Irradiation-induced Adduct Formation of RNA with Carcinogenic Arylamine Derivatives

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SYNOPSIS

Radiolysis of N2O-saturated solutions of transfer RNA (tRNA) and the arylacethydroxamic acids, N-hydroxy-N-2-acetylaminofluorene and N-hydroxy-N-4-acetylamino-biphenyl; their corresponding acetamides, 2-acetylaminofluorene and 4-acetylamino-biphenyl; or the O-glucuronide of N-hydroxy-N-2-acetylamino-biphenyl resulted in adduct formation of the nucleic acid with these carcinogenic arylamine derivatives. The yield of adducts on irradiation of the arylacethydroxamic acids with tRNA was greater than that for their corresponding acetamides or the O-glucuronide. The fluorenylacethydroxamic acid and acetamide were also more reactive than the biphenyl analogs. Adduct formation resulting from radiolysis of tRNA and the arylacethydroxamic acids or the O-glucuronide proceeded with retention of both the aromatic nucleus and the N-acetyl group.

The yields of adducts were much greater for irradiated mixtures than for irradiation of either component alone followed by mixing.

Evaluation of the data shows that initial modification of the tRNA or the carcinogen can lead to adduct formation. In the case of primary radical attack at the nucleic acid, it has been shown that short-lived reactive RNA intermediates are responsible for a major fraction of the observed yield of adducts in the irradiated mixtures.

Comparative studies showed that irradiation under conditions that favor reaction of oxidizing radicals enhanced formation of the adducts.

Oxygen was shown to protect RNA from irradiation-induced binding of the arylacethydroxamic acids due to competition of O2 with the carcinogen for the reactive RNA intermediates.

INTRODUCTION

Interactions of chemical carcinogens with nucleic acids and proteins may play an important role in the induction of tumors (33, 45). The aromatic amides constitute a class of carcinogens for which metabolic activation is a prerequisite to covalent combination with macromolecules. A primary step in this activation appears to be N-hydroxylation (33, 45) to give derivatives that are generally more carcinogenic than the parent amides. However, metabolic transformation of the aromatic acethydroxamic acids is necessary for adduct formation to occur.

Several enzymes have been identified that can activate these compounds (10, 22), and mechanisms by which these activation processes may occur have been proposed (3, 20).

Nonenzymatic activation of several carcinogenic aromatic acethydroxamic acids has been achieved by conversion to reactive esters (29, 30) and also by 1-electron oxidation with agents such as alkaline Fe(CN)63– or Ag2O (4, 5). Evidence from these studies suggests that oxidation of these materials proceeds via nitroxide radicals that dismutate to form active products.

Oxidative and reductive processes in general can often be conveniently studied using radiation-chemical techniques (1). By suitable choice of radical scavenger systems a solute can be exposed to oxidizing or reducing environments. In this paper we have demonstrated that ionizing irradiation can induce binding of tRNA to the arylacethydroxamic acids, N-hydroxy-AAF4 and N-hydroxy-AABP: their corresponding acetamides, AAF and AABP; and the O-glucuronide of N-hydroxy-AAF. It was shown that initial reaction of free radicals with either the nucleic acid or the carcinogen can result in adduct formation.

MATERIALS AND METHODS

The irradiations were performed with a linear accelerator at Michael Reese Hospital and Medical Center. The accelerator was operated in the continuous mode and provided 25-MeV electrons at a dose rate of approximately 2.5 kilorads/min. Solutions to be irradiated were bubbled with the desired gas for 10 min prior to and during irradiation. Each chart is derived from experiments carried out simultaneously. Research grade Matheson gases were used throughout. tert-Butyl alcohol was purified by multiple recrystallization.

