Persistent Binding of 2-Acetylaminofluorene to Rat Liver DNA in Vivo and Consideration of the Mechanism of Binding of N-Hydroxy-2-acetylaminofluorene to Rat Liver Nucleic Acids

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SUMMARY

Previous reports that radioactivity was bound to rat liver DNA in vivo after administration of 2-acetylaminofluorene (AAF)-9-14C were confirmed. In addition, administration of several other AAF derivatives, namely N-hydroxy-AAF-9-14C, N-acetoxy-AAF-9-14C, and the glucuronide of N-hydroxy-AAF-9-14C, led to the binding of higher levels of radioactivity to rat liver ribosomal RNA and DNA.

Maximum levels of bound radioactivity were found in rat liver RNA and DNA at 12-16 hr after a single injection of AAF-9-14C. Bound radioactivity disappeared rapidly from liver RNA, with approximately 3 days being required for one-half of the bound radioactivity to disappear. By four weeks after a single injection of AAF-9-14C, rat liver RNA no longer contained detectable radioactivity. On the other hand there was persistent binding of radioactivity to rat liver DNA following a single injection of AAF-9-14C. Approximately 10% of the bound radioactivity found at 12-16 hr remained associated with the DNA at 4-8 weeks after injection of AAF-9-14C.

There appeared to be at least two different mechanisms involved in the binding of N-hydroxy-AAF to rat liver ribosomal RNA and DNA in vivo since approximately 75% of the fluorene residues bound to ribosomal RNA after injection of N-hydroxy-AAF retained the N-acetyl group while the N-acetyl group was retained in only 35% of the fluorene residue bound to DNA.

INTRODUCTION

In the past few years there have been reports that a variety of chemical carcinogens, including aromatic hydrocarbons (2, 8), aromatic amines (5, 24, 31, 34), and azo dyes (3, 32) were covalently bound to DNA in vivo. Although it has been stated that there is no compelling evidence that chemical carcinogens affect changes in mammalian cellular DNA akin to mutagenesis (9, 33), there is probably less evidence favoring any other hypothesis. The importance of studies of the binding of chemical carcinogens to cellular DNA to an understanding of biochemical mechanisms of carcinogenesis has been discussed in excellent reviews by Farber (7) and by Miller and Miller (30).

Our preliminary report in 1964 (34) that some fluorene derivative was bound to rat liver DNA following the administration of the carcinogen AAF was confirmed in 1966 by Sporn and Dingman (31). More recently Kriek (24) and Farber and Liang have also indicated that DNA-bound radioactivity was found in rat liver after administration of AAF-9-14C. Perhaps more significant is the paper by Epstein et al. (5) in which they report the persistence of fluorene-bound derivatives in DNA from hyperplastic nodules in livers of rats fed AAF. Substantial evidence confirming our earlier report, and some data on the expansion of these studies in an attempt to establish the mechanism and the biologic significance of the binding of metabolite(s) of AAF to rat liver nucleic acids in vivo, are presented in this paper.

MATERIALS AND METHODS

Animals

Male albino rats, weighing 180-200 gm at the time of injection, were obtained from the Holtzman Company, Madison, Wisconsin. The rats were maintained on Purina laboratory chow and water.

The following abbreviations are used in this paper: AAF, 2-acetylaminofluorene; N-HO-AAF, N-hydroxy-2-acetylaminofluorene; N-G10-AAF, the glucuronide of N-HO-AAF; N-HO-AF, N-2-fluorenylhydroxylamine; GMP-AAF, 8-(N-2-fluorenylacetamido)guanosine-5'-phosphate; GMP-AF, 8-(N-2-fluorenylamino)guanosine-5'-phosphate; dGMP-AAF, 8-(N-2-fluorenylacetamido)deoxyguanosine-5'-phosphate; dGMP-AF, 8-(N-2-fluorenylamino)deoxyguanosine-5'-phosphate; rRNA, ribosomal RNA.

Cited by E. Farber in Reference 7, Table 1, p. 1864.

