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MODULATION OF THE ACCEPTANCE OF TRANSFER RIBONUCLEIC ACIDS FOR AMINO ACIDS BY ALKYL-ARYLTRIAZENES AND IMIDAZOLES

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Abstract—Selected triazene and imidazole compounds were incubated with postmitochondrial supernatants from rat liver, and tRNA isolated from these incubates was charged with thirteen different amino acids. The majority of tested compounds enhanced the acceptance of initiator tRNA but inhibited the formation of L-leucyl-tRNA. No unequivocal results were obtained with the other tRNA species. The direct alkylating monomethyltriazenes enhanced the acceptance of initiator tRNA when incubated alone with unfractionated tRNA whereas the procarcinogenic dialkyltriazenes showed this effect only after preincubation with postmitochondrial supernatant containing the activating enzymes. It appears that the reactive molecular species, that arise from monomethyltriazenes by heterolysis, or from dimethyltriazenes by enzymic activation, modify the structure of tRNA in a specific manner, possibly by a chemical reaction. The enhanced acceptance of initiator tRNA seems to be indicative of the generation of electrophilic (potentially carcinogenic) intermediates from the incubated compounds.

Interactions of chemical carcinogens with nucleic acids are thought to be the decisive prerequisite for the initiation of malignant transformation [1]. Although most intensive efforts have been expended on interactions of carcinogens with DNA, Magee and Farber [2] demonstrated almost twenty years ago that tRNA had a greater propensity for methylation by dimethynitrosamine than DNA. The administration of *N*-acetyl-aminofluorene to rats resulted in a preferential binding of this compound to tRNA [3], and specific modifications in the capacity for amino acid acceptance, codon recognition and ribosomal binding resulted from an *in vitro* interaction of *N*-acetoxy-2-acetylaminofluorene with tRNA [4].

More recently, carcinogenic polycyclic aromatic hydrocarbons were likewise shown to modify initiator tRNA in a specific manner, and the reaction was apparently mediated by enzymes of the microsomal fraction from rat liver [5]. Carcinogenic derivatives of 4-dimethylaminoazobenzene also stimulated the acceptance of initiator tRNA for methionine, and that of some other tRNA species, for their particular amino acids. Microsomal activation of these procarcinogens was invariably required for the manifestation of the enhancing effect. However, 4-hydroxy-4-dimethylaminoazobenzene, the putative active metabolite of 4-dimethylaminoazobenzene, was found to stimulate the acceptance of initiator tRNA without participation of any subcellular components, thus indicating no need of metabolic activation [6]. This finding suggested to us that the direct acting carcinogens, or the electrophilic intermediates (ultimate carcinogens) that arise from them are able to modify tRNA by a specific chemical reaction that leads to a specific change of its function.

In order to test the general validity of this finding, we have used selected acyclic triazene compounds, Ar–N=N–N(R_1R_2), for the present experiments. The reason for this choice was that the carcinogenic triazenes exist in two analogous series that differ only in substitution at 3N: the direct acting monomethyl aryltriazenes (R_1 = methyl, R_2 = H), and the procarcinogenic dimethyl aryltriazenes ($R_1 = R_2 = CH_3$) that require metabolic activation for their predominantly resorptive carcinogenic action. The test series was extended by the inclusion of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide, a clinically used cytostatic drug, and by two related imidazole derivatives.

We present evidence that all tested monomethyl aryltriazenes enhanced the acceptance of tRNA after preincubation without microsomal enzymes whereas the procarcinogenic dimethyl aryltriazenes required for their effect preincubation with, and therefore activation by a hepatic fraction containing microsomes.