4 The abbreviations used are: N-hydroxy-AAF, N-hydroxy-N-2-acetylaminofluorene; N-hydroxy-AABP, N-hydroxy-N-4-acetylamino-biphenyl; AAF, 2-acetylaminofluorene; AABP, 4-acetylamino-biphenyl; O-glucuronide of N-hydroxy-AAF, N-acetyl-N-2-fluorenylacethydroxamic-β-d-glucopyranosiduronic acid; 4-nitro-BP, 4-nitrobiphenyl; TLC, thin-layer chromatography; [C], carcinogen concentration.
Synthesis of Radioactive Carcinogenic Biphenylamine Derivatives. The starting material for these syntheses, 4-nitro-BP, was tritiated at the Amersham-Searle Corp., Arlington Heights, Ill., by catalytic exchange in aqueous media (26). The crude tritiated 4-nitro-BP was purified by silica gel column chromatography (Silic AR CC-7; Mallinckrodt Chemical Works, St. Louis, Mo.; 30% benzene in petroleum ether was used for elution). [3H]-N-Hydroxy-AABP (281 mCi/mmole) was prepared from [3H]-4-nitro-BP by a procedure similar to that described by Scribner and Naimy (42) for the preparation of N-hydroxy-2-acetamidophenanthrene. An impurity due to [3H]AABP, which was formed using this procedure, was removed by conversion of the [3H]-N-hydroxy-AABP to its sodium salt, extraction of the salt with ether, and precipitation of the sodium salt with acetic acid. [1-14C]N-Hydroxy-AABP (13.3 mCi/mmole) was prepared in 69% yield by the reaction of N-4-biphenylhydroxylamine with [1-14C]acetyl chloride (California BioNu-}

The O-glucuronide of [9-14C-2'-3H]-N-hydroxy-AAF (0.99 mCi 14C per mmole and 4.9 mCi 3H per mmole) was prepared biosynthetically. The bile ducts of 3 female Sprague-Dawley rats (200 g) were cannulated and a total of 10 mg of [9-14C-2'-3H]-N-hydroxy-AAF in 150 μl of dimethyl sulfoxide was administered i.p. The bile was collected and the O-glucuronide was isolated according to the method of Irving et al. [17]. A sample of the O-glucuronide was treated with β-glucuronidase (bacterial, type I: Sigma Chemical Co., St. Louis, Mo.) in Sorensen's phosphate buffer, pH 7, at 37° for 1.5 hr. Ether extraction of the incubation mixture and subsequent TLC and radioautographic analysis of the ether extract gave 1 fluorescent-quenching radioactive spot due to the aglycone N-hydroxy-AAF.

Radioisotopes of Carcinogen:tRNA Mixtures. Solutions of carcinogen and tRNA (yeast; Calbiochem, San Diego, Calif.) were irradiated under a variety of radical scavenger systems (see “Results and Discussion”) in mm potassium phosphate buffer, pH 7, at a concentration of 1 μg carcinogen per μg RNA per ml buffer, unless otherwise indicated. Nonirradiated samples were used as controls, and adduct formation in these cases was always less than 1%. Aliquots (1 ml) of the reaction mixture were removed after irradiation doses of 0, 5, 10, 30, and 80 kilorads and added to benzene (1 ml) to extract unreacted carcinogen and/or unbound products. In 1 experiment where the concentration of tRNA was reduced to 10 μg/ml, aliquots of the irradiated reaction mixture were added to tRNA (990 μg) to obtain a final tRNA concentration of 1 mg/ml. Binding of the carcinogen to RNA was determined as described previously (20) by precipitating the nucleic acid from 0.5-ml aliquots of the aqueous phase with approximately 6 ml of 2% potassium acetate in 95% ethanol. The precipitate was collected on glass fiber filters and then washed successively with 70 and 90% ethanol, acetone, and diethyl ether. The dry filters were placed in counting vials and moistened with 0.15 ml of water. A solubilizer (1 ml, NCS; Amersham-Searle, Arlington Heights, Ill.) and toluenes-based scintillator (10 ml) were added, and the vials were counted in a liquid scintillation counter.

Radioisolation of N-Hydroxy-AAF and N-Hydroxy-AABP Followed by Addition to tRNA. N2O-saturated solutions of [9-14C-2'-3H]-N-hydroxy-AAF or [ring-14C-2'-14C]-N-hydroxy-AABP (1 μg/ml) in mm phosphate buffer, pH 7, were irradiated at 0, 5, 10, 30, and 80 kilorads. Approximately 1 min after each dosage point, 1-ml aliquots were removed, added to tRNA (1 mg) in 0.1 ml mm phosphate buffer, and allowed to stand at room temperature for approximately 20 hr. Adduct formation was determined as described above. In controls using the nonirradiated aryl-}

