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CARCINOGENIC AZO DYES MODIFY THE ACCEPTANCE OF TRANSFER RIBONUCLEIC ACID FOR SOME AMINO ACIDS

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Abstract—Postmitochondrial supernatant fractions of rat liver were incubated with 11 carcinogenic and non-carcinogenic derivatives of 4-dimethylaminoazobenzene and tRNA was isolated from this material. Only initiator tRNA from preparations pretreated with carcinogenic compounds showed a significantly enhanced acceptance for methionine. This effect was dependent from the dose and time of preincubation. Also the formation of lysyl- and alanyl-tRNA was stimulated after pretreatment of postmitochondrial supernatant fractions with carcinogenic derivatives whereas the synthesis of glycyl-tRNA was only slightly affected. No correlations were found between the carcinogenic activity of azo dyes and the charging of tRNA with phenylalanine, isoleucine, valine and arginine. All derivatives tested significantly inhibited the formation of leucyl-tRNA, carcinogenic compounds being more effective in this respect than non-carcinogenic ones. No changes in the charging of tRNA with amino acids were found if cytosols in the absence of microsomes were pretreated with the compounds tested. Only the presumed metabolite of 4-dimethylaminoazobenzene, 4'-hydroxy-4-dimethylaminoazobenzene, enhanced significantly the charging of initiator tRNA if preincubated with cytosol or with pure tRNA. Parent carcinogenic azo dyes are apparently converted into active intermediates by microsomal enzymes present in postmitochondrial supernatant fractions and these ultimate carcinogens react with initiator tRNA and some other tRNA species and modify their acceptance.

Interaction of chemical carcinogens or their active metabolites with macromolecules in the target cell seems to be an essential event in chemical carcinogenesis (see Ref. 1 for review). At least some carcinogens exhibit a greater affinity in this respect for RNA than for DNA or proteins [2]. Several reports indicate that tRNA may be a preferential target molecule for carcinogenic compounds and that the extent of binding of these compounds to tRNA may exceed that to the other RNA species [3–7].

Because of the important role of tRNA in the translation of the genetic message, the pattern of individual tRNA species was studied in various physiological as well as pathological conditions and evidence has been presented that changes in the abundance of different tRNA species are associated with metabolic regulation, cellular differentiation and neoplastic transformation and growth [2, 8, 9]. Interaction of chemical carcinogens with tRNA results in apparently specific alterations of its acceptance for amino acids, codon recognition and the binding to ribosomes [10]. Evidence has been presented that *N*-nitroso carcinogens and triazenes enhance the charging of tRNA with L-leucine [11]. Only carcinogenic but not similar non-carcinogenic polycyclic aromatic hydrocarbons were shown to enhance the acceptance of initiator tRNA for L-methionine [12].

Experiments described in this paper were performed to find out whether the effect of carcinogenic hydrocarbons is specific for this class of compounds only or is a general property of carcinogenic compounds. Evidence is presented that only carcinogenic derivatives of DAB‡ enhance the formation of Met-tRNA$_F^{Met}$ in a similar way as carcinogenic hydrocarbons, that non-carcinogenic derivatives are free from this effect and that this influence is mediated by microsomal enzymes.

MATERIALS AND METHODS

Animals. Wistar rats of both sexes (120–150 g) bred in this laboratory and fed a standard pelleted diet were used.

Chemicals and radiochemicals. Azo dyes were prepared by the reaction of arenediazonium salts with *N,N*-dimethylaniline [13]. All other chemicals were described by Hradec *et al.* [12].

L-[^{35}S]Methionine (1155 Ci/mmole) was purchased from the Radiochemical Centre, Amersham, Bucks., U.K., L-[U-^{14}C] arginine (240 mCi/mmole), L-[U-^{14}C] alanine (120 mCi/mmole), L-[U-^{14}C] phenylalanine (360 mCi/mmole), [U-^{14}C]glycine (80 mCi/mmole), L-[U-^{14}C]isoleucine (240 mCi/mmole), L-[U-^{14}C]leucine (240 mCi/mmole), L-[U-^{14}C]lysine (240 mCi/mmole) and L-[U-^{14}C]valine (200 mCi/mmole) were products of the Institute for Research, Production and Uses of Radioisotopes, Prague, Czechoslovakia.