MATERIALS AND METHODS

Chemicals and radiochemicals. ATP (sodium salt) was a product of Boehringer (Mannheim, West Germany). L-[^{35}S]Methionine (1155 Ci/mmol) purchased from the Radiochemical Centre (Amersham, U.K.) was diluted with non-radioactive L-methionine to a

Abbreviations used: AIC, 5-aminoimidazole-4-carboxamide; DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; DZIC, 5-diazoimidazole-4-carboxamide; DMPT, 3,3-dimethyl-1-phenyltriazene; DMTBA, 4-(3,3-dimethyl-1-triazeno)benzoic acid; DMTSA (Na salt), 4-(3,3-dimethyl-1-triazeno)sulphonic acid sodium salt; 4-Cl-PDMT, 1-(4-chlorophenyl)-3,3-dimethyltriazene; 4-Br-PDMT, 1-(4-bromophenyl)-3,3-dimethyltriazene; 2,4,6-Cl_3-PDMT, 1-(2,4,6-trichlorophenyl)-3,3-dimethyltriazene; MPT, 3-methyl-1-phenyl-triazene; 4-Cl-PMT, 1-(4-chlorophenyl)-3-methyl-triazene; 4-Br-PMT, 1-(4-bromophenyl)-3-methyltriazene; 2,4,6-Cl_3-PMT, 1-(2,4,6-trichlorophenyl)-3-methyltriazene.

specific radioactivity of 250 mCi/mmol. L-[U-^{14}C]Alanine (120 mCi/mmol), L-[U-^{14}C]arginine (240 mCi/mmol), L-[U-^{14}C]aspartic acid (160 mCi/mmol), [U-^{14}C]glycine (80 mCi/mmol), L-[U-^{14}C]-histidine (240 mCi/mmol), L-[U-^{14}C]isoleucine (240 mCi/mmol), L-[U-^{14}C]leucine (240 mCi/mmol), L-[U-^{14}C]lysine (240 mCi/mmol), L-[U-^{14}C]phenylalanine (360 mCi/mmol), L-[U-^{14}C]threonine (160 mCi/mmol), L-[U-^{14}C]tyrosine (360 mCi/mmol) and L-[U-^{14}C]valine (200 mCi/mmol) were obtained from the Institute for Research, Production and Uses of Radioisotopes (Prague, Czechoslovakia).

Test compounds. 5-(3,3-Dimethyl-1-triazeno)-imidazole-4-carboxamide (DTIC) was generously supplied by Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD), 5-aminoimidazole-4-carboxamide hydrochloride (AIC) was purchased from Heinrich Mack Nachf. (Illertissen, West Germany). 5-Diazoimidazole-4-carboxamide (DZIC) was prepared by diazotization of AIC according to a published procedure [7]. The isolated product decomposed explosively at 205–210° and gave a positive azo coupling test with *N*-ethyl-1-naphthylamine reagent [8]. The dimethyl aryltriazenes were prepared by known methods through coupling of the corresponding arenediazonium chlorides with dimethylamine in the presence of excess base [9], whereas 4-(3,3-dimethyl-1-triazeno)benzoic acid was obtained from a reaction of 4-carboxybenzenediazonium fluoroborate with dimethylamine [10]. The monomethyl aryltriazenes were synthesized by reacting methylmagnesium iodide with the corresponding aryl azide [11].

Isolations. Isolation of the postmitochondrial supernatant from rat liver, its preincubation with the test compounds and the isolation of tRNA from this material have been described by Hradec *et al.* [5] and by Stiborová *et al.* [6]. After the isolation, tRNA was deacylated [12]. Rat liver tRNA for studies on direct interaction with the test compounds was purified as described by Rogg *et al.* [13] and deacylated as reported by Stanley [12].

Incubations. Incubation mixtures for the direct reaction of test compounds with tRNA contained in the final volume of 0.5 ml: 10 mM Tris–HCl buffer, pH 7.5, 0.5 mg of tRNA and the required amount of test compound (dissolved in ethanol). They were incubated for 60 min at 37°. After addition of 1/10 volume of 20 per cent sodium acetate, pH 5.0, tRNA was precipitated with 2 volumes of chilled ethanol, and the precipitate was separated by low-speed centrifugation. tRNA was redissolved in water and reprecipitated with ethanol under the same conditions. The final precipitate was dried under a stream of N_2 and dissolved in water.