Radioisolation of tRNA Followed by Addition to [9-14C-2'-
N_{2}O-saturated solution of tRNA (2 mg/ml) in mM potassium phosphate buffer, pH 7, was irradiated at doses of 0, 5, 10, 30, and 80 kilorads. Samples (0.5 ml) of the reaction mixture were removed after each dose point and approximately 1 min later added to \([9\text{-}^{14}C\text{-}2\text{-}^3H\text{-}}\text{N-hydroxy-AAF}(1 \mu\text{g})\) in 0.5 ml mM phosphate buffer. The mixtures were processed as described for the preirradiated arylacethydroxamic acids. A control sample using tRNA that was not irradiated resulted in approximately 0.2% binding of N-hydroxy-AAF to the nucleic acid after 20 hr.

RESULTS AND DISCUSSION

When dilute aqueous solutions are exposed to ionizing radiation, all of the energy is absorbed in the water. This results in the production of the 3 primary species: OH, the hydroxyl radical; e^{-}_{aq}, the hydrated electron; and H, the hydrogen atom. The hydroxyl radical and hydrated electron are produced in 4-fold greater yield than the hydrogen atom.

\[ \text{H}_{2}\text{O} \rightarrow \text{OH}, e^{-}_{\text{aq}}, H \] (A)

The radical scavenger systems used to create the oxidizing or reducing environments used in this study are given in Table 1. In N_{2}O-saturated solution, the hydrated electrons react rapidly with this solute to form OH radicals (12).

\[ \text{N}_{2}\text{O} + e^{-}_{\text{aq}} \rightarrow \text{N}_{2} + \text{OH} + \cdot\text{OH} \] (B)

Addition of 10 mM tert-butyl alcohol to this system results in a removal of the reactive hydroxyl radical (44) to form the relatively unreactive tert-butyl alcohol radical,

\[ \text{OH} + \text{CH}_3\text{(CH}_3)_2\text{COH} \rightarrow \text{CH}_3\text{(CH}_3)_2\text{COH} + \text{H}_2\text{O} \] (C)

leaving H atoms as the sole reactive species.

Scavenger-free solutions (N_{2}O-saturated) allow all 3 primary radicals to be available for reaction with solute. Addition of 10 mM tert-butyl alcohol in this situation again results in OH radical scavenging via Reaction C. However, since e^{-}_{aq} and H are both unreactive with this alcohol (2, 37) they are available for further reaction with other added solutes.

In O_{2}-saturated solutions e^{-}_{aq} and H are both rapidly scavenged to form O_{2}^{2} leaving OH as the sole reactive radical.

The hydroxyl radical is a strong oxidizing agent and can act by several mechanisms, e.g., addition, and H atom or electron abstraction. The hydrated electron is a strong reducing agent generally acting by addition. The H atom is anomalous in that it can abstract H atoms (oxidation) or add (reduction).

Two carcinogenic arylacethydroxamic acids, N-hydroxy-AAF and N-hydroxy-AABP, have been studied in detail. Irradiation of each of the tRNA:carcinogen mixtures has been carried out where [RNA] > [C] using each of the scavenger systems listed in Table 1. The limited solubility of the arylacethydroxamic acids precluded study of the condition [C] > [RNA] for irradiation of the mixture, except by reducing the nucleic acid concentration. This method proved to be unsatisfactory from a quantitative viewpoint but was of qualitative value.

In an attempt to help understand basic mechanisms in these studies, preirradiation of the arylacethydroxamic acids in N_{2}O-saturated solutions and addition to tRNA, and the reverse procedure, have been investigated. Also, the carcinogenic acetamide analogs, AAF and AABP, and the O-glucuronide of N-hydroxy-AAF have been studied under oxidizing conditions (N_{2}O) where [RNA] > [C] and, in the case of the O-glucuronide, where [C] > [RNA].

Radiolysis of Carcinogen:tRNA Mixtures in N_{2}O-saturated Solution (Oxidizing Environment). The irradiation-induced binding of the ring-labeled arylacethydroxamic acids, N-hydroxy-AAF, N-hydroxy-AABP, and their corresponding acetamides to tRNA was determined in N_{2}O-saturated solutions containing mM phosphate buffer, pH 7. Chart 1 shows the percentage of radiolabel bound to tRNA as a function of radiation dose.

