Metabolism of 2-Aminofluorene and 2-Acetylaminofluorene to Mutagens by Rat Hepatocyte Nuclei

Daniel L. Stout and Frederick F. Becker

Department of Anatomic and Research Pathology, M. D. Anderson Hospital and Tumor Institute, The University of Texas System Cancer Center, Houston, Texas 77030

ABSTRACT

The Ames assay was used to determine the ability of rat hepatocyte nuclei to metabolize 2-aminofluorene (AF) and 2-acetylaminofluorene (AAF) to mutagens. When hepatocyte nuclei and hepatic postmitochondrial fraction (S9) were compared, it was found that the distribution of these metabolic activities was unique for each fraction. Nuclei produced approximately 1260% more revertants with AF than with AAF, whereas S9 produced 1640% more mutants with AF than with AAF. Pretreatment with phenobarbital or methylcholanthrene, however, altered metabolic activity in a manner which was specifically unique for each fraction. Phenobarbital pretreatment increased nuclear activation of AF and AAF to mutagens by 400 and 460%, respectively. Methylcholanthrene pretreatment increased nuclear activation of AF by 125%. Methylcholanthrene pretreatment increased nuclear activation of AAF by 69%, whereas AAF activation increased by 375%. S9 activation of AAF was not altered by methylcholanthrene pretreatment, but AF activation was increased by 41%. In a similar group of rats exposed to a carcinogenic regimen of AAF diet, nuclear activation of AF and AAF to mutagens was increased during the first month of feeding by 28 and 85%, respectively. However, after 16 weeks on the carcinogen regimen, nuclear activation of AF and AAF was reduced to 30 and 19% of control values, respectively. Thus, as has been demonstrated for other carcinogens, the hepatocyte nuclei are capable of activating aromatic amines to their mutagenic forms. The data suggest strongly that the metabolic pathways for the activation of AF and AAF differ and that AF may play a prominent role in mutagenesis.

INTRODUCTION

Carcinogenic aryl hydrocarbons are oxidized to quinones, dihydrodiols, and phenols by a monooxygenase system associated with the endoplasmic reticulum. Their carcinogenicity is attributed to the formation of electrophilic intermediates which alkylate DNA and other critical macromolecules in the cell (6, 11, 13). The carcinogenicity of the arylamines AF and AAF is dependent upon the formation of N-hydroxy derivatives by the microsomal monooxygenase system (13, 14). The N-hydroxy derivatives can also form electrophilic species which alkylate macromolecules (13, 14, 16, 21). Because of this capacity to convert exogenous substances into carcinogens and mutagens, the monooxygenase system has been studied extensively. Of particular interest is the observation that the activity of the system and the spectrum of metabolites may be modified by a variety of chemical substances (8). This phenomenon is thought to be an important factor in determining individual risk or tissue susceptibility to chemical carcinogens.

It was recently demonstrated that hepatic nuclei have a monooxygenase system similar to that of the microsomal system (5, 9, 10). Incubation of nuclei with benzo(a)pyrene resulted in DNA alkylation products identical with those produced by the microsomal arylhydroxylases (5). The nuclear system also responded to arylhydroxylase-inducing agents, but not in parallel with the microsomal system (9, 10). Whereas microsomal arylhydroxylases were induced by both PB and MC, only MC stimulated the nuclear arylhydroxylases. Thus, regulation of the nuclear monooxygenase system appears to be independent of the microsomal system. Although the nuclear membrane activity represents only a small fraction of the total cellular capacity for carcinogen activation (5), its intimate association with the genetic apparatus suggests that it could play an important role in chemical carcinogenesis.

It has been demonstrated with the Ames assay that reversion of Salmonella typhimurium TA1538 with AF or AAF is dependent upon microsomal N-hydroxylation (2, 12, 17). We previously used this assay to study the alteration of microsomal capacity to produce mutagen from AAF during hepatocarcinogenesis (18). In this study, we again used the Ames assay to determine whether or not whole hepatocyte nuclei could produce mutagen from AF and AAF. The influence of PB and MC on nuclear and S9 activation of AF and AAF to mutagens was examined. Hepatocyte nuclei were also obtained from rats during hepatocarcinogenesis to determine whether change in nuclear activity occurred in a manner similar to that previously seen with S9.