Incubation mixtures for the assay of charging of initiator tRNA in the presence of aminoacyl–tRNA synthetases from *E.coli*, and mixtures for the other species of tRNA that were studied in the presence of aminoacyl–tRNA synthetases from rat liver, were composed and incubated as described by Stiborová *et al.* [6].

RESULTS

The results clearly show that specific alterations in the functional properties of unfractionated tRNA from postmitochondrial supernatants from rat liver were brought about by preincubation with the majority of test compounds. After charging with L-methionine in the presence of aminoacyl-tRNA synthetases from *E. coli*, the incubates containing treated tRNA enhanced the acceptance of initiator tRNA more efficiently than untreated controls. This

Table 1. Effect of triazene and imidazole pretreatment of initiator tRNA on its acceptance for L-methionine

No.	Compound	Initiator pretreated in S-30 fraction (Per cent of controls)	tRNA alone (Per cent of controls)
1	5-Aminoimidazole-4-carboxamide (AIC)	290	90
2	5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC)	213	99
3	5-Diazoimidazole-4-carboxamide (DZIC)	203	96
4	3,3-Dimethyl-1-phenyltriazene (DMPT)	245	106
5	4-(3,3-Dimethyl-1-triazeno)benzoic acid (DMTBA)	378	104
6	4-(3,3-Dimethyl-1-triazeno)sulphonic acid sodium salt (DMTSA Na salt)	142	107
7	1-(4-Chlorophenyl)-3,3-dimethyltriazene (4-Cl-PDMT)	194	119
8	1-(4-Bromophenyl)-3,3-dimethyltriazene (4-Br-PDMT)	161	136
9	1-(2,4,6-Trichlorophenyl)-3,3-dimethyltriazene (2,4,6-Cl_3-PDMT)	120	97
10	3-Methyl-1-phenyltriazene (MPT)	174	232
11	1-(4-Chlorophenyl)-3-methyltriazene (4-Cl-PMT)	198	170
12	1-(4-Bromophenyl)-3-methyltriazene (4-Br-PMT)	245	236
13	1-(2,4,6-Trichlorophenyl)-3-methyltriazene (2,4,6-Cl_3-PMT)	133	182

Unfractionated tRNA isolated from postmitochondrial supernatants and either preincubated with compounds tested, or pretreated alone with these compounds, was charged with aminoacyl–tRNA synthetases from *E.coli*. Controls were preincubated with the solvent (ethanol) alone. Charging of tRNA in the control mixtures was 1.24 pmol of L-methionine/nmol of unfractionated tRNA. Test compounds were preincubated at 10^{-4} mg/ml for 60 min at 37° in both experimental systems used.

finding indicated that the formation of Met-tRNA$_f^{Met}$ was enhanced. If unfractionated tRNA from eukaryotic cells is used as substrate, bacterial synthetases selectively charge this tRNA species but not tRNA$_m^{Met}$ [12].

Although the majority of test compounds enhanced the acceptance of initiator tRNA for its amino acid, the most significant effect was found with several DMPT derivatives: 4-(3,3-dimethyl-1-triazeno)benzoic acid (DMTBA) showed the highest stimulation whereas 1-(2,4,6-trichlorophenyl)-3,3-dimethyltriazene (2,4,6-Cl_3-PDMT) — the only exception — was almost inactive (Table 1).

Significantly different effects from those demonstrated with tRNA isolated from postmitochondrial supernatants pretreated with the test compounds were found if purified unfractionated tRNA from rat liver was preincubated with triazenes directly, i.e. in the absence of any subcellular fractions or cofactors. In these experiments only monomethyl aryltriazenes enhanced the charging of tRNA$_f^{Met}$ with L-methionine whereas dimethyl aryltriazenes were without effect in the same dose range that was effective in experiments with the postmitochondrial supernatants (Table 1).

