INTRODUCTION

Binding of carcinogens, or their metabolites, to nucleic acid is of particular interest in view of the current trend toward attempting to relate aberrant conditions of an organism or system to genetic abnormalities. In addition to well-known theories on the biologic significance and the mechanism of this interaction, part of the reason for this has been the relatively large amount of nonspecific binding of the carcinogen, and some of its noncarcinogenic metabolites, to protein in both susceptible and nonsusceptible species (8, 26).

Attention of investigators was turned to the binding of carcinogens to RNA and the possible role of this interaction in the mechanism of carcinogenesis, with the preliminary report of Marroquin and Farber (16) which was later expanded (17) and confirmed by other investigators (2, 18, 27), that AAF was bound to liver RNA in species susceptible to AAF carcinogenesis.

This communication describes in detail results of an earlier report (27) on the binding of AAF and some related compounds to liver RNA in vivo, with further observations on the possible biologic significance and the mechanism of this interaction.

MATERIALS AND METHODS

Animals. Albino rats, weighing 180–200 gm at the time of injection, were obtained from the Holtzman Company, Madison, Wisconsin. Hartley strain albino guinea pigs, weighing 400–500 gm, were purchased from Abrams Small Stock Breeders, Chicago, Illinois, and the New Zealand white rabbit (1.7 kg) was obtained from Colonial Acres, Monett, Missouri. Male animals were used in all experiments. Rats were maintained on Purina laboratory chow and water ad libitum and the guinea pigs and rabbits were kept on Purina rabbit chow Checkers and water. The diet of the guinea pigs was supplemented with fresh lettuce daily.

Radioactive Compounds. The high-specific-activity AAF-9-14C (11.7 mc/mmmole) was synthesised in our laboratory from biphenyl-2-carboxylic-14C acid (25) which was obtained from New England Nuclear Corporation. The AAF-9-14C was purified by chromatography on basic aluminum oxide using benzene:

\[ \text{AAF-9-14C (11.7 mc/mmol)} \]

The abbreviations used are: AAF, 2-acetylaminofluorene; \( N \)-hydroxy-AAF, \( N \)-hydroxy-2-acetylaminofluorene; 1-hydroxy-AAF, 1-hydroxy-2-acetylaminofluorene; \( N \)-hydroxy-AAF, \( N \)-hydroxy-2-aminofluorene.

CHARLES C. IRVING, RICHARD A. VEAZEY, AND ROBERT F. WILLIARD

Veterans Administration Hospital, Memphis, Tennessee 38115

SUMMARY

Previous reports that radioactivity was bound to liver RNA after administration of 2-acetylaminofluorene-9-14C or \( N \)-hydroxy-2-acetylaminofluorene-9-14C in species susceptible to the carcinogenic action of 2-acetylaminofluorene have been confirmed. Injection of 1-hydroxy-2-acetylaminofluorene-1-14C, a noncarcinogenic metabolite of 2-acetylaminofluorene, did not result in any binding of radioactivity to rat-liver RNA. On the other hand, 2-acetylaminofluorene-9-14C was bound to liver RNA of the rabbit, a species which does not contract liver cancer upon administration of 2-acetylaminofluorene. In addition, \( N \)-hydroxy-2-acetylaminofluorene-9-14C was bound to liver RNA of the guinea pig, which does not develop liver cancer after administration of \( N \)-hydroxy-2-acetylaminofluorene.

Sucrose density gradient sedimentation studies of purified rat-liver RNA, isolated after injection of 2-acetylaminofluorene-9-14C of high specific radioactivity, revealed that there was uniform labeling of the ribosomal RNA but that the soluble RNA had a specific radioactivity which was 2–2.5 times that of ribosomal RNA.

Following administration of either \( N \)-hydroxy-2-acetylaminofluorene-9-14C or \( N \)-hydroxy-2-acetylaminofluorene-1-14C, it was observed that an equal amount of radioactivity was bound to rat-liver RNA. Since the radioactivity bound to the RNA after injection of \( N \)-hydroxy-2-acetylaminofluorene-1-14C represented the acetyl group of the compound injected, these data exclude the participation of the metabolite \( N \)-hydroxy-2-aminofluorene in the mechanism of binding of \( N \)-hydroxy-2-acetylaminofluorene to rat-liver RNA to any significant extent in vivo.