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Table 1: Primary water radicals and their yields produced in the radical scavenger systems used in this study

<table>
<thead>
<tr>
<th>Scavenger system</th>
<th>Primary radicals</th>
<th>Radical yield (molecules/100 eV)</th>
<th>Nature of environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>N_{2}O</td>
<td>OH, H</td>
<td>G(OH) = 5.4, G(H) = 0.7</td>
<td>Oxidizing</td>
</tr>
<tr>
<td>N_{2}O + 10 mM tert-butyl alcohol</td>
<td>H</td>
<td>G(H) = 0.7</td>
<td>Reducing and/or oxidizing</td>
</tr>
<tr>
<td>N_{2}O + 10 mM tert-butyl alcohol</td>
<td>e^{-}_{aq} H</td>
<td>G(e^{-}_{aq}) = 2.7, G(H) = 0.7</td>
<td>Reducing</td>
</tr>
<tr>
<td>O_{2}</td>
<td>OH</td>
<td>G(OH) = 2.7</td>
<td>Oxidizing</td>
</tr>
</tbody>
</table>

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Chart 1. Irradiation-induced binding of ring-labeled carcinogenic fluorenyl- and biphenylacethydroxamic acids and their corresponding acetamides (1 \muG/ml) to tRNA (1 mg/ml) in N_{2}O-saturated mM phosphate buffer, pH 7. 0, [9\text{-}^{14}C\text{-}N-hydroxy-AAF; •, [9\text{-}^{14}C\text{-}AABP; ◦, [3H\text{-}N-hydroxy-AABP; ❋, [3H\text{-}AAFP. krad, kilorads.
as a function of irradiation dose. The concentrations of carcinogen and tRNA used were 1 µg/ml and 1 mg/ml, respectively. The molarities of the hydroxamic acids and their acetamides fell within the range 4.2 to 4.7 µM. Based on a molecular weight of 25,000 to 30,000 (24), the tRNA concentration was 33 to 40 µM. Although the rate of reaction of OH with the arylacethydroxamic acids and the acetamides is unknown, it is unlikely to be greater than that for OH with nucleic acid which is diffusion controlled (41). It would therefore be reasonable to assume that the majority of OH radical reaction will take place with the tRNA under our experimental conditions.

\[
\text{RNA} + \cdot \text{OH} \rightarrow \text{RNA(OH)} \rightarrow \text{products} \quad (D)
\]

and that subsequent binding of carcinogen must be due to reaction with the unstable RNA(OH) radical and/or reactive final products. There is also the possibility that H atom-induced RNA intermediates and/or final products could be responsible for some of the observed adduct formation.

Additional evidence that the incorporation of label into tRNA represented the formation of covalent adducts came from experiments in which the nucleic acid was subjected to ion-exchange chromatography. Adducts formed by irradiation of N₂O-saturated solutions containing tRNA and N-hydroxy-AAF or N-hydroxy-AABP with a dose of 10 kilorads were isolated as described in “Materials and Methods,” dissolved in 0.05 M Tris:NaCl buffer (pH 7.4), and adsorbed to DEAE-cellulose. Greater than 70% of the radioactivity applied to the column was associated with the tRNA eluted with linear NaCl gradients (35).

The amount of radiolabel bound to tRNA increased linearly with irradiation dose up to ~10 kilorads and appeared to plateau after ~30 kilorads (Chart 1). The adduct formation for the arylacethydroxamic acids with tRNA was greater than that for their corresponding acetamides over the whole dose range. The fluorenylacethydroxamic acid and acetamide were also more reactive than the biphenyl analogs.

The difference in the reactivity between the fluorenyl and biphenyl derivatives is in general agreement with previous metabolic activation studies (3, 9, 14, 25, 26). The greater carcinogenicity of the arylacethydroxamic acids compared to their respective acetamides (33, 45) also parallels the formation of the irradiation-induced adducts.