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Radioactive Compounds

AAF-9-14C (17), N-HO-AAF-9-14C (17), N-G10-AAF-9-14C (10), and 1-HO-AAF-1-14C (18) were obtained by the published methods indicated.

N-Acetoxy-AAF-9-14C was prepared by the acetylation of N-HO-AAF-9-14C (25.9 mg) with acetic anhydride (0.25 ml) in dry pyridine (0.25 ml). After standing overnight at 24°C, ice was added to the solution, and the N-acetoxy-AAF-9-14C was collected and washed thoroughly with cold water; the yield was 25.0 mg (88%) of N-acetoxy-AAF-9-14C, m.p. 110-111°C [reported m.p. 109-111°C (27)].

N-HO-AAF-9-14C-2',3'H was synthesized as follows: N-HO-AAF-9-14C (160 mg), prepared from 2-nitrofluorene-9-14C, was acetylated with acetic-3H anhydride exactly as described by Irving (12); the yield was 113 mg (58%) of N-HO-AAF-9-14C-2',3'H with specific radioactivities of 21.9 mc/mmole (3H) and 4.23 mc/mmole (14C).

The radiochemical purities of each of these compounds was established by paper or thin-layer chromatography (13, 19).

Isolation of Nucleic Acids

In some early experiments (reported in Chart 2) liver RNA was isolated by method A of Irving et al. (17). In all other experiments, rRNA and DNA were isolated from rat liver by the procedure of Irving and Veazey (14). The liver was removed and homogenized in 15 ml of ice-cold 6% sodium p-aminosalicylate per gm of liver. The homogenate was collected and washed thoroughly with cold water; the ice was added to the solution, and the N-acetoxy-AAF-9 -14C was precipitated with 10% trichloracetic acid at 100°C for 30 min; the hydrolysate was cooled and diluted with 20 ml of counting solution containing Cab-O-Sil (17). DNA samples were prepared for counting by heating 2.4 mg of the DNA in one ml of 5% trichloracetic acid at 100°C for 30 min; the hydrolysate was collected and redissolved in 9 ml of 0.015

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\text{NaCl-0.0015 M sodium citrate, pH 7.0.} \]

The DNA was free of detectable RNA and protein (14).

Radioactivity Determinations

In general, radioactivity determinations were carried out by liquid scintillation counting in appropriate scintillator solutions (19) using a Packard Model 3375 liquid scintillation spectrometer. Counting efficiency was determined by means of the automatic external standardization ratio. Whole liver RNA and rRNA were prepared for counting by dissolving 3.5 mg of the RNA in 0.3 ml of 0.3 N KOH at 37°C for 1 hr. This hydrolysate was diluted with 20 ml of counting solution containing Cab-O-Sil (17). DNA samples were prepared for counting by heating 2.4 mg of the DNA in one ml of 5% trichloracetic acid at 100°C for 30 min; the hydrolysate was cooled and diluted with 20 ml of counting solution containing Cab-O-Sil (17).

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\text{DNA isolated from rat liver after injection of N-HO-AAF-9-14C has been found to contain bound radioactivity. In one experiment, summarized in Chart 1, liver DNA (containing 1.920 dpm/mg DNA) which had been isolated from a rat at 16 hr after i.p. injection of N-HO-AAF-9-14C (11.2 mc/mmole; dose, 30 mg/kg) was banded in CsCl by density equilibrium centrifugation. All of the radioactivity in the isolated DNA was associated with the DNA, which banded at a density of 1.70 (14). This experiment has been repeated several times with similar results being obtained.}
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\text{The rat liver rRNA isolated by the procedure described (14) also contained bound radioactivity after administration of N-HO-AAF-9-14C. The association of the radioactivity with the rRNA was demonstrated by sucrose density gradient centrifugation studies as shown in an earlier publication (17).}
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\text{RESULTS}
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\text{Evidence for Binding of Radioactivity to Nucleic Acids in Vivo after Administration of N-HO-AAF-9-14C}
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DNA isolated from rat liver after injection of N-HO-AAF-9-14C was found to contain bound radioactivity. In one experiment, summarized in Chart 1, liver DNA (containing 1.920 dpm/mg DNA) which had been isolated from a rat at 16 hr after i.p. injection of N-HO-AAF-9-14C (11.2 mc/mmole; dose, 30 mg/kg) was banded in CsCl by density equilibrium centrifugation. All of the radioactivity in the isolated DNA was associated with the DNA, which banded at a density of 1.70 (14). This experiment has been repeated several times with similar results being obtained.