On the Significance and Mechanism of the Binding of 2-Acetylaminofluorene and \( N \)-Hydroxy-2-acetylaminofluorene to Rat-Liver Ribonucleic Acid in Vivo

CHARLES C. IRVING, RICHARD A. VEAZEY, AND ROBERT F. WILLIARD

Veterans Administration Hospital, Memphis, Tennessee 38115

SUMMARY

Previous reports that radioactivity was bound to liver RNA after administration of 2-acetylaminofluorene-9-14C or \( N \)-hydroxy-2-acetylaminofluorene-9-14C in species susceptible to the carcinogenic action of 2-acetylaminofluorene have been confirmed. Injection of 1-hydroxy-2-acetylaminofluorene-1-14C, a noncarcinogenic metabolite of 2-acetylaminofluorene, did not result in any binding of radioactivity to rat-liver RNA. On the other hand, 2-acetylaminofluorene-9-14C was bound to liver RNA of the rabbit, a species which does not contract liver cancer upon administration of 2-acetylaminofluorene. In addition, \( N \)-hydroxy-2-acetylaminofluorene-9-14C was bound to liver RNA of the guinea pig, which does not develop liver cancer after administration of \( N \)-hydroxy-2-acetylaminofluorene.

Sucrose density gradient sedimentation studies of purified rat-liver RNA, isolated after injection of 2-acetylaminofluorene-9-14C of high specific radioactivity, revealed that there was uniform labeling of the ribosomal RNA but that the soluble RNA had a specific radioactivity which was 2–2.5 times that of ribosomal RNA.

Following administration of either \( N \)-hydroxy-2-acetylaminofluorene-9-14C or \( N \)-hydroxy-2-acetylaminofluorene-1-14C, it was observed that an equal amount of radioactivity was bound to rat-liver RNA. Since the radioactivity bound to the RNA after injection of \( N \)-hydroxy-2-acetylaminofluorene-1-14C represented the acetyl group of the compound injected, these data exclude the participation of the metabolite \( N \)-hydroxy-2-aminofluorene in the mechanism of binding of \( N \)-hydroxy-2-acetylaminofluorene to rat-liver RNA to any significant extent in vivo.
methanol (99:1) as an eluant. The solvent was evaporated from the fractions containing AAF-9-14C, and the compound was dissolved in ethanol:benzene (1:1) at a concentration of 2–3 mg/ml and stored at —20°C. This was essential in order to prevent radiation decomposition of the high-specific-activity AAF-9-14C. AAF-9-14C, having a lower specific activity, was either prepared from 2-nitrofluorene-9-14C (obtained from Tracerlab) or obtained by dilution of the high-specific-radioactivity AAF-9-14C with unlabeled AAF. N-Hydroxy-AAF-9-14C was obtained from Tracerlab and diluted with unlabeled N-hydroxy-AAF, then recrystallized from water before use. N-Hydroxy-AAF-1-14C (6) and 1-hydroxy-AAF-1-14C (7) were synthesized as previously described. The radiochemical purity of each of the compounds was established by both paper and thin-layer chromatography (5, 8).

**Administration of Radioactive Compounds.** In most cases, the radioactive compounds were dissolved in corn oil (2–3 mg/ml) and administered i.p. at a dose level of 20 mg/kg body weight. In some experiments, the compounds were dissolved or suspended in 0.9% NaCl for the injections. The animals were allowed food and water after the injections and were killed at 24 hr for the isolation of RNA from the liver.

**Extraction of Liver RNA.** Our initial studies were begun using the procedure of Kirby (10, 11) before it was known or reported that ribonuclease survivors the phenol extraction when RNA is isolated by this method. This procedure has been designated Method K. Following the reports that the RNA isolated by this procedure was degraded (3, 12), other methods were used to isolate RNA from the total liver (Method A) and from the postmitochondrial cytoplasmic fraction of liver (Method B). A third procedure was used for the direct extraction of ribosomal RNA from liver (Method C). Since the methods were adapted and modified from several published procedures (1, 12, 13, 24), each will be briefly described.