Radiolysis of Arylacethydroxamic Acid: tRNA Mixtures in the Presence of N₂O and Other Scavenger Systems. In an attempt to evaluate the mechanism of irradiation-induced formation of the arylacethydroxamic acids N-hydroxy-AAF and N-hydroxy-AABP, the experiments in N₂O-saturated solution were repeated with compounds labeled in both the ring and acetyl group. This technique has been proven useful in determining the nature of the arylamine derivatives bound to nucleic acid following metabolic activation in vivo (15, 25, 27) and in vitro (10, 22). Further, the effect of the irradiation-induced chemical environment, e.g., oxidizing or reducing, was tested by measuring binding in the presence of other scavenger systems, i.e., N₂O:10 mM tert-butyl alcohol; N₄:10 mM tert-butyl alcohol; and O₂.

The results obtained upon irradiating both the fluorene and biphenyl double-labeled acethydroxamic acids in the presence of tRNA under each of the conditions listed in Table 1 are shown in Chart 2. Irrespective of the scavenger systems used, binding occurred with retention of both ring and acetyl radiolabels.

It is apparent from Chart 2 that the greatest amount of adduct formation occurred under N₂O-saturated conditions (oxidizing environment), where OH radicals predominate. However, when one takes into account the initial yield of primary radicals in the different scavenger systems (Table 1), it can be shown that H atoms are 2 to 3 times more efficient at inducing binding than OH. Further, it would appear that hydrated electrons, e⁻aq, do not react to form intermediates that can lead to binding.

In oxygen-saturated conditions incorporation of radiolabel was dramatically reduced for both compounds. Although the primary radical present is ·OH, which we have seen is effective at inducing binding, the small amount of binding can be explained by the fact that ·OH-induced intermediates will rapidly react with O₂ to form peroxo...
radicals (43) which are apparently less reactive in terms of binding than their precursors.

The results obtained here are in general agreement with those of Chapman et al. (7) who in a study of irradiation-induced binding of nitrofurazone to DNA found that OH is an efficient initiator of binding whereas $e_{aq}^-$ is not, and that $O_2$ suppresses binding, probably through peroxo radical formation. These data are in contrast, however, to previous studies that demonstrated that X-ray irradiation of physical complexes of 3,4-benzpyrene with DNA was equally effective in air or nitrogen in promoting the covalent combination of the hydrocarbon with the DNA (39).

The enzyme-induced binding of the arylacetohydroxamic acids to RNA is thought to involve enzyme-catalyzed chemical changes in the arylacetohydroxamic acid followed by interactions with the nucleic acid (9, 10, 22, 23). It was therefore of interest in the radiolysis experiments to determine whether the initial free radical damage could be channelled to the hydroxamic acid rather than the RNA. In principle this can be approached by altering the ratio of concentrations of carcinogen to RNA such that $[C] > [RNA]$. However, in practice this was limited by the low water solubility of the arylamine derivatives. Therefore, in order to achieve this condition ($[C] > [RNA]$) the [RNA] was reduced to 10 $\mu$g/ml (0.3 to 0.4 $\mu$M). The percentage binding of double-labeled N-hydroxy-AAF to tRNA under this concentration condition was much lower than that achieved when $[RNA] > [C]$ (Chart 3). Only in $N_2O$-saturated solutions was binding greater than 1% observed. In contrast to experiments at [RNA] of 1 mg/ml, adduct formation at high irradiation doses proceeded with considerable loss of the acetyl group. This difference in the relative incorporation of the 2 isotopes suggests that 2 or more reaction mechanisms are responsible for adduct formation. At the low [RNA]:[C] ratio it is possible that a certain fraction of OH and H radicals are reacting with the hydroxamic acid. This may account for the decrease in $^3H$-acetyl incorporation in the adduct relative to the $^1C$ ring.

Radiolysis of Arylacethydroxamic Acids Followed by Addition to tRNA. In order to determine whether or not binding could result from initial OH and H reaction with the arylacetohydroxamic acids, experiments were performed where the carcinogens were preirradiated and then added to tRNA. These experiments may be significant since binding of N-hydroxy-AAF and N-hydroxy-AABP to tRNA in vitro and in vivo is generally thought to involve enzymatic modification of the hydroxamic acid to some reactive form that will bind to the nucleic acid. On this basis double-labeled N-hydroxy-AAF (1 $\mu$g/ml) and N-hydroxy-AABP (1 $\mu$g/ml) were irradiated in $N_2O$-saturated solutions and added to tRNA to give a final tRNA concentration of 1 mg/ml. The adduct formation as a function of irradiation dose is shown in Chart 4. This binding is due primarily to reactive final products since the relatively long interval between irradiation and mixing (1 min) minimizes contributions due to carcinogen radicals. These results are in contrast to the observation that irradiation of nitrofurazone followed by addition to protein or polynucleotides did not result in binding (8).