Isolations. Postmitochondrial supernatant fractions (the S-30 fraction) from rat liver were isolated and incubated with compounds to be tested as

‡ Abbreviations used: DAB, 4-dimethylaminoazobenzene; DA-1-N, dimethylaminobenzene-1-azo-1'-naphthalene; DA-2-N, dimethylaminobenzene-1-azo-2'-naphthalene.

described by Hradec *et al.* [12]. Cytosol was prepared by centrifuging the S-30 fraction at 300,000 *g* and 4° for 60 min in a Beckman 70 Ti rotor using a L5-65 ultracentrifuge. Total tRNA was isolated from the cytosol by phenol extraction [14] and deacylated as described by Stanley [15]. Crude aminoacyl-tRNA synthetases from *E. coli* were isolated as described by Matthaei *et al.* [14] and aminoacyl-tRNA synthetases from rat liver were partially purified using the method of Stanley [15].

Incubations. Mixtures for the charging of initiator tRNA with L-methionine were composed and incubated as described by Hradec *et al.* [12]. Charging of tRNA with the other amino acids in the presence of aminoacyl-tRNA from rat liver was assayed in mixtures described by Hradec and Dušek [16]. Samples for the assay of radioactivity were prepared and the radioactivity was assayed as described by Hradec *et al.* [12].

RESULTS

If postmitochondrial supernatant fractions were preincubated with DAB, tRNA isolated from these preparations was charged with L-methionine in the presence of bacterial aminoacyl-tRNA synthetases significantly more efficiently than in control mixtures, indicating an enhanced acceptance of initiator $tRNA_F^{Met}$ [15] for this amino acid. This effect was dose-dependent and doses of DAB required for the maximum stimulating effect were in the range of 10 pmoles DAB/ml of the S-30 fraction. Higher as well as lower doses of the compound tested were considerably less effective. Essentially the same dose-dependence was found if tRNA isolated from the preparations preincubated with the azo dye was charged with L-alanine in the presence of aminoacyl-tRNA synthetases from rat liver (Fig. 1).

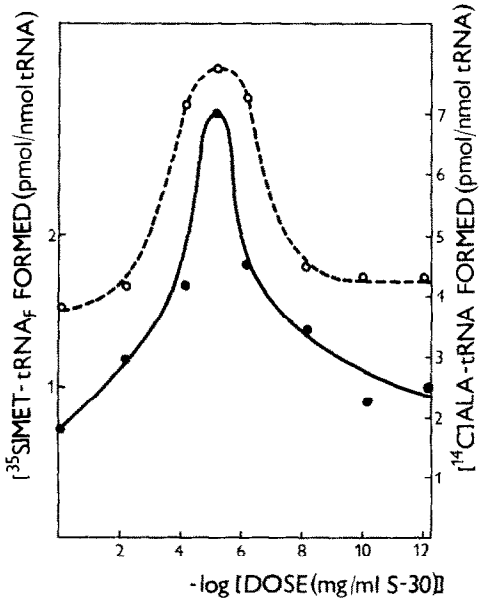

Fig. 1. Effect of different doses of DAB on the acceptance of unfractionated tRNA of rat liver for L-methionine (●) and L-alanine (○). Preincubation with DAB of the postmitochondrial supernatant fraction was for 60 min at 37° as described in Materials and Methods.

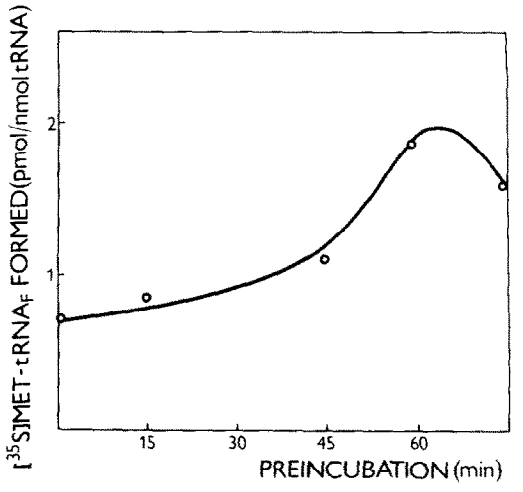

Fig. 2. Effect of different preincubation times on the charging of $tRNA_F^{Met}$ with L-[^{35}S]methionine. The dose used for the preincubation was 30 pmoles DAB/ml postmitochondrial supernatant fraction.