**Method A.** Rapidly frozen liver (7–8 gm) was homogenized at 10–15°C with 80 ml of phenol:creosol mixture (13) and 80 ml of 0.5% 1,5-naphthalenedisulfonic acid disodium salt in a Servall Omnimixer at top speed for 2 min. The mixture was transferred to a flask, stirred for 20 min at 25°C, and then centrifuged at 40,000 × g for 25 min. The supernatant solution was removed and made 1% in sodium dodecyl sulfate, then reextracted with 0.5 volume of the phenol:creosol mixture for 15 min at 25°C. Following recentrifugation at 40,000 × g for 25 min, the supernatant solution was removed, made 0.1 M with respect to NaCl, and then 2 volumes of ethanol were added (1). After the mixture had stood at —10°C for 2 hr, the crude RNA was collected by centrifugation and then purified at 2–4°C according to a modification of the procedure of Ralph and Bellamy (24). The cetyltrimethylammonium salt of RNA was dissolved in 3 ml of water, then 2 ml of 0.3 M sodium acetate and 10 ml of ethanol were added. The sodium salt of the RNA was collected by centrifugation, and this procedure was repeated 2 more times. The purified RNA was finally washed with 75% ethanol, then twice with 95% ethanol, and dried in vacuo over calcium chloride.

**Method B.** RNA was isolated from the postmitochondrial cytoplasmic fraction of liver exactly as described by DiGirolamo et al. (1). However, since the animals were not fasted in our studies, the RNA contained considerable amounts of glycojen, which was removed by the procedure of Ralph and Bellamy as modified above.

**Method C.** Ribosomal RNA was isolated by direct extraction of the liver according to Method 2 described by Kirby (13) except that the centrifugations during the phenol extractions were carried out at 40,000 × g for 25 min.

The average yields of purified RNA (from 7 to 8 gm of liver) were 20 mg using Methods A and C and 10–12 mg with Method B. The mean F1% values at 260 μM (in water) for the RNA samples isolated by the 3 methods were: Method A, 205; Method B, 203; and Method C, 196.

**Sucrose Density Gradient Sedimentation Studies.** The RNA samples (2–4 mg)4 were layered on 28-ml linear sucrose gradients (5–20%) containing 0.1 M NaCl and 0.01 M sodium acetate buffer, pH 5.0. Following centrifugation in the Spinco model L centrifuge (SW 25.1 rotor) at 25,000 rpm for 13 hr, the samples were fractionated into 1-ml fractions taken from the bottoms of the tubes. The absorbance of each fraction at 260 μM was measured and the amount of radioactivity in the entire fraction was determined as described below.

**Radioactivity Determinations.** Radioactivity determinations were carried out as previously described (8). In initial studies, the radioactivity of the RNA samples was determined by the Schoniger combustion technic (22), but later a simpler

![Table 1](https://cancerres.aacrjournals.org/)

<table>
<thead>
<tr>
<th>Species</th>
<th>Method for RNA Isolation</th>
<th>Binding to RNA (μmoles/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AAF-9-14C</td>
</tr>
<tr>
<td>Rat</td>
<td>K</td>
<td>36.6 ± 3.3 (4)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>A</td>
<td>27.4 ± 2.8 (5)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>K</td>
<td>12.8 (1)</td>
</tr>
</tbody>
</table>

*a AAF, 2-acetylaminofluorene; N-hydroxy-AAF, N-hydroxy-2-acetylamino fluorene; 1-hydroxy-AAF, 1-hydroxy-2-acetylamino fluorene.

*b For the experiments in which RNA was isolated from rat liver by Method K, livers from 2 animals were worked up together. This was repeated using 2 additional rats and the S.D. given is that of the mean value of the 2 experiments using the 4 rats. In all other experiments in this table and the others, the livers of individual animals were worked up separately, and the values given are the mean ± S.D., using the number of animals indicated in parentheses.

*The total radioactivity in 4–5 mg of RNA was less than 10 cpm above background (30 cpm) with a counting efficiency of 50%.