MATERIALS AND METHODS

Animals and Diets

Male CFE Sprague-Dawley rats were obtained at approximately 100 g body weight from Charles River Farms (Wilmington, Mass.). Those rats receiving the carcinogen regimen were fed a fully nutritious synthetic diet (no. 101; Bio-Serv, Inc., Frenchtown, N.J.) for 1 week prior to the regimen. The regimen, consisting of cycles of 3 weeks of carcinogen (0.06% AAF) followed by 1 week of normal diet with 4 cycles representing a fully carcinogenic exposure.

1 Supported by Research Grant CA 20657 from NIH. Presented originally, in part, at the Sixty-ninth Annual Meeting of the American Association for Cancer Research in Washington, D.C., April 5 to 8, 1978 (19).
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: AF, 2-aminofluorene; AAF, 2-acetylaminofluorene; PB, phenobarbital; MC, 3-methylcholanthrene; S9, postmitochondrial fraction; S150, postmicrosomal fraction.

Received February 13, 1978; accepted December 27, 1978.
Nuclear Activation of Arylamines

determined by incubating fixed amounts of nuclei and S9 with 112.5, 225, and 450 nmol of AF and AAF per plate. Approximately $9 \times 10^6$ nuclei suspended in S150 were added per plate, while 1.5 mg of S9 protein per plate were incubated with AF, and 3.0 mg S9 protein per plate were incubated with AAF. The results are shown in Charts 1 and 2. No significant increase in reversion occurred when AF or AAF were used at concentrations greater than 225 nmol/plate with nuclei and S9. AF alone was found to increase the reversion frequency about 20 times above the spontaneous reversion frequency when used at a concentration of 450 nmol/plate (data not shown). Therefore, the concentration of 225 nmol/plate of AF or AAF was chosen for the remaining studies to minimize reversion caused directly by AF itself.

Using AF and AAF at a concentration of 225 nmol/plate, various amounts of nuclei and S9 were added to determine a suitable working range for each of the two liver fractions. With both AF and AAF, the number of nuclei used ranged from 55 to 170 $\times 10^6$ per plate. S9 was incubated with AF over the range of 0.5 to 2.0 mg protein per plate, whereas 0.8 to 5.0 mg S9 protein per plate were incubated with AAF. The results are shown in Charts 3 and 4. With AF, a near-linear relationship between the number of revertants per plate and the number of nuclei per plate was maintained.

and resultant tissue alterations, have been described in detail (4, 18, 20).

Tissue Preparation

**Microsomal Fraction (S9).** Animals were sacrificed by decapitation, and the livers were removed aseptically, chilled, weighed, and homogenized in a Thomas glass-Teflon tissue homogenizer with 3 volumes (w/v) of cold sterile 0.15 M KCl. After centrifugation at 9,000 $\times g$ for 10 min, a portion of the supernatant fraction (S9) was decanted into sterile tubes and frozen for use the following day. A second portion of S9 was centrifuged at 150,000 $\times g$ for 1 hr. The supernatant fraction was passed through sterile filter membranes, 0.45-$\mu$m pore size, and refrigerated until it was used to prepare nuclear suspensions. Utilizing this method, approximately 81 mg of S9 protein were obtained per g of liver.

**Hepatocyte Nuclei.** Nuclei were prepared aseptically by the procedure of Becker (3). Livers were chilled and minced in 0.9% NaCl solution with scalpels. After rinsing and weighing, the fragments were homogenized briefly in homogenizing medium (0.25 M sucrose—0.025 M MgCl2—0.01 M Tris-HCl, pH 7.4). The homogenate was filtered through gauze and diluted 1:1 with homogenizing medium. The dilute filtrate was spun at 2000 rpm on an International PR2-269 head for 15 min at 2°C. The pellet was then suspended in 3 volumes of dense sucrose (4 molal sucrose—0.01 M Tris-HCl, pH 7.4—0.005 M KCl—0.025 M MgCl2). Fifteen ml of the suspension were layered over 23 ml of dense sucrose in polyallomer tubes suitable for use in the Beckman SW27 rotor and centrifuged at 23,000 rpm for 1 hr. The supernatant was aspirated off, and the sides of the tubes were cleaned with cotton swabs. Utilizing this method, approximately 300 $\times 10^6$ nuclei were obtained per g of liver.

