyde were formed. Incubation of phenyldiazonium fluoroborate as substrate with liver microsomes gave no indication of aniline formation. This shows aniline to be a metabolite of (I) and not of phenyldiazonium ion, a possible hydrolysis product of (I).

(3) *Dealkylation of* 1-(*pyridyl*-3)-3,3-*dialkyltriazenes*

1-(Pyridyl-3)-3,3-dimethyltriazene (II) is a potent carcinogen with the same neurotropic carcinogenic action as (I).[2] Likewise this compound was also demethylated, as shown in Table 3. Formaldehyde yields are significantly lower as with (I). With higher substrate concentrations demethylation rate was higher. *N*-Oxidation of the pyridine moiety to form the *N*-oxide (III) gave a compound which was only very weakly demethylated. On the other hand pyridyl-diethyltriazene (IV) was dealkylated to form acetaldehyde in almost the same yield as (I). In this series there is an indication of decreasing dealkylation with increasing water solubility: (III) is 30% soluble, (II) 10% and (IV) 0·5%.

(4) *Variation in the alkyl residue*

Phenyl-diethyltriazene (V), the next higher homologue of (I), was de-ethylated to form acetaldehyde in 55% yield (Table 4). The other derivatives VI–IX, bearing one methyl group and one alkyl group with a functional substituent resp. a hydroxy-group (IX), were not demethylated. The possible dealkylation of the second, substituted alkyl group in (VI)—(VIII) was not investigated.

(5) *Variation in the phenyl residue*

Results of substitution of (I) with functional groups in the phenyl residue are summarized in Table 5. Of all investigated triazenes, the p-nitro-(XIV) and the p-chloro-(XV) derivatives of (I) were the best substrates for dealkylation with a medium formaldehyde yield of 85% of the theory. The sodium-salt of (XVI) (p-SO_3Na) is practically not lipoid soluble and was not demethylated. Also negative was (XIII) (p-Carbethoxy). In the case of the three, *o*-, *m*- and *p*-methyl-substituted derivatives it is striking that only the *m*-methyl compound (XI) was demethylated.

DISCUSSION

The experiments presented here have shown that isolated microsome fraction from liver in the presence of a NADPH-generating system and oxygen dealkylates the carcinogenic phenyldimethyltriazene (I) and certain other triazenes. The dealkylated group is trapped and determined as aldehyde. It has been made certain that dealkylation and aniline formation are not preceded by hydrolysis of (I) to form dialkylamine and phenyldiazonium ion. Dimethylamine is not dealkylated and phenyldiazonium is not reduced to form aniline under the reaction conditions used. Enzymatic degradation therefore occurs on the intact triazene.

The investigation of 16 triazenes has shown a surprising structure specificity for microsomal dealkylation. Variations in the aliphatic as well as in the aromatic part of the triazene molecule have been examined. Generally it can be stated that higher water solubility decreases dealkylation *in vitro*; this has been found also by other authors.[21] In the case of functional groups the reason for decreased dealkylation may also be enzymatic oxidation of the functional group, which takes up the enzymatic activity. The same may be true in the case of the ring methyl-substituted derivatives of (I); we think that enzymatic oxidation of the tolyl group to form a benzoic acid derivative in the case of the *o*- and *p*-methyl-phenyltriazenes (X) and (XII) is possible. The m-derivative (XI) is not oxidized at the ring methyl group but on the aliphatic methyl group in position 3 of the triazene structure. These problems must be investigated in further experiments.

Unfortunately at present we cannot give a quantitative correlation of dealkylation with carcinogenic activity, since some carcinogenesis experiments with triazenes are not yet finished. However it must be emphasized that the potent carcinogens (I) and (II) are dealkylated, and there is experimental evidence of a carcinogenic activity of XV and XVI[2] which are also easily dealkylated.

We are well aware of the difficulties of extrapolating *in vitro* results with isolated microsomal fractions to the intact organ or even to the whole organism. Nevertheless, on the basis of the results presented we feel entitled to propose a mechanism of action for carcinogenesis with phenyldimethyltriazene (I) and other carcinogenic triazenes:

Animal experiments with (I) have indicated that an enzymatic activation of the carcinogen is necessary *in vivo*. We have shown in this paper that dealkylation occurs with microsomal enzymes of the type of drug metabolizing enzymes. There is growing agreement that many of these enzymes are *hydroxylases*.[22] Applied to (I) formation of 1-phenyl-3-methyl-3-hydroxymethyl-triazene (Ib) (Fig. 1) should be the first activation product. Compounds of this type are not known at present, their stability and reactivity can only be compared with similar structures, hydroxymethylurea or methylazoxymethanol[23] for example. In aqueous solution, especially in slightly acid medium, the hydroxymethyl-triazene (Ib) should be hydrolysed more or less readily to give formaldehyde and 1-phenyl-3-monomethyltriazene (Ic).