Irradiation of N-hydroxy-AABP (Chart 4b) yields reac-

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**Chart 3.** Irradiation-induced binding of [9-$^1C-2'$-$^3H$]-N-hydroxy-AAF (1 $\mu$g/ml) to tRNA (10 $\mu$g/ml) in $N_2O$-saturated mM phosphate buffer, pH 7. 0. $^1C$ labeling of tRNA; 3$H$ labeling of tRNA. krads, kilorads.

**Chart 4.** a. radiolysis of [9-$^1C-2'$-$^3H$]-N-hydroxy-AAF (1 $\mu$g/ml) in $N_2O$-saturated mM phosphate buffer, pH 7, followed by addition to tRNA (1 mg). $O$, $^1C$ labeling of tRNA; $\bullet$, $^3H$ labeling of tRNA. b. radiolysis of [ring-$^3H-1'$-$^14C$]-N-hydroxy-AABP (1 $\mu$g/ml) followed by addition to tRNA (1 mg) in mM phosphate buffer, pH 7. Experimental conditions and designation of symbols are as described for a. krads, kilorads.

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tive products that react with tRNA to give mainly acetylated biphenylamine:RNA adducts, whereas N-hydroxy-AAF yields both acetylated and nonacetylated fluorenlylamine:RNA adducts (Chart 4a). The similarity in the relative incorporation of the 2 isotopes observed in the preirradiation of N-hydroxy-AAF (Chart 4a) to that in the irradiated tRNA:N-hydroxy-AAF mixture (Chart 3) supports the conclusion that a certain fraction of radical damage occurred at the hydroxylamine acid when the mixture was irradiated at the low [RNA]:[C] ratio.

It has been previously demonstrated that oxidation of a number of carcinogenic aromatic acetyhydroxamic acids, including N-hydroxy-AAF and N-hydroxy-AABP, by alkaline Fe(CN)$_6^-$, Ag$_2$O, and peroxidative enzymes yield nitrooxide free radicals which dismutate to the corresponding N-acetoxy-$N$-acyethylaminooxides and nitrosooxygenes (4, 5). N-Acetoxy-$N$-acyethylaminooxides readily react with RNA to give $N$-acyethylaminooxide:RNA adducts (28, 30). Similarly, incubation systems containing peroxidase, hydrogen peroxide, and N-hydroxy-AAF yielded $N$-acyethylaminolone:fluorene-substituted RNA (21).

It is possible that oxidation by OH free radicals, generated under our experimental conditions, could also give rise to the formation of reactive $N$-acetoxy derivatives. These reactive esters could account for formation of acetylaminooxid- and acetylated RNA adducts, but they could not be responsible for the aminoarene-substituted nucleic acids. However, attempts to identify $N$-acetoxy-AAF and $N$-acetoxy-AABP by irradiation of their corresponding acetyhydroxamic acids (1 $\mu$g/ml) followed by benzene extraction of the irradiated mixtures and subsequent TLC analysis of the benzene-soluble material were unsuccessful. In view of the instability of the $N$-acetoxy derivatives in water solutions (33), their presence as final products still remains a possibility.

Addition of the tRNA after irradiation of the fluorenyl- or biphenylhydroxamic acids reduced the labeling of the nucleic acid by more than 80% as compared to adduct formation induced on irradiation of RNA:carcinogen mixtures. This observation makes it seem unlikely that the incorporation of carcinogen into tRNA that occurred on irradiation of the mixtures resulted from the formation of carcinogen polymers that were coprecipitated with the nucleic acid.