Also, the time for which the postmitochondrial supernatant fraction had been treated with the compound tested was of considerable importance and a preincubation for 1 hr at 37° was found to be the most satisfactory period (Fig. 2). Preincubation had no significant effect on the charging with any amino acids used of tRNA isolated from control mixtures containing only the solvent.

Pretreatment of the postmitochondrial supernatant fraction with the azo dyes prior to the isolation of tRNA revealed a high degree of correlation between the carcinogenic activity of these compounds and their enhancing effect on the acceptance of initiator tRNA. Thus both 3′-methyl- and 3′-methoxy-substituted DAB, which exhibit a similar carcinogenic activity to the parent DAB, enhanced the acceptance to a very similar extent. Moreover, 3′-chloro- and 3′-nitro-derivatives of a considerably lower carcinogenic activity also showed a lower effect on initiator tRNA. Non-carcinogenic derivatives did not affect the charging of initiator tRNA. A particularly significant difference in this respect was found with both naphthalene derivatives tested. Whereas the 1′-substituted compound is non-carcinogenic and without effect on the charging of tRNA, the derivative substituted at the 2′position is strongly carcinogenic and exhibited also a significant enhancing effect on the acceptance of initiator tRNA. The presumed metabolites of DAB [17], 4′-hydroxy-DAB, showed a stimulating effect in the system used similarly as the 2′-hydroxy derivative (Table 1).

Carcinogenic azo dyes did enhance the acceptory activity of unfractionated tRNA not only for L-methionine but also for L-alanine and L-lysine if total tRNA isolated from the S-30 fraction pretreated with these compounds was charged with these amino acids in the presence of aminoacyl-tRNA synthetases from rat liver. The acceptance of both $tRNA^{Ala}$ and $tRNA^{Lys}$ was affected by individual azo dyes tested

Table 1. Charging of $tRNA_F^{Met}$ pretreated with DAB and its derivatives with L-[^{35}S]methionine*

No.	Compound	Carcinogenicity	Reference	Met-$tRNA_F^{Met}$
1	DAB	Strong	18	250.6
2	3′-CH_3-DAB	Strong	18	301.2
3	3′-COOH-DAB	Weak	18	108.8
4	3′-Cl-DAB	Intermediate	18	141.0
5	3′-OCH_3-DAB	Strong	18	257.3
6	3′-NO_2-DAB	Intermediate	18	148.8
7	3′-Br-DAB	Non-carcinogenic	18	101.0
8	4′-CH_3-DAB	Non-carcinogenic	18	100.2
9	4′-OH-DAB	Weak	27	159.7
10	2′-OH-DAB	Weak	27	161.1
11	DA-1-N	Non-carcinogenic	29	91.5
12	DA-2-N	Strong	30	200.0

* All values are per cent of controls (postmitochondrial supernatant fractions pretreated with the solvent only) obtained with saturating amounts of unfractionated tRNA isolated from the S-30 fraction preincubated with 30 pmoles of compounds tested/ml postmitochondrial supernatant fraction. Charging was performed using aminoacyl-tRNA synthetases from *E. coli* as described in Materials and Methods. In control mixtures $tRNA_F^{Met}$ was charged with 0.717 pmole methionine/nmole tRNA.

essentially in the same way as that of $tRNA_F^{Met}$. The same holds true also for $tRNA^{Gly}$, although much less significant differences were found between individual derivatives of DAB in this respect.

No correlation, however, could be found between the carcinogenic activity and the acceptance of $tRNA^{Arg}$, $tRNA^{Phe}$, $tRNA^{Ile}$ and $tRNA^{Val}$ for their amino acids. In contrast to the results obtained with all other species of tRNA studied, the acceptance of $tRNA^{Leu}$ for its amino acid was significantly decreased following the pretreatment of the S-30 fraction with any of the azo dyes tested, irrespective of their carcinogenic activity, although carcinogenic compounds seemed to be a little more active in this respect (Table 2).

No changes in the acceptory activity of tRNA for any amino acid tested were found if cytosols free of microsomes were preincubated with azo dyes instead of the postmitochondrial supernatant fractions containing microsomes (results not shown). The single exception in this respect was 4′-hydroxy-DAB, the preincubation of which with the cytosol significantly enhanced (by 64 per cent) the acceptory activity of initiator tRNA. Moreover, this compound was also the only one which enhanced the acceptance of $tRNA^{Met}$ if incubated with purified unfractionated tRNA in the absence of any other cellular constituents or cofactors. Doses required for this effect were, however, higher than those necessary for the maximum effect with the S-30 fraction or cytosol (Fig. 3).

