Protein Modifications by Activated Carcinogens

I. THE ACETYLATION OF RIBONUCLEASE BY N-ACETOXY-2-FLUORENYLACETAMIDE*

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SUMMARY

The postulated fragmentation of RNase by N-acetoxy-2-fluorenylacetamide (N-acetoxy-2-FAA), an activated metabolite of the carcinogen, N-hydroxy-2-fluorenylacetamide, has been investigated. The interaction of N-acetoxy-2-FAA with RNase resulted in the formation of two new proteins separable from RNase by electrophoresis and ion exchange chromatography. These proteins contained no NH₂-terminal amino acids, and their amino acid composition was identical with that of native RNase, indicating that the protein had not been modified by cleavage of the peptide chain. The alternative possibility that the formation of the additional proteins was due to a decrease in the positive charge of RNase was examined by the determination of the incorporation of ³H and ¹⁴C from N-[³H]acetoxy-[⁹⁻¹⁴C]2-FAA into RNase. The ratio of bound ³H to bound ¹⁴C, which measures the relative extent of acetylation and arylamidation in the modified proteins, suggested that acetylation rather than arylamidation was the predominant reaction modifying RNase. The conclusion that the acetyl group of N-[³H]acetoxy-[⁹⁻¹⁴C]2-FAA had been transferred to RNase was confirmed (α) by the recovery of [³H]acetic acid from the hydrolysates of the modified protein and (β) by the isolation of ε-N-acetyl-L-lysine from enzymatic hydrolysates of the modified protein. The isolation of the N-acetylated amino acid accounted in part for the decrease in the positive charge of RNase after reaction with N-acetoxy-2-FAA. The results indicate that N-acetoxy-2-FAA is an acetyl donor and modifies proteins primarily by acetylation rather than arylamidation.

The biological activity of certain hepatocarcinogenic arylamides appears to depend on a two-step mechanism of metabolic activation. The arylamide is first N-hydroxylated (2) and then esterified to an N-sulfate (3), and possibly also to an N-phosphate (4) or N-acetate (5). These esters are highly unstable and decompose spontaneously to an electrophilic arylamidonium ion which arylamidates DNA, RNA, and proteins (6, 7). Although these macromolecular interactions have been documented in detail, the critical reaction that initiates the malignant transformation of the cell is not known. In a previous study, we have described the arylamidation of nuclear proteins, including histones, of rat liver by N-2-fluorenylacetamide in vivo (8). These data prompted us to suggest that arylamidation might alter the histones in such a way that they were no longer able to inhibit the transcription of nuclear DNA (9). However, the arylamidation of the nuclear proteins under these conditions was of such a low order that the elucidation of the structural modifications of the nuclear proteins by presently available analytical techniques was not feasible. We have reinvestigated the problem of the structural modification of proteins by arylamidation in a model system in which we reacted N-acetoxy-2-fluorenylacetamide (N-acetoxy-2-FAA) with RNase. This model system was chosen because it had been reported that N-acetoxy-2-FAA, an ester that is well characterized and available in pure form, reacts through arylamidation with methionine residues of RNase (10). Our objective was to determine whether this would cleave the peptide chain of RNase as had been suggested (7, 11), and, if so, whether histones that had been modified by arylamidation were fragmented in a similar manner. Although we were able to demonstrate arylamidation of RNase by N-acetoxy-2-FAA, to a minor extent, no cleavage of the protein was observed. Further examination of the modified protein showed that acetylation, rather than arylamidation, appeared to be the predominant reaction between N-acetoxy-2-FAA and RNase and that it accounted, at least in part, for the altered properties of RNase that had been exposed to the ester. The experiments leading to these conclusions form the basis of this report.