Radiolysis of tRNA in $N_2O$-saturated Solution Followed by Addition to N-Hydroxy-AAF. Under the conditions of most of the experiments on RNA:carcinogen mixtures described above, the initial attack of the primary radical would be on the tRNA. Following this attack, binding could possibly occur from reaction of the RNA radical intermediates and/or final RNA radiolysis products with the carcinogen. The contribution of final products to adduct formation was determined by irradiating the tRNA under optimal conditions, i.e., $N_2O$-saturated, and adding [9-$^{14}C$-$^2$-$^{3}H$]-N-hydroxy-AAF, the most reactive carcinogen. At doses of 80 kilorads or less, no more than 2.5% of the carcinogen was incorporated into the nucleic acid. Further, as in the irradiation of the mixture (Chart 2a), no deacetylation occurred. These results strongly indicate that a majority of the binding obtained upon radiolysis of the mixture is due to reactions of reactive RNA free radical intermediates with the carcinogen.

Radiolysis of Mixtures of the O-Glucuronide of N-hydroxy-AAF and tRNA in $N_2O$-saturated Solutions. The O-glucuronide of N-hydroxy-AAF is a major metabolite of the parent acetamide and hydroxamic acid that is excreted in the urine and bile of several species (18, 19, 32). This conjugate has been considered as a possible candidate for modification of tissue macromolecules. This modification is thought to occur primarily by a 2-step reaction involving deacetylation and subsequent reaction of the O-glucuronide of N-2-fluroenylhydroxylamine with nucleophiles (13, 16, 17, 31). In view of the lability of the N-acetyl group, experiments were carried out with the O-glucuronide of the acetyhydroxamic acid labeled with $^{14}C$ in the ring and $^3$H in the acetyl group. The water solubility of the O-glucuronide of N-hydroxy-AAF permitted the study of the radiolysis of solutions where [C] > [RNA] by increasing [C] rather than decreasing [RNA].

The irradiation-induced binding of the O-glucuronide to tRNA was studied at 2 concentrations of the conjugate so that [C]:[RNA] was 0.065 or 2.5. Chart 5a shows the percentage of binding of the radiolabels upon radiolysis of these glucuronide:RNA mixtures. The incorporation of both $^{14}C$ and $^3$H was equivalent at each dose point. The percentage of binding was less for the higher value of [C]:[RNA]. However, a comparison of absolute amounts...
bound (Chart 5b) shows that there were ~10 times more bound at each dose point at the higher glucuronide concentration. The fact that increasing the glucuronide concentration by a factor of 39, which means more initial -OH attack at the glucuronide (and therefore less at RNA), resulted in only a 10-fold increase in binding implies that the efficiency of adduct formation following initial damage of the glucuronide was less than that following initial damage at the RNA. This parallels the observation in the irradiation-induced binding of nitrofurazone to DNA, where initial damage at the nucleic acid was more efficient in terms of adduct formation (7).

Comparison of adduct formation for N-hydroxy-AAF and its glucuronide, under conditions where [RNA] > [C], shows that the glucuronide was not as efficiently bound to tRNA as its aglycone N-hydroxy-AAF. These results may reflect the presence, in the glucuronide, of more potential sites for reactions with radicals that do not lead to adduct formation.

In conclusion, substantial adduct formation of tRNA with carcinogenic arylamine derivatives by ionizing radiation was demonstrated. Experiments designed to define their relationship to enzyme-induced RNA-arylamine adducts formed in vitro and in vivo have been initiated. Preliminary results indicate that irradiation-induced adduct formation occurs with each of the homopolymers of the major ribonucleotides and with DNA (J. L. Redpath, M. R. Thissen, and C. M. King, unpublished observations).

Although metabolic transformation of arylamine derivatives has been considered necessary for macromolecule modification, the present study suggests that free radical damage to macromolecules can also lead to combination. Irradiation-induced formation of arylamine nucleic acid adducts may offer an alternative method for the study of macromolecule modification in biological systems that are not capable of activating arylamine derivatives metabolically.

In the present experiments, measurable adduct formation was achieved with irradiation dose levels that have been used previously to study DNA repair in mammalian cells (11, 40). Chapman et al. (8) have reported the formation of trichloroacetic acid-insoluble derivatives of nitrofurazone on irradiation of intact mammalian cells. Consequently, it is not unreasonable to expect to be able to demonstrate nucleic acid adduct formation in intact cells. Such studies may be useful in attempting to understand the mechanism of the synergistic carcinogenic effects observed in vivo with ionizing radiation and N,N'-2,7-fluorenylenebisacetamide (36). It is not known whether similar synergistic effects exist for other aromatic amines.

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