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\text{The rat liver rRNA isolated by the procedure described (14) also contained bound radioactivity after administration of N-HO-AAF-9-14C. The association of the radioactivity with the rRNA was demonstrated by sucrose density gradient centrifugation studies as shown in an earlier publication (17).}
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\text{Kinetics of Binding}
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These experiments were designed to determine the time of

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\text{A}_{259\text{nm}}/A_{231\text{nm}} = 2.42; \quad T_m = 69.7^\circ C, \text{with a hyper-}
\]

maximum binding of radioactivity to rat liver nucleic acids after a single injection of AAF-9-\(^{14}\)C and to obtain an estimate of the rate of disappearance of radioactivity from the nucleic acids.

Total liver RNA was isolated by Method A of Irving et al. (17); the maximum level of bound radioactivity was found at 12-16 hr after i.p. injection of AAF-9-\(^{14}\)C (Chart 2). The specific radioactivity of the rat liver RNA then decreased with the time after injection, falling from a maximum of 514 dpm/mg at 12 hr to 73 dpm/mg by 7 days after injection (Chart 2). The biologic half-life of the RNA-bound radioactivity was 2.9 days. The rate of decrease in specific radioactivity of the RNA between Days 2 and 7 was used for this calculation.

These studies were repeated and extended after the development of a method for the isolation of rRNA and DNA from a single rat liver (14). The results are shown in Chart 3. The maximum level of radioactivity bound to either rRNA or to DNA after a single i.p. injection of AAF-9-\(^{14}\)C was found at 12-16 hr. The specific radioactivities of the rRNA and DNA then declined rapidly, and the initial (up to 48 hr after injection) rate of loss of radioactivity from DNA roughly paralleled the rate of loss of radioactivity from rRNA. Until about 48 hr after injection, the level of radioactivity bound to DNA was about 50% of that bound to the rRNA. The biologic half-life of the radioactivity associated with the rRNA was 3.1 days. Some radioactivity disappeared from the DNA during the first week after injection (between Days 2 and 7) with a half-time estimated to be 3.5 days\(^4\). By one week after injection of AAF-9-\(^{14}\)C, there was an equal amount of radioactivity associated with rRNA and DNA, and by 4 weeks rat liver rRNA no longer contained any detectable radioactivity. However, the DNA still contained low levels of radioactivity at 4 weeks after the single injection of AAF-9-\(^{14}\)C, and this level was not further diminished at 8 weeks after the injection (Chart 3).

\(^4\)In the case of DNA, the specific radioactivities between Days 2 and 7 after injection were corrected for the constant low amount of radioactivity which persisted at 4-8 weeks, before calculating the rate of decrease during this first week.
Comparison of Levels of Nucleic Acid-bound Radioactivity in Rat Liver after Injection of AAF-9-14C and Related 14C-labeled Fluorene Derivatives