APRIL 1967

721
Charles C. Irving, Richard A. Veazcy, and Robert F. Williard

CHART 1. Sedimentation patterns of rat-liver RNA isolated after injection of 2-acetylaminofluorene-9-\(^{14}\)C (11.7 mc/mmole). Details concerning the injections are described in the legend for Table 2. Centrifugations were through 28 ml of linear 5-20% sucrose gradients containing 0.10 M NaCl-0.01 M sodium acetate, pH 5.0, at 25,000 rpm for 13 hr in the Spinco model L centrifuge (SW 25.1 rotor). Fractions of 1 ml were collected from the bottom of the tube, which is to the left of each figure. A, Total liver RNA, isolated by Method A; 1380 dpm/mg. B, Postmitochondrial cytoplasmic RNA, isolated by Method B; 776 dpm/mg. C, Ribosomal RNA, isolated by Method C; 726 dpm/mg.

RESULTS

Attempted Correlation of the Binding of \(^{14}\)C-labeled AAF and Metabolites to Liver RNA and Carcinogenicity. The binding of radioactivity to rat liver RNA after administration of \(N\)-hydroxy-AAF-9-\(^{14}\)C was 2-4 times greater than that observed after giving AAF-9-\(^{14}\)C and isolation of the RNA under comparable conditions (Table 1). These data confirm the observations of Marroquin and Farber (17) on the in vivo binding of AAF-9-\(^{14}\)C and \(N\)-hydroxy-AAF-9-\(^{14}\)C to rat-liver RNA. Although the RNA isolated by Method K was degraded, as shown by sucrose density gradient centrifugation, there was no significant difference in the level of radioactivity bound to the RNA after administration of AAF-9-\(^{14}\)C or \(N\)-hydroxy-AAF-9-\(^{14}\)C as compared to that found in the undegraded RNA isolated by Method A.

In marked contrast to the results obtained after administration of \(^{14}\)C-labeled AAF or \(N\)-hydroxy-AAF to rats, radioactivity was not bound to rat-liver RNA after i.p. injection of equivalent doses of 1-hydroxy-AAF-1-\(^{14}\)C, a noncarcinogenic metabolite of AAF in the rat (21) (Table 1). Identical results were obtained using either Method K or Method A for isolation of the RNA. Bound radioactivity was not detectable in RNA isolated from guinea-pig liver 24 hr after injection of AAF-9-\(^{14}\)C (Table 1). On the other hand, after the i.p. injection of \(N\)-hydroxy-AAF-9-\(^{14}\)C in guinea pigs, bound radioactivity equivalent to 20 \(\mu\)moles of \(N\)-hydroxy-AAF per mg of liver RNA was found. In a single experiment, a small level of bound radioactivity was found in rabbit liver RNA after the i.p. injection of AAF-9-\(^{14}\)C (Table 1).

Sucrose Density Gradient Centrifugation Studies of Rat Liver RNA Isolated after Injection of High Specific Activity AAF-9-\(^{14}\)C. Twenty-four hr after the i.p. injection of AAF-9-\(^{14}\)C (specific radioactivity, 11.7 mc/mmole) in rats, RNA was isolated from the liver or a liver cell fraction by 1 of 3 methods and subjected to centrifugation in linear gradients of 5-20% sucrose as described under Materials and Methods. The results, shown in Chart 1, revealed that radioactivity was bound to all components of the RNA samples obtained from the total liver (Method A), from the postmitochondrial cytoplasmic fraction of the liver (Method B), or by direct extraction of ribosomal RNA from total liver (Method C). Quantitative data on the binding of radioactivity to RNA isolated by the three methods are given in Table 2. The apparently higher binding to RNA (52 \(\mu\)moles/mg) isolated from the total liver by Method A (Table 2) was not statistically significant with the number of animals used. Other experiments in which RNA was isolated from the total liver by Method A, using a larger number of rats (Table 1), but with AAF-9-\(^{14}\)C of lower specific radioactivity, showed levels of binding to RNA comparable to those obtained.
TABLE 2

Binding of Radioactivity of 2-Acetylaminofluorene-9-14C to Rat Liver RNA Fractions in Vivo

The specific radioactivity of the 2-acetylaminofluorene-9-14C was 11.7 mc/m mole and the amount injected in each experiment was: total liver RNA, 201 µc; postmitochondrial cytoplasmic RNA, 105 µc; and ribosomal RNA, 108 µc. The RNA was isolated 24 hr after the injection by the method indicated and the specific radioactivity (µmoles/mg) was determined. The RNA was subjected to sucrose density gradient centrifugation as described in Chart 1 and the relative specific radioactivities of the 28 S, 18 S, and 4 S components were calculated. Two rats were used in each experiment.