For more detailed studies on the dose-dependence of the test compounds 1-(4-bromophenyl)-3-methyltriazene (4-Br-PMT) was used as a model compound since it showed a high activity in the S-30 fraction as well as in direct incubations with tRNA, and is relatively stable. Experiments using incremental doses of this compound revealed that the stimulating effect of this triazene on the formation of Met–tRNA$_f^{Met}$ was dependent on the dose of the test compound that had been added to the postmitochondrial supernatant before the isolation of tRNA. The most effective dose was found to be approximately 10^{-4} mg/ml of postmitochondrial supernatant.

The same concentration of 4-Br-PMT also most efficiently enhanced the formation of Val–tRNA if unfractionated tRNA pretreated with this triazene was charged in the presence of aminoacyl–tRNA synthetases from rat liver. However, the most effective dose for the synthesis of Lys–tRNA was lower (10^{-5} mg/ml of the S-30 fraction). The dose-dependence curve for Ala-tRNA showed two peaks at 10^{-4} and 10^{-7} mg of 4-Br-PMT/ml of the S-30 fraction indicating that apparently two different isoaccepting species of tRNA^{Ala} were affected by these doses of the test compound (Fig. 1).

The most effective doses of 4-Br-PMT in experiments with purified tRNA in the absence of postmitochondrial supernatants were lower than those required for the maximum effect with the postmitochondrial supernatants (Fig. 2). Even the short-lived 2,4,6-Cl_3-PMT showed the enhancing effect on the charging of initiator tRNA with L-[^{35}S]methionine (Fig. 3).

The time for which the postmitochondrial supernatant had been treated with 4-Br-PMT was found to be important for the enhancing effect on the charging of initiator tRNA. Incubations for 60 min at 37° were most effective, short preincubations (up to about 15 min) were virtually ineffective, and longer treatments led to a significant decrease of the stimulating effect (Fig. 4). However, in the absence of postmitochondrial supernatants, preincubation for 15 min was adequate for obtaining the maximum effect. Preincubations up to 75 min were equally effective as those for 15 min (results not shown).

Preincubation with the test compounds affected not only the acceptance of initiator tRNA but also that of some other tRNA species when the assay was carried out with rat liver aminoacyl–tRNA synthe-

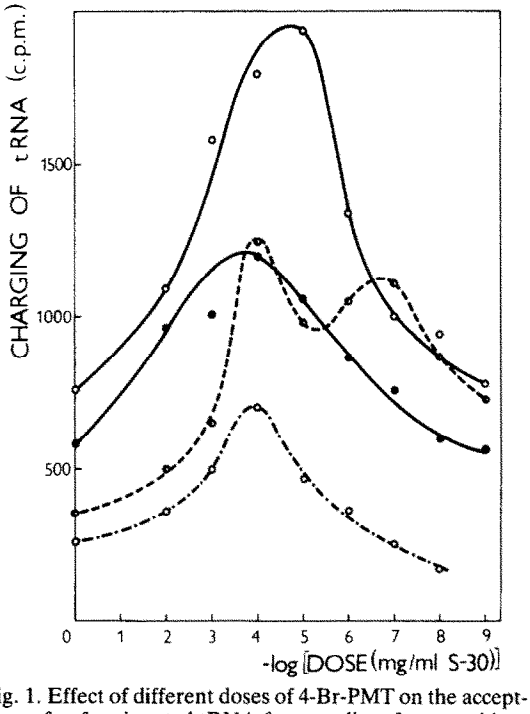

Fig. 1. Effect of different doses of 4-Br-PMT on the acceptance of unfractionated tRNA from rat liver for L-methionine (—●—), L-alanine (--◐--), L-lysine (—○—) and L-valine (-·-○-·-). Preincubation with the compound tested in the postmitochondrial supernatants was 60 min at 37° as described in the Materials and Methods section. All values are cpm/incubation mixture.