Because a soluble enzyme is required to convert the N-hydroxy derivative of AAF to mutagen (12, 17, 21), nuclei were suspended in a postmicrosomal supernatant fraction. Two ml of the postmicrosomal supernatant were layered over the pellets, and the tubes were placed in the refrigerator overnight. The following morning the nuclei were suspended by gentle vortexing. The number of nuclei in suspension was determined by counting on a hemacytometer.

Mutagenesis Assay

Salmonella typhimurium TA1538 was used according to the procedure described by Ames et al. (1). Except where described in the text, nuclei were used at concentrations of 80 to 125 $\times 10^6$ per plate and S9 was used at concentrations of 1 to 3 mg of protein per plate. AF and AAF were used at a concentration of 225 nmol/plate.

RESULTS

AF and AAF Activation by Hepatocyte Nuclei and S9. Under the phase-contrast microscope, the nuclei appeared to be free of cytoplasmic halos, and rare RBC were the only contaminants seen. Nuclear activity was determined as revertants per million nuclei, and S9 activity was determined as revertants per mg of protein.

Optimal conditions for measuring the capacity of liver nuclei and S9 to activate AF and AAF to mutagens were determined by incubating fixed amounts of nuclei and S9 with 112.5, 225, and 450 nmol of AF and AAF per plate. Approximately $9 \times 10^6$ nuclei suspended in S150 were added per plate, while 1.5 mg of S9 protein per plate were incubated with AF, and 3.0 mg S9 protein per plate were incubated with AAF. The results are shown in Charts 1 and 2. No significant increase in reversion occurred when AF or AAF were used at concentrations greater than 225 nmol/plate with nuclei and S9. AF alone was found to increase the reversion frequency about 20 times above the spontaneous reversion frequency when used at a concentration of 450 nmol/plate (data not shown). Therefore, the concentration of 225 nmol/plate of AF or AAF was chosen for the remaining studies to minimize reversion caused directly by AF itself.

Using AF and AAF at a concentration of 225 nmol/plate, various amounts of nuclei and S9 were added to determine a suitable working range for each of the two liver fractions. With both AF and AAF, the number of nuclei used ranged from 55 to 170 $\times 10^6$ per plate. S9 was incubated with AF over the range of 0.5 to 2.0 mg protein per plate, whereas 0.8 to 5.0 mg S9 protein per plate were incubated with AAF. The results are shown in Charts 3 and 4. With AF, a near-linear relationship between the number of revertants per plate and the number of nuclei per plate was maintained.
D. L. Stout and F. F. Becker

response relationships were also determined with these preparations as described above. The number of revertants as a function of the concentration of AF and AAF with nuclei and S9 are shown in Charts 1 and 2, respectively. With these preparations, a plateau occurred at 225 nmol of AF or AAF per plate. Despite the fact that a plateau occurred at this concentration, a considerable increase in the number of revertants was obtained with some of the preparations from treated rats. For example, it can be seen from Chart 1A that PB treatment increased the yield of revertants from nuclear activation of AF (225 nmol/plate) about 3-fold. This suggests that this concentration is nonlimiting.

Nuclei and S9 from MC- and PB-treated rats were added to plates containing 225 nmol of AF or AAF in the range of concentrations described above to determine their relative activities. These data are shown in Charts 3 and 4. Nuclei from PB-treated rats showed a considerable increase in AF-

using up to $125 \times 10^8$ nuclei per plate. Fewer revertants were produced from AAF by nuclei, and a linear response was seen up to the highest concentration of nuclei used. S9, when incubated with AF, produced large numbers of revertants at the lowest concentration used. Above 0.75 mg S9 per plate, the response began to decrease rapidly. Fewer revertants were obtained when S9 was incubated with AAF. However, a linear relationship persisted with as much as 4.5 mg S9 protein per plate.