<table>
<thead>
<tr>
<th>RNA Fraction</th>
<th>Method for RNA isolation</th>
<th>Binding to RNA (µmoles/mg)</th>
<th>Relative specific radioactivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>28 S</td>
<td>18 S</td>
</tr>
<tr>
<td>Total liver RNA</td>
<td>A</td>
<td>52.0 ± 1.0 ²</td>
<td>1.2</td>
</tr>
<tr>
<td>Postmitochondrial cytoplasmic RNA</td>
<td>B</td>
<td>27.1 ± 0.9 ²</td>
<td>1.0</td>
</tr>
<tr>
<td>Ribosomal RNA</td>
<td>C</td>
<td>33.0 ± 3.0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Specific radioactivities of the RNA components are expressed relative to the 18 S component.

² Standard deviation.

by Methods K, B, and C. Calculation of the relative specific radioactivities of the ribosomal RNA and the soluble RNA of rat liver isolated after injection of AAF-9-14C revealed that there was almost uniform labeling of the 28 S and 18 S components of the ribosomal RNA (Table 2). However, the soluble RNA (4 S) had a specific radioactivity which was 2.2-2.5 times that of the ribosomal RNA. These findings are similar to those reported by Henshaw and Hiatt (2).

Preliminary Observations on the Identification of the Fluorene Moiety Bound to Liver RNA. After the i.p. injection of N-hydroxy-2-acetylaminofluorene-9-14C (labeled in the fluorene ring) and of N-hydroxy-AAF-1-14C (labeled in the N-acetyl group) of approximately equal specific radioactivity to rats and isolation of the RNA from the total liver (Method A), it was found that there was no significant difference in the amount of radioactivity bound to the RNA in each case (Table 3). The radioactivity found in the RNA after administration of N-hydroxy-AAF-1-14C could have been due to: (a) binding of a metabolite of N-hydroxy-AAF which retained the N-acetyl group of the compound injected; or (b) incorporation of 14CO2 into the RNA by de novo synthesis of the purine or pyrimidine bases. Therefore, an experiment was devised to evaluate the relative contributions of these two alternatives to the labeling of the RNA. Since 14CO2 could arise from N-hydroxy-AAF-1-14C by the oxidation of acetate-1-14C formed either by direct deacetylation of the N-hydroxy-AAF-1-14C or by reduction of N-hydroxy-AAF-1-14C followed by deacetylation of the AAF-1-14C, the extent of incorporation of equivalent amounts of sodium acetate-1-14C into RNA was measured. Injection of 61 µc of sodium acetate-1-14C (4.52 mc/m mole) under identical conditions used for the administration of N-hydroxy-AAF-1-14C led to the binding of radioactivity equivalent to only 5 µmoles of sodium acetate per mg of RNA; this was less than 1/20th the binding found after the injection of 70 µc of N-hydroxy-AAF-1-14C (4.52 mc/m mole)

TABLE 3

Binding of Radioactivity to Rat-Liver RNA in Vivo after Injection of N-Hydroxy-2-acetylaminofluorene-9-14C, N-Hydroxy-2-acetylaminofluorene-1-14C or Sodium Acetate-1-14C

The specific radioactivities of the compounds used were: N-hydroxy-AAF-9-14C, 4.01 mc/m mole; N-hydroxy-AAF-1-14C, 4.52 mc/m mole; and sodium acetate-1-14C, 4.18 mc/m mole. The RNA was isolated by Method A at 24 hr after injection of the labeled compound.