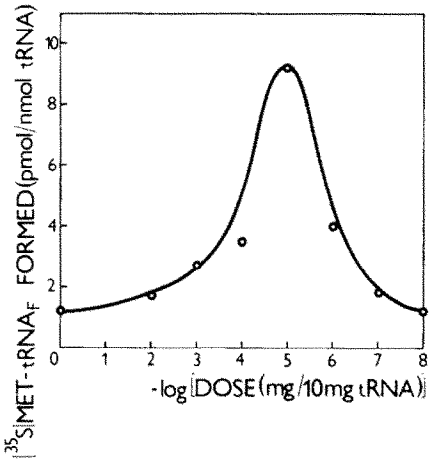

Fig. 2. Effect of different doses of 4-Br-PMT on the charging of tRNA$_f^{Met}$ with L-[^{35}S]methionine. The test compound was preincubated with purified unfractionated tRNA from rat liver without any further additions for 60 min at 37°, as described in the Materials and Methods section.

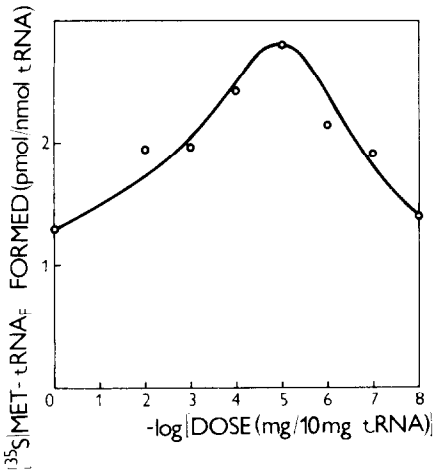

Fig. 3. Effect of different doses of 2,4,6-Cl_3-PMT on the charging of initiator tRNA with L-[^{35}S]methionine. Conditions of the experiment were the same as in legend for Fig. 2.

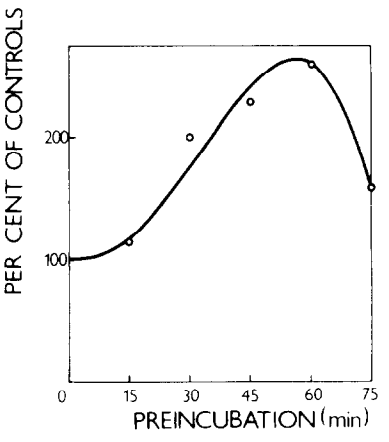

Fig. 4. Effect of different preincubation times on the charging of $tRNA_f^{Met}$ with L-[^{35}S]methionine. The dose of 4-Br-PMT was 10^{-4} mg/ml of postmitochondrial supernatant.

Table 2. Effect of triazenes and imidazoles on the charging of tRNA with various amino acids

No.	Compound	Amino acid: Ala	Arg	Asp	Gly	His	Ile	Leu	Lys	Phe	Thr	Tyr	Val
		Per cent of controls											
1	AIC	76.4	524.4	120.5	77.5	87.1	188.2	28.1	505.5	131.1	185.4	108.7	99.2
2	DTIC	65.6	61.5	69.6	121.4	110.6	107.8	22.6	80.4	134.1	107.8	122.8	323.1
3	DZIC	100.3	186.9	143.2	209.5	270.7	223.4	18.5	208.5	103.8	60.7	198.2	313.0
4	DMPT	189.5	201.2	–	209.5	–	188.2	36.9	277.9	101.6	–	319.2	260.8
5	DMTBA	167.9	106.9	82.6	84.5	216.2	164.0	19.1	264.9	124.1	94.8	103.5	123.1
6	DMTSA Na s.	135.1	184.9	98.4	221.1	109.4	123.1	25.1	237.5	130.8	110.3	104.3	188.4
7	4-Cl-PDMT	123.3	93.6	141.4	200.0	93.8	213.2	18.7	223.3	148.8	86.4	244.7	197.1
8	4-Br-PDMT	86.8	73.5	184.1	142.2	75.3	165.6	25.6	377.5	118.6	85.4	125.4	139.8
9	2,4,6-Cl_3-PDMT	99.3	157.2	143.2	123.9	43.9	131.2	22.9	162.8	95.2	109.8	93.8	157.9
10	MPT	97.7	110.1	108.7	231.0	–	96.8	13.4	266.3	136.9	68.5	233.3	153.6
11	4-Cl-PMT	79.2	232.7	137.9	187.3	69.1	109.3	27.4	252.0	113.8	55.0	97.3	131.8
12	4-Br-PMT	181.6	73.1	103.5	180.2	131.8	103.1	30.3	208.3	98.8	108.3	169.2	137.6
13	2,4,6-Cl_3-PMT	198.2	93.4	202.5	142.2	97.5	156.2	27.4	578.4	108.6	173.5	158.7	62.3