N-HO-AAF, which is more carcinogenic than AAF when tested under comparable conditions (29), also gave rise to greater levels of bound radioactive activity in both rRNA and DNA of rat liver after a single i.p. injection of each of the corresponding 14C-labeled compounds (Table 1). N-Acetoxy-AAF-9-14C and N-G10-AAF-9-14C also yielded more bound radioactivity in rat liver rRNA and DNA than did AAF-9-14C (Table 1). There are no published data on the carcinogenicity of N-acetoxy-AAF for rat liver although this compound does produce local sarcomas and gives rise to protein-bound derivatives at the site of injection when given s.c. to the rat (30). We do not yet have any data on the carcinogenicity of N-G10-AAF in the male rat. Repeated s.c. injections of N-G10-AAF in the female rat have thus far yielded only a low level of sarcomas and delayed incidence of sarcomas (17, 28). The administration of a single dose of 1-HO-AAF resulted in the binding of only traces of radioactivity to rat liver rRNA and DNA than did AAF-9-14C (Table 1). We do not yet have any data on the carcinogenicity of N-acetoxy-AAF for rat liver although this compound does produce local sarcomas and gives rise to protein-bound derivatives at the site of injection when given s.c. to the rat (30). We do not yet have any data on the carcinogenicity of N-G10-AAF in the male rat. Repeated s.c. injections of N-G10-AAF in the female rat have thus far yielded only a low level of sarcomas and delayed incidence of sarcomas (17, 28). The administration of a single dose of 1-HO-AAF resulted in the binding of only traces of radioactivity to rat liver rRNA and DNA; 1-HO-AAF is not carcinogenic in the rat (29).

Nature of the Fluorene Moiety Bound to Liver rRNA and DNA

In order to determine whether the fluorene species which was bound to rRNA and DNA after a single injection of N-G10-AAF retained the N-acetyl group, N-G10-AAF labeled with 14C in the 9-position of the fluorene ring and with 3H in the N-acetyl group was given to rats. The liver rRNA and DNA were isolated at 16 hr after injection. In confirmation of earlier results using N-HO-AAF-9-14C labeled with 14C in the N-acetyl group (20), the acetyl-3H group was retained to a major extent in the fluorene moiety which was bound to rRNA (Table 2). Assuming that all of the 3H in the rRNA was of the N-acetyl group of the bound fluorene moiety (17), 75% of the fluorene residues bound to the rRNA retained the acetyl-3H group (Table 2) On the other hand, more 3H was lost from the fluorene moiety which was bound to liver DNA; only 35% of the fluorene residues bound to DNA retained the acetyl-3H group (Table 2).

### Table 1

<table>
<thead>
<tr>
<th>14C</th>
<th>Compound injected</th>
<th>No. of animals</th>
<th>µmoles 14C bound/mg</th>
<th>% of residues with acetyl-3H</th>
<th>% of residues with 14C in 9-position</th>
<th>Ratio 3H/14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAF</td>
<td>4</td>
<td>39 ± 3</td>
<td>22 ± 0.2</td>
<td>35.0 ± 0.3</td>
<td>15.7 ± 1.2</td>
<td>1.84 ± 0.02</td>
</tr>
<tr>
<td>N-HO-AAF</td>
<td>4</td>
<td>119 ± 7</td>
<td>64 ± 7</td>
<td>75.0 ± 0.6</td>
<td>44.3 ± 3.8</td>
<td>1.84 ± 0.02</td>
</tr>
<tr>
<td>N-acetoxy-AAF</td>
<td>6</td>
<td>84 ± 15</td>
<td>56 ± 9</td>
<td>75.0 ± 0.6</td>
<td>44.3 ± 3.8</td>
<td>1.84 ± 0.02</td>
</tr>
<tr>
<td>N-G10-AAF</td>
<td>3</td>
<td>58 ± 14</td>
<td>44 ± 5</td>
<td>75.0 ± 0.6</td>
<td>44.3 ± 3.8</td>
<td>1.84 ± 0.02</td>
</tr>
<tr>
<td>1-HO-AAF</td>
<td>2</td>
<td>&lt;1</td>
<td>5 ± 3</td>
<td>75.0 ± 0.6</td>
<td>44.3 ± 3.8</td>
<td>1.84 ± 0.02</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Liver nucleic acid</th>
<th>rRNA</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific radioactivity of nucleic acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dpm 3H/mg</td>
<td>3,470 ± 720</td>
<td>764 ± 55</td>
</tr>
<tr>
<td>dpm 14C/mg</td>
<td>892 ± 178</td>
<td>416 ± 35</td>
</tr>
<tr>
<td>Ratio 3H/14C</td>
<td>3.88 ± 0.03</td>
<td>1.84 ± 0.02</td>
</tr>
</tbody>
</table>

Difference in the binding of 3H and 14C to rat liver rRNA and DNA after injection of N-G10-AAF-9-14C. The N-HO-AAF was labeled with 3H in the acetyl group (specific radioactivity, 21.9 mc/mmmole) and with 14C in the 9-position of the fluorene ring (specific radioactivity, 4.23 mc/mmmole). The ratio of the specific radioactivities (3H/14C) was 5.18. The rats were killed at 16 hr after the compound was injected intraperitoneally (84 µmoles per kg of body weight), and the liver RNA and DNA were isolated. See Footnote 2 for abbreviations.