<table>
<thead>
<tr>
<th>Compound injected</th>
<th>Vehicle for injection</th>
<th>Dose (µc) injected</th>
<th>Binding of 14C to RNA (µmoles/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Hydroxy-AAF-9-14C</td>
<td>Corn oil</td>
<td>61</td>
<td>114 ± 5* (3)²</td>
</tr>
<tr>
<td>N-Hydroxy-AAF-1-14C</td>
<td>Corn oil</td>
<td>70</td>
<td>106 ± 24 (3)²</td>
</tr>
<tr>
<td>Sodium acetate-1-14C</td>
<td>0.9% NaCl</td>
<td>61</td>
<td>5.1 ± 0.0 (2)</td>
</tr>
<tr>
<td>Sodium acetate-1-14C</td>
<td>0.9% NaCl</td>
<td>81</td>
<td>14.4 ± 0.2 (2)</td>
</tr>
<tr>
<td>Sodium acetate-1-14C</td>
<td>Corn oil</td>
<td>600</td>
<td>93 (1)</td>
</tr>
<tr>
<td>N-Hydroxy-AAF-1-14C plus unlabeled sodium acetate</td>
<td>Corn oil</td>
<td>52</td>
<td>71 (1)</td>
</tr>
</tbody>
</table>

* The N-hydroxy-AAF-1-14C was labeled with 14C in the carboxyl carbon of the N-acetyl group.

² N-Hydroxy-AAF, N-hydroxy-2-acetylaminofluorene.

³ Standard deviation.

Table 3. Even upon using saline as a vehicle for the injection instead of corn oil, radioactivity equivalent to only 14 µmoles of sodium acetate per mg of RNA was found after administration of the 14C-labeled sodium acetate. Only after administration of 10 times as much sodium acetate-1-14C, on a molar basis, did the level of radioactivity in rat-liver RNA approach that found after administration of N-hydroxy-AAF-1-14C. Furthermore, the administration of 500 µmoles of unlabeled sodium acetate at 30 min prior to the injection of N-hydroxy-AAF-1-14C (11.5 µmoles) did not significantly reduce the level of radioactivity which became bound to rat-liver RNA. Thus it appeared that only a small fraction of the radioactivity found in rat-liver RNA after administration of N-hydroxy-AAF-1-14C could have arisen by incorporation of 14CO2 into the RNA, the major fraction having resulted from the interaction of some fluorene metabolite.
which retained the labeled N-acetyl group, with the RNA. This was more clearly demonstrated by the results of the experiments presented in Table 4. The 14C-labeled RNA sample obtained from rat liver after injection of 75 μC of N-hydroxy-AAF-14C was subjected to hydrolysis by refluxing in 6 N H2SO4 overnight. Upon steam distillation of the hydrolysate, 70% of the radioactivity was found in the distillate. These findings were consistent with the hypothesis that the labeled RNA isolated after the injection of N-hydroxy-AAF-14C contained a 14C-labeled acetyl group. On the other hand, acid hydrolysis of the 14C-labeled RNA sample isolated after injection of 600 μC of sodium acetate-14C, followed by steam distillation of the hydrolysate, revealed that only 1% of the radioactivity was carried over in the distillate. This was consistent with the fact that the labeled carbon, in this case, was in the purine or pyrimidine ring of the bases of the RNA. This experiment was repeated on additional samples of RNA with identical results.

DISCUSSION

The sucrose density gradient centrifugation analyses of the purified RNA obtained from rat liver by 3 different methods following the administration of AAF-9-14C of high specific radioactivity afford corroboration evidence that some metabolite of AAF is indeed bound to liver RNA after administration of the carcinogen to rats (2, 16–18, 27). There was uniform labeling of the ribosomal RNA but the soluble RNA had a specific radioactivity (dpm/mg RNA) 2–2.5 times that of the ribosomal RNA; this was also reported by Henshaw and Hiatt (2). These investigators indicated that the pattern of labeling which they observed suggested attachment of the fluorene derivative to preformed RNA rather than incorporation during synthesis. One approach available for determining the nature of the interaction of the fluorene derivative with rat-liver RNA involves studying the reactivity of known and of hypothetic metabolites with nucleic acid in vitro and attempting to determine the site and mechanism of any interaction observed. Preliminary reports of such studies have recently been made by Kriek (14) and by Miller et al. (19).