DISCUSSION

Results presented in this paper clearly indicate that the presence of microsomes in the subcellular fraction used for the preincubation with azo dyes is a prerequisite absolutely necessary for the effect of carcinogenic compounds of this class on the acceptory activity of tRNA for amino acids. Carcinogenic

Table 2. Charging of unfractionated tRNA pretreated with DAB and its derivatives with various amino acids*

No.	Compound	tRNA charged with Ala	Arg	Phe	Gly	Ile	Leu	Lys	Val
1	DAB	185.6	136.5	95.2	128.1	136.8	26.2	246.8	171.0
2	3′CH_3-DAB	183.5	99.9	100.1	130.3	144.0	24.0	269.8	74.3
3	3′-COOH-DAB	182.9	54.2	147.8	89.6	104.6	59.3	149.0	95.7
4	3′-Cl-DAB	132.7	89.6	103.7	61.7	133.9	22.1	164.0	63.3
5	3′-OCH_3-DAB	173.6	91.2	109.8	120.7	142.2	28.4	261.4	222.7
6	3′-NO_2-DAB	127.6	72.8	114.9	118.3	105.1	37.3	230.3	96.7
7	3′-Br-DAB	96.5	91.2	105.1	100.2	71.1	26.4	100.9	144.0
8	4′-CH_3-DAB	109.6	76.2	104.9	99.8	80.1	54.6	100.0	105.3
9	4′-OH-DAB	120.3	97.2	99.4	139.1	114.6	38.8	179.1	153.4
10	2′-OH-DAB	136.7	105.1	76.9	76.4	114.2	48.9	138.8	155.9
11	DA-1-N	99.0	78.5	85.8	102.2	99.5	46.5	184.5	109.7
12	DA-2-N	258.7	83.5	377.3	368.0	89.9	37.6	236.8	165.9

* All values represent per cent of controls (preincubation with ethanol only) pretreated with compounds tested as described in legend for Table 1. Unfractionated tRNA was charged in the presence of aminoacyl-tRNA synthetases from rat liver. The following chargings were obtained in control mixtures (pmoles amino acid/nmoles tRNA): Ala, 3.86; Arg, 2.89; Phe, 0.36; Gly, 2.02; Ile, 0.88; Leu, 0.57; Lys, 1.05; Val, 0.55.

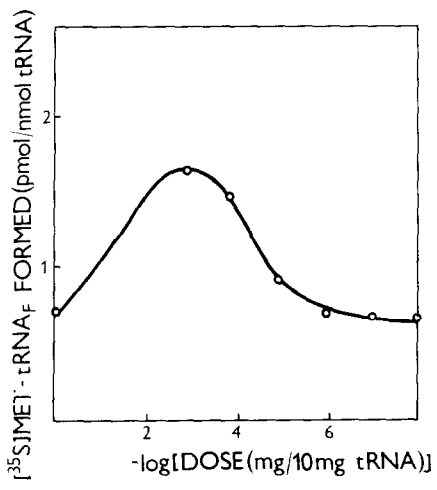

Fig. 3. Effect of the incubation of 4'-hydroxy-DAB with tRNA on the acceptance of $tRNA_F^{Met}$ for L-[^{35}S]methionine. Unfractionated tRNA (10 mg) from rat liver in 1 ml of Tris–HCl buffer, pH 7.5, was incubated with 0.1 ml of ethanol containing the quantities of 4'-hydroxy-DAB as indicated for 60 min at 37°. After that, tRNA was precipitated with ethanol [14] and its acceptance was tested in the presence of aminoacyl-tRNA synthetases from *E. coli* as described in Materials and Methods

azo dyes are typical procarcinogens requiring microsomal enzymes for their conversion into active ultimate carcinogens [18] which are apparently only able to modify the acceptance of tRNA for amino acids. In agreement with this opinion, 4'-hydroxy-DAB, which is a metabolite of the parent DAB [17], was the only compound in our experiments capable of modifying the acceptance of $tRNA_F^{Met}$ in the absence of microsomes. Because this compound is able to affect initiator tRNA even by incubation with tRNA alone in the absence of any further additions, it would seem that at least some metabolites of DAB are able to react with tRNA directly by chemical rather than by enzymic mechanisms. Possible binding of these compounds to tRNA [3–7] or reactions with certain nucleotides should be considered in this respect. From this point of view, the presence of microsomes would be essential only for the conversion of parent inactive compounds to active metabolites and not for their interaction with tRNA.