### DISCUSSION

Reports that radioactivity was bound to rat liver DNA in vivo after administration of AAF-9-14C (24, 31, 34) have been conclusively confirmed in these studies by direct isolation of DNA. Furthermore, it was shown (Table 1) that some other fluorene compounds derived from AAF are also bound to rat liver DNA and RNA in vivo following their administration. Radioactivity disappeared rapidly from liver RNA, with approximately 3 days being required for one-half of the radioactivity to disappear. By four weeks after the single injection, the liver RNA no longer contained any detectable radioactivity. There are no data available on the mechanism involved in the disappearance of the fluorene-bound derivatives from liver RNA. However, it does not seem likely that the disappearance was due to normal turnover of liver RNA since a number of investigators have shown that total rat liver RNA, nuclear RNA, rRNA, and transfer RNA each have half-lives of 5 days (1, 6, 11, 26).

The kinetics of disappearance of radioactivity from rat liver DNA following injection of a single dose of AAF-9-14C differed from those obtained with RNA. Between 1-3 weeks after injection of the AAF-9-14C, the level of radioactivity bound to the rat liver DNA stopped decreasing and remained constant at 4-8 weeks after injection. Approximately 10% of the radioactivity found at 12 hr remained associated with the DNA at 4-8 weeks after injection of AAF-9-14C. This figure is probably somewhat low, because it does not take into account
the fact that the labeled DNA became diluted with unlabeled DNA (due to liver growth) during the 8 week period. Even though the gain in liver weight was not considered in these studies, it is doubtful that such consideration would alter the conclusion that there is persistent binding of some metabolite of AAF to rat liver DNA in vivo. Our results with AAF differ somewhat from those obtained with p-dimethylaminobenzene by Warwick and Roberts (32). Following the injection of a single dose of p-dimethylaminobenzene-\(^3\)H, the specific radioactivity of the liver DNA dropped only very slightly during the first week after injection, then the level of DNA-bound radioactivity remained constant for a period of up to 3 months (32). Warwick and Roberts concluded that the DNA in differentiated liver cells not undergoing division was not repaired or was repaired only slowly after modification by covalent interaction with foreign molecules such as p-dimethylaminobenzene. It is not likely that cell division was sufficient to account for the rapid rate of decay in the specific radioactivity of the rat liver DNA which we observed during the first week after administration of AAF-\(^9\)\(^{14}\)C. The mitotic index for normal rat liver cells, quoted in the paper by Warwick and Roberts, is 0.005-0.01%. A major portion of the rat liver DNA which was modified by the interaction of some metabolite of AAF-\(^9\)\(^{14}\)C appeared to be capable of being repaired. An alternate explanation is that cell death accounted for the rapid loss of radioactivity which was observed. A small fraction of the modified DNA persisted at 8 weeks after injection of AAF-\(^9\)\(^{14}\)C. Repeated injections or continuous feeding of AAF might result in the accumulation of this modified DNA (repair-resistant?) and, as suggested by Warwick and Roberts (32), this latent damage might be manifested as a chromosome aberration during subsequent cell division. Epstein et al. (5) have reported the persistent binding of metabolites of AAF to liver DNA after feeding rats a diet containing AAF. No experimental data were given in their paper although the binding of AAF metabolites to DNA was reported to be based upon spectrophotometric evidence. DNA from hyperplastic nodules of liver, but not from normal liver or from liver surrounding these nodules, was reported to have an absorption spectrum similar to that of normal rat liver DNA or calf thymus DNA obtained after injection of N-acetoxy-AAF in vivo. These authors suggested that the absence of repair mechanisms, as evidenced by the persistence of a carcinogen bound to DNA, might be an essential step in carcinogenesis with such compounds as AAF (5).