Unfractionated tRNA was isolated from postmitochondrial supernatants preincubated with the compounds tested and charged by aminoacyl-tRNA synthetases from rat liver. Controls were preincubated under the same conditions with the solvent (ethanol) only. The following chargings were obtained in control mixtures (pmol amino acid/nmol tRNA): Ala - 3.99, Arg - 1.02, Asp - 0.66, Gly - 0.45, His - 0.84, Ile - 1.45, Leu - 0.13, Lys - 1.21, Phe - 0.50, Thr - 1.04, Tyr - 0.16, Val - 0.67. Postmitochondrial supernatants were incubated for 60 min at 37° with 10^{-4} mg/ml of the compound tested.

tases. In these experiments most test compounds enhanced the formation of Val–tRNA and Lys–tRNA, and several stimulated also the acceptance of $tRNA^{Ala}$. Only some compounds affected the acceptance of $tRNA^{Tyr}$, $tRNA^{Ile}$ and $tRNA^{Gly}$. Essentially no effects were found with $tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Phe}$, $tRNA^{Thr}$, and $tRNA^{Asp}$. Preincubation of the postmitochondrial supernatant with any of the test compounds resulted in a significant inhibition of Leu–tRNA formation (Table 2).

DISCUSSION

In agreement with previous experience [5, 6], the findings provide supporting evidence that the enhancing effect on the acceptance of initiator tRNA is probably associated with the carcinogenic activity of the test compound.

Both monomethyl aryltriazenes (Ar–N=N–NH–CH_3) and the dimethyl aryltriazenes [Ar–N=N–$N(CH_3)_2$] are potent carcinogens [14]. Although the chemical reactivity and the mode of carcinogenic action differ markedly in each series, the derivatives of both classes stimulated effectively the formation of the Met-$tRNA_f^{Met}$ complex. The monomethyl aryltriazenes are alkylating agents that act as direct carcinogens whereas the dimethyl analogues must be first enzymatically converted (by dealkylation) into their carcinogenic form. In fact, these structural and functional differences were the main reason for testing representative compounds of both series in our system.

The stability of the monoethyl aryltriazenes to hydrolysis in a 0.15 M phosphate buffer, pH 7.4, at 37° (F. Delben *et al.*, to be published) reflected the observed stimulative efficiency of this class: The

relatively stable 4-Br-PMT ($t_{0.5}$ 201 sec) and 4-Cl-PMT (175 sec) were significantly more active than the very labile 2,4,6-Cl_3-PMT (15 sec) or even MPT (68 sec). It appears that monomethyl triazenes with short half-lives, such as 2,4,6-Cl_3-PMT, hydrolyze faster than they are able to modify tRNA.