Since MC and PB treatment have been shown to alter the capacity of S9 preparations to activate AAF to mutagen, the effects of MC and PB treatment on nuclear and S9 activation of AF and AAF were compared. Rats were given MC (80 mg/kg) i.p. in corn oil 24 hr prior to sacrifice or 0.1% PB in their drinking water for 6 days prior to sacrifice. The dose-

Chart 2. Effect of concentration on AF (A) and AAF (B) mutagenicity mediated by liver S9 fractions from untreated (■), MC-treated (○), and PB-treated (●) rats. S9 protein concentrations were 1.5 mg/plate (A) and 3.0 mg/plate (B). Values are the mean of 2 experiments.

Chart 3. AF (A) and AAF (B) mutagenicity mediated by liver nuclei from untreated (■), MC-treated (○), and PB-treated (●) rats. AF and AAF were used at a concentration of 225 nmol/plate. Values are the mean of 2 experiments.
Nuclear and 59 preparations from MC- and PB-treated rats were incubated with AF and AAF as described in "Materials and Methods." Specific activities were determined from the slopes of the straight portion of the curves in Charts 3 and 4. Revertants/10^6 nuclei Revertants/mg 59

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AF</th>
<th>AAF</th>
<th>AF</th>
<th>AAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>33.06</td>
<td>2.44</td>
<td>12,727</td>
<td>733</td>
</tr>
<tr>
<td>MC</td>
<td>56.03</td>
<td>11.50</td>
<td>18,000</td>
<td>733</td>
</tr>
<tr>
<td>PB</td>
<td>165.88</td>
<td>13.74</td>
<td>20,000</td>
<td>1,649</td>
</tr>
</tbody>
</table>

Nuclear Activation of Arylamines

to mutagens

Nuclei and microsomes suspended in S150 or Tris-sucrose buffer were incubated with AF and AAF as described in "Materials and Methods." Specific activities were determined from the slopes of the straight portion of the curves in Charts 3 and 4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AF</th>
<th>AAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei in S150</td>
<td>24.05 ± 2.74</td>
<td>1.32 ± 0.18</td>
</tr>
<tr>
<td>Nuclei in buffer</td>
<td>6.26 ± 1.94</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AF</th>
<th>AAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei in S150</td>
<td>5670 ± 9</td>
<td>3035 ± 151</td>
</tr>
<tr>
<td>Nuclei in buffer</td>
<td>5900 ± 102</td>
<td>40 ± 6</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 2 experiments.
rats on a carcinogenic regimen of AAF at the times shown in Table 3. During the first 4 weeks of feeding, the capacity of nuclei to activate AF was increased to a maximum of 35% above controls, nuclear activation of AF was less than control level, and at 16 weeks, when feeding was terminated, the capacity of nuclei to activate AF had diminished by 71%. The capacity of nuclei to activate AF was also altered during feeding. Maximum activity for AAF also occurred at 4 weeks and was 85% above controls. At 16 weeks, the capacity of nuclei to activate AAF had been reduced from control values at 81%.

DISCUSSION

The major findings of this study demonstrate that rat hepatocyte nuclei possess the capability of activating AF and AAF to mutagens, and that AF may play a prominent role in mutagenesis. The former result is similar to that reported for aryl hydrocarbons, and together they suggest that nuclei possess a monooxygenase system as complex and competent as that of the microsomes. The data presented in Table 2 reveal that the soluble enzymes play a major role in the conversion of AAF to a mutagen with nuclei and S9. However, microsomes alone are competent in metabolizing AF to a mutagen, whereas the capacity of nuclei in buffer was severely reduced. Although it is possible that soluble enzymes may assist in nuclear conversion of AF to mutagen, it is also possible that other factors are involved. During isolation, nuclei lose some of their soluble components to the surrounding medium, and some of these may be recovered by the nuclei when they are resuspended in S150. Those nuclei resuspended in buffer remain in a depleted condition.

DNA alkylation products resulting from the metabolism of carcinogens have been attributed previously to the activity of microsomal enzymes. Using the values obtained for the amount of S9 protein and number of nuclei per g of liver, we estimate that approximately 1.3% of the capacity of hepatocytes to form mutagen from AF, and 1.6% of the total capacity to activate AAF, resides with the nucleus. Although this is a relatively minor contribution in comparison to that of the microsomes, the intimate association of DNA with the nuclear membrane raises the possibility that this source of activation could be significant.