AAF-\(^{\text{N}}\)-sulfate (4, 30), N-G\(^{\text{10}}\)-AAF (15, 16, 28), and N-HO-\(^{\text{AF}}\) (22-24) have each been proposed to be involved in the biochemical mechanism of binding of N-HO-\(^{\text{AF}}\) to rat liver nucleic acids in vivo. Based upon data obtained on the reactivity of AAF-\(^{\text{N}}\)-sulfate (4, 30) and reactive synthetic esters such as N-acetoxy-\(^{\text{AF}}\) (30, 28), i.e., the retention of the N-acetyl group of the \(^{\text{AF}}\) residue in the products of the reaction with methionine and guanosine, one would expect the N-acetyl group to be retained on the bound fluoresce moiety if AAF-\(^{\text{N}}\)-sulfate and N-acetoxy-\(^{\text{AF}}\) were involved in the binding of AAF or N-\(^{\text{HO}}\)-\(^{\text{AF}}\) to rat liver nucleic acids in vivo. On the other hand, the major products of the reaction of N-G10-\(^{\text{AF}}\) with methionine and with guanosine in vitro were the deacetylated derivatives (30). N-G10-\(^{\text{AF}}\) also reacts with RNA and DNA in vitro (15), and we have recently shown that the products of these reactions contain a major fraction of bound fluoresce residues which do not have the N-acetyl group on them (16). Consequently, if N-G10-\(^{\text{AF}}\) were involved in the biochemical mechanism of binding of AAF or N-HO-\(^{\text{AF}}\) to rat liver nucleic acids in vivo, then one might expect to find a significant loss of the N-acetyl group on the fluoresce moiety bound to the nucleic acids. After injection of N-HO-\(^{\text{AF}}\)-\(^{9}\)\(^{14}\)C-\(^2\)\(^\text{H}\) in the rat, the N-acetyl group was retained on most of the fluoresce moieties bound to rat liver rRNA, whereas a large fraction of the N-acetyl groups was lost on the moieties bound to rat liver DNA (Table 2). This finding was surprising, and it suggested that there might be at least two different metabolic pathways for the binding of radioactivity to rat liver nucleic acids after administration of N-HO-\(^{\text{AF}}\)-\(^{9}\)\(^{14}\)C. Based upon evidence obtained by an independent method, Kriek has suggested that there might be two different mechanisms also involved in the binding of AAF-\(^{9}\)\(^{14}\)C to rat liver nucleic acids (24). Rat liver RNA and DNA, isolated after injection of AAF-\(^{9}\)\(^{14}\)C, were subjected to enzymatic hydrolyses, and the hydrolysates were chromatographed on Sephadex G-25 with synthetic GMP-AAF and GMP-AF (for the RNA hydrolysates) or with synthetic dGMP-AAF and dGMP-AF (for DNA). The N-acetyl group was reported to be lost from the fluoresce moiety bound to the DNA but was retained on the fluoresce moiety bound to RNA. Kriek concluded that there were at least two different mechanisms of bindings in vivo: the reaction of an ester of N-HO-\(^{\text{AF}}\) with RNA in the cytoplasm and a reaction of N-HO-\(^{\text{AF}}\) which was being favored in the nucleus. However, we feel that the more stable, but yet slightly reactive, N-G10-\(^{\text{AF}}\) might account for most of the binding of N-HO-\(^{\text{AF}}\)-\(^{9}\)\(^{14}\)C to rat liver DNA in vivo. The binding of AAF or N-HO-\(^{\text{AF}}\) to cytoplasmic nucleic acids might be attributed to the gener-
tion in the cytoplasm of more reactive esters, such as AAF-N-sulfate, which have a very short half-life.8

REFERENCES


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8 The half-life of synthetic AAF-N-sulfate (50% purity) was given to be less than one minute (30).
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