In addition to stimulation of the initiator tRNA, several but not all triazenes enhanced the acceptance of $tRNA^{Ala}$. This effect was previously observed only in carcinogenic derivatives of 4-dimethylaminobenzene. However, the synthesis of Gly–tRNA and Ile–tRNA, that was essentially unaffected by azo dyes [6], was significantly enhanced by some triazenes. Contrary to effects exerted by chemical carcinogens on the initiator tRNA, these agents induced no unequivocal response in other species of tRNA. Moreover, it appears that the individual classes of chemical carcinogens, such as azo dyes or triazenes, may utilize different species of tRNA as their targets, Thus the acceptance of only some tRNAs was reported to be affected by *N*-acetoxy-2-acetylaminofluorene *in vitro* [4] and, similarly, Lys–$tRNA_2$ was demonstrated to be the major target for carcinogenic L-ethionine [15].

Results obtained with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) are consistent with the finding that its enhancing effect on the acceptance of initiator tRNA may be specific for compounds that must be enzymatically activated. This dimethyltriazene cytostatic compound was found to stimulate the formation of Met–$tRNA_f^{Met}$ only after preincubation with the S-30 fraction but not without it. Moreover, $tRNA^{Val}$ is the only other tRNA species the acceptance of which was significantly stimulated by DTIC whereas the acceptance of other tRNA species was hardly affected.

The only tRNA species, the acceptance of which was significantly decreased by preincubation with triazenes or derivatives of 4-dimethylaminoazobenzene—irrespective of their carcinogenic activity, was $tRNA^{Leu}$ [6]. However, the results of our previous experiments showed that the formation of Leu–tRNA in the isolated fraction of pH 5 enzymes was significantly enhanced by some *N*-nitroso carcinogens and triazenes [16]. It cannot be excluded that $tRNA^{Leu}$ may be exceptionally sensitive to the action of exogenous chemicals and that this effect may be modulated by microsomes. The postmitochondrial supernatants used in the present experiments contained substantial quantities of microsomes whereas only traces of these organelles were present in the pH 5 enzyme fraction used previously. It is therefore possible that some important enzymic systems of the postmitochondrial supernatant that mediate the inhibition of Leu-tRNA synthesis may have been missing from the pH 5 fraction.

Previous results already indicated that the acceptance of the initiator tRNA by procarcinogenic aromatic hydrocarbons [5] and azo dyes [6] could be stimulated only when these chemically rather inert procarcinogens were first incubated with hepatic systems containing microsomes. This implies that these procarcinogens had to be first converted into as yet unknown intermediates that finally react with tRNA and thus modify its acceptance properties. Since the hepatic enzymes are also known to generate the (ultimately) carcinogenic species from the procarcinogenic compounds, there appears to be a distinct analogy between both types of biological response that can be detected after microsomal activation of the procarcinogenic compounds. We have concluded that the same reactive species may be responsible for the induction of both biological effects.

The results of the present experiments not only confirm that microsomal activation is essential for the induction of the enhancing effect by the procarcinogenic dimethyltriazenes but also that similar stimulation could be attained by the direct action of the alkylating monomethyl aryltriazenes, i.e. without any activation by the microsomal enzymes.

Since the monomethyl aryltriazenes yield reactive (electrophilic) intermediates (methyldiazonium and arenediazonium cations) by heterolysis, it is conceivable that the enhanced acceptance of initiator tRNA exhibited by this class of carcinogens is probably due to a chemical reaction of these electrophilic species with tRNA.

DMPT has long been known to methylate extrahepatic nucleic acids *in vivo* [17], and it has been suggested that this dimethyltriazene carcinogen is metabolically converted into the direct-acting MPT which methylates the crucial sites in nucleic acids [18].

It is tempting to speculate that the reactive intermediates released from monomethyltriazenes by heterolysis, or generated from dimethyltriazenes by enzymic activation, react with nucleophilic receptors of tRNA and thus specifically modify those sites that are recognized by its corresponding synthetase. Such chemical alteration of tRNA may lead to an enhanced combination of the enzyme with its substrate. This interpretation is consistent with recent findings that different optimal synthetase–tRNA ratios were found with preparations of tRNA pretreated with triazenes (M. Stiborová, unpublished results).

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