It cannot be excluded that the difference between modified and control tRNA may depend on a different degree of uncompensated -CCA and shortening, as incubation mixtures containing aminoacyl-tRNA synthetases from rat liver were not supplemented with CTP. However, this does not apply for the charging of tRNA with methionine by synthetases from *E. coli* where CTP was present [12, 14].

Alterations of the acceptory activity of tRNA may result from the changes in tRNA structure induced by active metabolites of carcinogens. Administration of 3'-methyl-4-aminoazobenzene, ethionine and some other carcinogens resulted in a significant change of chromatographic profile of tRNA in the liver [7, 19, 20]. A complex of the azo dye with $tRNA^{Phe}$ was demonstrated in the liver of rats fed 3'-methyl-DAB [21]. Convincing evidence has been presented to show that the major dye-containing components obtained by the hydrolysis of liver RNA from rats given *N*-methyl-4-aminoazobenzene is *N*(guanosin-8-yl)-methyl-4-aminoazobenzene and the same compound has been also identified if *N*-benzoyloxy-methyl-4-aminoazobenzene was reacted with RNA *in vitro* [22–24].

Different carcinogens are apparently able to react with or to become bound to different species of tRNA. The administration of *N*-acetoxy-2-acetylaminofluorene to rats stimulated the synthesis of arginyl-tRNA but inhibited the formation of several other aminoacyl-tRNAs [10]. On the other hand, ethionine seems to react preferentially with $tRNA^{Lys}$ [25]. *N*-Nitroso carcinogens and triazenes enhanced the formation of Leu-tRNA *in vitro* [11]. Both carcinogenic hydrocarbons [12] and azo dyes in the present experiments seem to have $tRNA_F^{Met}$ as a common target.

Both hydroxy derivatives of DAB used in our experiments enhanced the acceptance of initiator tRNA to an extent characteristic for compounds with an intermediate carcinogenic activity. However, the ring hydroxylation of DAB is believed to represent a detoxification mechanism leading to inactive compounds [26]. On the other hand, both these hydroxy derivatives were demonstrated to be weakly carcinogenic [27]. It has been suggested that the 4'-hydroxylation of aminoazo dyes might be related in some obscure way to their carcinogenicity [28]. This may well correlate with the unique effect of this compound found in our experiments. Moreover, it should be considered that the carcinogenicity of azo dyes is tested mostly by feeding these compounds and it seems reasonable to suppose that natural metabolites administered in this way may be excreted without reaching the target liver cell. This assumption can help to explain the disagreement between the reported carcinogenic activity and the effect of both hydroxy derivatives on the charging of initiator tRNA in our experiments.

Striking similarities exist between the results of our earlier experiments with carcinogenic hydrocarbons [12] and those with azo dyes reported in this paper. In both instances $tRNA_F^{Met}$ was affected, an identical period of preincubation was required and the presence of microsomes was absolutely necessary for the effect. The influence of both classes of compounds was strictly dose-dependent, although optimum doses for azo dyes are somewhat lower than those of polycylic aromatic hydrocarbons. Even more important, the enhanced acceptance of initiator tRNA was induced only by carcinogenic compounds whereas closely chemically related non-carcinogenic derivatives were ineffective.

From the chemical point of view, scarcely any similarities can be found between polycyclic aromatic hydrocarbons and azo dyes. The requirement for a prior metabolic activation seems to be the only common property for both these classes of compounds. Thus it would seem that the interaction of carcinogenic compounds with tRNA, in particular with initiator tRNA, may be characteristic for carcinogens irrespective of their chemical nature, although further experiments with compounds of different struc-

tures are undoubtedly required for a more generalized conclusion.

At the moment it is not possible to see any correlation between the effect of azo dyes on the charging of tRNA and the *in vivo* carcinogenic activity of these compounds. It is not known whether they directly affect translation either by enhancing protein synthesis or by inducing synthesis of abberant proteins.

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