Phenylmethyltriazene (Ic) is a known compound and can be synthesized. Chemically it reacts like a typical *alkylating agent*. Dimroth described this property as early as 1903 and stated that "phenylmethyltriazene reacts like a stabilized diazomethane".[24] In water it is hydrolysed, especially in acid medium, it methylates carbonic acids and phenols in high yields.[24,25] Succinimide is *N*-methylated,[26] alcohols are methylated to form ethers in the presence of a Al $(O\,CH_3)_3$ catalyst.[26] Acid decomposition of a monoalkyl triazene, 1-(4-Nitrophenyl)-3-carbethoxymethyl-triazene, has been shown recently by Baumgarten[27] to be a preparative method for diazo acetic ester. After reaction of phenylmonomethyl-triazene nitrogen and *aniline* are formed as the only other reaction products.

As has been shown in this paper, aniline is also formed after dealkylation of (I) with liver microsomes *in vitro*. In acute toxicity experiments with several triazenes we have observed methemoglobinaemia *in vivo* (S. Ivankovic, unpublished). This can be explained best with the formation of aniline resp. aromatic amines *in vivo*, known methemoglobin forming compounds.[28]

Phenylmethyltriazene has been tested for carcinogenic activity in rats by our group (unpublished experiments): it is a powerful carcinogen. On oral administration *all* animals died with "local" carcinoma in esophagus and forestomach. Subcutaneous application produced predominantly local carcinoma at the site of injection, but also some neurogenic malignant tumours. This demonstrates that the compound is sufficiently stable to have also distant effects. Compared with (I) phenylmethyltriazene fulfills conditions for a proximate carcinogen:[13] It is more effective than the parent compound and produces predominantly local tumours.

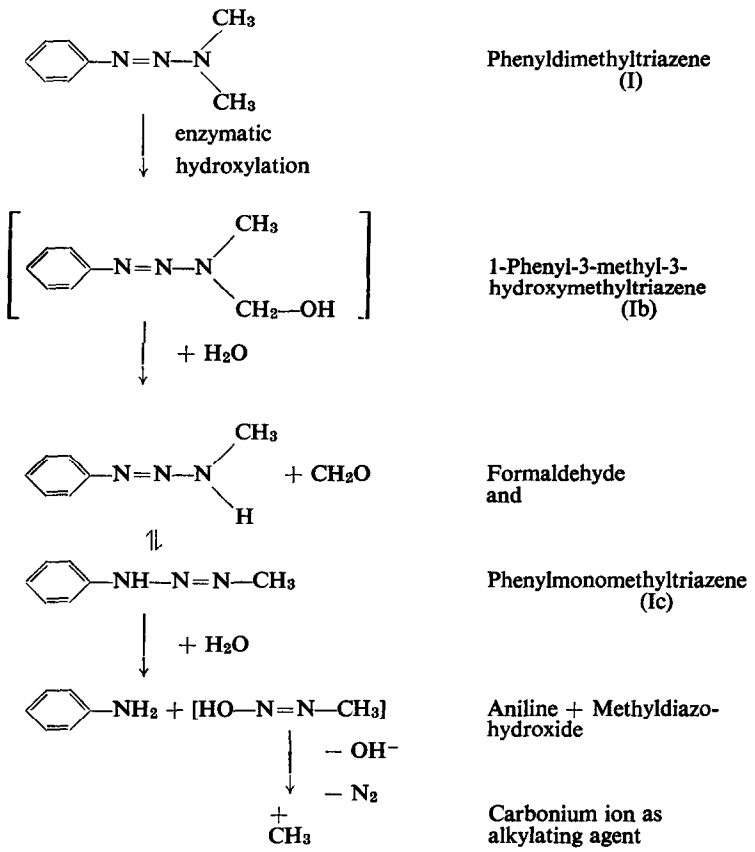


FIG. 1. Proposed activation mechanism of carcinogenic phenyldimethyltriazene to form phenylmonomethyltriazene as proximate carcinogen and carbonium ion as ultimate alkylating agent. Formaldehyde and aniline as metabolites of (I) have been detected in the present study.