However, because of the high level of metabolic activity associated with microsomes, a small degree of contamination might contribute significantly to the activity found in association with nuclei. Several findings mitigate this possibility. The method of nuclear purification used has been shown previously to result in negligible levels of membrane contamination (3). Further, the response of the 2 systems to PB and MC pretreatment was strikingly different. Nuclear activation of AF was stimulated approximately 400 and 69%, respectively, by PB and MC pretreatment. The respective increases observed with S9 were 57 and 0%. Nuclear activation of AAF was increased 460% by PB and 375% by MC, whereas PB increased S9 activity by 125%, and MC was inactive. It appears from these results that the metabolizing activity of hepatocyte nuclei was in the main inherent and not the result of microsomal contamination. In support of this contention, it has been reported recently that mouse hepatic nuclei can metabolize AAF and N-hydroxy-2-acetylaminofluorene to mutagens (16). When mice were pretreated with MC, nuclear activation of AAF to mutagen was markedly increased.

We previously reported the alteration in the ability of hepatic microsomes to form mutagen from AAF during hepatocarcinogenesis (18). S9 activity was found to increase on the initiation of AAF feeding and to peak during the fourth week. Thereafter, activity declined steadily and at 16 weeks was only 43% of control level. The difference between the maximum activity achieved and the activity at termination of feeding was approximately 700%. S9 activation of AF was elevated only 10% when AAF feeding was initiated, and it persisted at that level for 8 weeks. During the following 2 weeks this activity declined to approximately 90% of control activity. However, at 16 weeks S9 activation of AF was again about 10% greater than control S9 activation.

In the current study it was found that the capacity of hepatocyte nuclei to metabolize AAF closely paralleled that of hepatic S9 during hepatocarcinogenesis. During early exposure to AAF, the ability of nuclei and S9 to activate AAF to mutagen was increased; it peaked at 1 month and then declined. At the conclusion of the feeding regimen, nuclei, like S9, suffered a drastic loss in their capacity to activate AAF to mutagen. However, S9 activation of AF was relatively constant throughout feeding, whereas nuclear activation was depressed to a degree similar to that with AAF.

Decreased microsomal oxidative capacity during hepatocarcinogenesis has been reported by several investigators and is commonly ascribed to diminution of P-450 content (7, 15). That nuclear activation of AAF during hepatocarcinogenesis was seen to parallel S9 activation of AAF suggests that both monooxygenase systems are affected by a similar mechanism during chemical carcinogenesis. However, it is not yet possible to determine how closely related are the nuclear and microsomal monooxygenases. The differential response of the AF- and AAF-activating enzymes in nuclei and microsomes to AAF, PB, and MC supports the view that these systems are independently regulated (9, 10). The activation of AF by S9 was constant throughout the feeding cycle, whereas that of nuclei fell. This phenomenon may indicate that the nuclear AF and AAF enzyme activities and the microsomal AAF enzyme activity have something in

<table>
<thead>
<tr>
<th>Wk</th>
<th>Control</th>
<th>AF</th>
<th>AAF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25.33 ± 2.25a (8)</td>
<td>2.23 ± 0.34 (8)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>32.64 ± 1.50 (2)</td>
<td>4.13 ± 0.69 (2)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>32.37 ± 2.57 (4)</td>
<td>3.23 ± 0.33 (4)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>16.98 ± 14.76 (2)</td>
<td>2.84 ± 0.05 (2)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>19.14 ± 4.51 (2)</td>
<td>1.63 ± 0.47 (2)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>16.50 ± 3.50 (2)</td>
<td>0.84 ± 0.32 (2)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>7.36 ± 3.36 (2)</td>
<td>0.43 ± 0.01 (2)</td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± S.E.

b Numbers in parentheses, number of animals tested.
common not shared by the microsomal AF-activating enzyme.

ACKNOWLEDGMENTS

The authors thank Margaret Bahou and Bonnie Wolder for their technical assistance.

REFERENCES

Metabolism of 2-Aminofluorene and 2-Acetylaminofluorene to Mutagens by Rat Hepatocyte Nuclei

Daniel L. Stout and Frederick F. Becker


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/39/4/1168

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.