Kriek showed that the highly reactive metabolite N-hydroxy-AAF, which can be derived from N-hydroxy-AAF by enzymatic deacetylation (6), reacted nonenzymatically at pH 4–5 with yeast-soluble RNA. However, the relevance of this observation to the mechanism of interaction of fluorene derivatives with RNA in vivo is not clear, since no significant interaction of N-hydroxy-AAF with the yeast RNA occurred at physiologic pH. Our data on the comparative binding of N-hydroxy-2-acetylaminofluorene-14C and N-hydroxy-2-acetylamino-fluorene-1-14C would exclude N-hydroxy-AAF from having a significant role in the mechanism of binding of N-hydroxy-AAF to RNA in vivo. The results would further indicate that any mechanism which is postulated for the binding of N-hydroxy-AAF to rat-liver RNA in vivo must take into account the fact that the acetyl group of the compound injected is retained in the interaction.

Lotlikar et al. (15) reported on the nonenzymatic interaction of N-acetyloxy and N-aroyloxy derivatives of AAF with methionine in vitro. The mechanism proposed for the interaction of the acyloxy derivatives with methionine, or proteins containing methionine, as well as the product observed in the interaction (3-SCH3-AAF) resulted in the retention of the N-acetyl group of the fluorene derivative. Evidence that this type of reaction might occur in vivo has been described by J. A. Miller. Upon treatment of liver protein, isolated after administration of AAF to rats, with alkali, 3-SCH3-AAF was obtained as a product. This could only be interpreted to indicate that an N-acetyl group was on the fluorene moiety which was bound to the protein in vivo. More recently, Miller et al. (19) described the reaction of N-acetoxy-AAF with DNA and RNA in vivo. Although these reactive esters of N-hydroxy-AAF (15, 19) have not been identified as metabolites of either AAF or N-hydroxy-AAF, there is one known O-substituted derivative of N-hydroxy-AAF which is found in the urine and bile of susceptible species as a major excretory product, namely the O-glucuronide of N-hydroxy-AAF. The potential role of the chemical reactivity of C—O—N glucuronides of N-hydroxy metabolites in the mechanism of carcinogenesis should be considered (9).

The biological significance of the observed interaction of AAF and N-hydroxy-AAF with rat liver RNA in vivo remains to be evaluated. In general, the findings are consistent with the supposition that binding of carcinogenic fluorene derivatives to RNA is related to the mechanism of action of these compounds, but obviously no causal relationship has been established, or claimed, between the two phenomena. Thus, a number of conditions in which the degree of binding of AAF or N-hydroxy-AAF to liver RNA in vivo has been correlated with the incidence of liver cancer after feeding these compounds have been provided in these studies and by those of Marroquin and Farber (17). On the other hand, data obtained from several experiments do not support the hypothesis that where there is binding of isotope to liver RNA after administration of 14C-labeled fluorene compound, liver cancer has been shown to be induced in that species by that compound and vice versa. For example, radioactivity was bound to liver RNA after administration of N-hydroxy-AAF-9-14C to the guinea pig, a species in which liver cancer is not induced by administration of N-hydroxy-AAF (20). Furthermore, a detectable amount of radioactivity was found in rabbit liver RNA following the administration of AAF-9-14C. The rabbit, which is capable of N-hydroxylation of AAF (4, 5), does not get liver cancer after feeding AAF or N-hydroxy-AAF (9).

Although we reported preliminary data on the binding of radioactivity to rat liver DNA after administration of AAF-9-14C or N-hydroxy-AAF-9-14C (27) and the observations of others would seem to confirm this (17), we do not yet have convincing evidence that the DNA was entirely free of RNA and protein. This will be the subject of another report. Recently, Sporn4 also indicated that radioactivity became bound to rat-liver DNA after the administration of AAF-9-14C.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Edgar C. Henshaw for supplying, prior to publication, some details of their experiments on the binding of AAF to specific rat-liver RNA fractions.

REFERENCES


On the Significance and Mechanism of the Binding of 2-Acetylaminofluorene and \( N \)-Hydroxy-2-acetylaminofluorene to Rat-Liver Ribonucleic Acid \textit{in Vivo}

Charles C. Irving, Richard A. Veazey and Robert F. Williard