Based on the results presented here we feel justified to postulate that aryldialkyltriazenes are dealkylated *in vivo* to form aryl-monoalkyl-triazenes as proximate carcinogens and that the carcinogenic activity of triazenes is caused by *in vivo* alkylation of biopolymers, probably nucleic acids. This places carcinogenic triazenes in one line with other groups of carcinogens, which are metabolized *in vivo* to form alkylating agents: the organic *N*-nitroso-compounds[11,29] and aliphatic hydrazo-, azo- and azoxy-compounds.[29,30] Carcinogenic activity of ethionine can be related

to the ethylating effect of enzymatically formed S-adenosylethionine[31] and the toxicity and carcinogenicity of certain pyrrolizidine alcaloids has recently been shown to be connected with the formation of alkylating agents by Mattocks.[32] Carcinogenic effects of several directly alkylating agents are well known.[13,33,2]

The proposed reaction mechanism does not exclude that certain triazenes may act by a purely chemical heterolysis to form aryldiazonium ions as reactive intermediate. The very few local sarcomas after s.c. injection of (I) may well be explained on this basis.

A main difficulty in the interpretation of the results presented is the following: *In vitro* metabolism of (I) occurs only with liver and lung microsomal fraction (Table 2). In the carcinogenicity experiments on the other hand we never observed tumours in these organs, but always in brain, neurogenic tissue and also in kidney. Microsomal fractions of these organs did not dealkylate *in vitro*. The exact mechanism of organotropism is also unsolved in the groups of organic *N*-nitroso- and aliphatic hydrazo-, azo-, and azoxy compounds, while the general activation mechanism seems to be quite clear.[11,29]

In this relation we want to discuss the following possibilities:

(1) The demonstrated dealkylation *in vitro* of certain carcinogenic triazenes may have nothing to do with carcinogenic action. We think this improbable. All biological, biochemical and chemical data, available at present and discussed in detail above, indicate that the principal activation mechanism is as proposed. Furthermore there is ample evidence that carcinogenic nitrosamines and azo- and azoxy-compounds are activated *in vivo* by similar mechanisms.

(2) The activity of microsomal hydroxylating enzymes in brain and kidney may be much higher in the intact organ than *in vitro* and both organs can dealkylate triazenes *in vivo*. This seems possible, but it does not explain the fact that in liver and lungs *no* tumours have been observed after treatment with (I) or (II).

(3) Activation may be effected not by "drug metabolizing enzymes", which have highest activity in liver, but by "specific hydroxylases" in organs, in which tumours have been observed, and which cannot be assayed by the methods used. We are not aware of experimental evidence for such "specific hydroxylases" if we do not take into consideration highly specific adrenal steroid hydroxylases for example, which metabolize physiologic substrates. Such a hypothesis also does not explain the absence of tumours in liver and lungs, where the substrate is activated even *in vitro*.

(4) Microsomal hydroxylases of liver and/or lungs metabolize the substrate to form a *pre-activated* product and this has sufficient stability and can be transported to other organs, where the final activation takes place. At present we consider this the most probable hypothesis and discuss two possibilities:

(a) The α-hydroxyalkyl-triazene as the first postulated reaction product according to Fig. 1 may have enough stability for a transport to other organs. It even seems possible that it is stabilized by some conjugation process and is transported in conjugated form. Compounds of this type are unknown at present, but determination *in vivo* seems possible and synthesis of compounds of this type are under way.

(b) Monodealkylation occurs in the liver and the formed mono-alkyltriazene is transported to other organs. In order to evaluate such a possibility we have determined the stability of phenylmethyltriazene at 37° and pH 7·4, measuring the nitrogen release in a Warburg apparatus. The half time t_{50} under these conditions is 3·5 min.

Addition of serum to the buffer solution (1:4) almost doubled the half time to 6 min and shows a significant stabilizing effect of serum. This warrants sufficient half life for circulation of the monoalkyltriazene. The fact that we obtained also distant tumours with this compound on subcutaneous application also shows that its stability under physiologic conditions is sufficient for transport phenomena.

Our investigations will be continued in the indicated directions.

Acknowledgements—Our work has been made possible by the Deutsche Forschungsgemeinschaft. We thank Prof. Dr. H. Druckrey for his support and many discussions.

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