EXPERIMENTAL PROCEDURE

Labeled and Unlabeled Compounds—N-Acetoxy-[⁹⁻¹⁴C]2-FAA† was prepared by the acetylation of a mixture of N-hydroxy-[⁰⁻¹⁴C]2-FAA (10.3 mCi, lot 485-120, 10 mCi per mmole, New England Nuclear) and N-hydroxy-2-FAA (403 mg) in 10 ml of pyridine with 0.2 ml of acetic anhydride (12). The compound

† The following abbreviations are used: N-acetoxy-2-FAA, N-acetoxy-2-fluorenylacetamide; N-hydroxy-2-FAA, N-hydroxy-2-fluorenylacetamide; 3-CH₃-S-2-FAA, 3-methylthio-2-fluorenylacetamide; TEMED, N,N,N',N'-tetramethylethylenediamine; FDNB, 2,4-dinitrofluorobenzene; α CH₂, α 2-FAA, α methylthio-2-fluorenylacetamide.
was crystallized from ethanol-water (400 mg, 82% yield, m.p. 110-111.5\(^\circ\), 0.23 \(\mu\)Ci per \(\mu\)mole). \(\text{N}[\text{III}]\) Acetoxy-2-FAA was prepared by reacting \(\text{N}\)-hydroxy-2-FAA (100 mg) with \(\text{H}^+\) acetic anhydride (25.0 mg, 25 \(\mu\)Ci per \(\mu\)mole, New England Nuclear) in pyridine (10 ml) (19). The ester (97 mg, 82% yield, m.p. 110-111\(^\circ\), 16.5 \(\mu\)Ci per \(\mu\)mole) gave an ultraviolet absorption spectrum superimposable on that of an authentic sample. 3-Methylthio-2-FAA (m.p. 167-168\(^\circ\)) was obtained by reacting DL-methionine with \(\text{N}\)-acetoxy-2-FAA (10).

\(\epsilon\)-\(N\)-Acetyl-L-lysine (m.p. 247-249\(^\circ\)) was prepared by the acetylation of the copper complex of L-lysine (13). \(\alpha\)-\(N\)-Acetyl-L-lysine (m.p. 249-251\(^\circ\)) was obtained from \(\epsilon\)-\(N\)-benzoyloxy-carbonyl-\(\epsilon\)-N-acetyl-L-lysine (13, 14). The infrared spectrum of the product was identical with that of an authentic sample.

\(\alpha\)-Acetyl-L-serine was prepared by the acetylation of L-serine (2.0 g, 19 mmoles, Calbiochem) in glacial acetic acid saturated with HCl (15). The acetylation of the amino acid was carried out in suspension, instead of in solution as reported (15) because we found that L-serine is only very slightly soluble in glacial acetic acid saturated with HCl. The hydrochloride of \(\alpha\)-acetyl-L-serine in ethanol was treated with triethylamine and \(\alpha\)-acetyl-\(\epsilon\)-lysine (0.70 g, m.p. 163-165\(^\circ\), \([\alpha]\)\(^{20\circ}\) = +17.2\(^{\circ}\), \(c\) = 2\% in 0.1 \(\text{N}\) HCl, 1235 (\(\text{NH}_3^+\), 1790 (-C-\(\text{O}\)-), 1600 (-C-\(\text{O}\)-), 1500 (\(\text{NH}_3^+\)), 1255 (\(\text{CH}_2\text{C}==\text{O}\)) cm\(^{-1}\)) was obtained after recrystallization from water-ethanol (0.97 g, m.p. 163-165\(^\circ\), \([\alpha]\)\(^{20\circ}\) = +20.8\(^{\circ}\), \(c\) = 2\% in 0.1 \(\text{N}\) HCl, \(\gamma_{\text{max}}\) 3180, \(\lambda_{\text{max}}\) 3000 (\(\text{NH}_3^+\)), 1790 (-\(\text{C}\)-\(\text{O}\)-), 1600 (-\(\text{C}\)-\(\text{O}\)-), 1500 (\(\text{NH}_3^+\)), 1255 (\(\text{CH}_2\text{C}==\text{O}\)) cm\(^{-1}\)).

\[
\begin{align*}
\text{C}_4\text{H}_9\text{O}_2\text{N} & = 27.8^{\circ}, c = 2\% \text{ in H}_2\text{O}\).^4
\end{align*}
\]

\[
\text{C}_4\text{H}_9\text{O}_2\text{N}^+\text{H}_2\text{O}^-
\]

Calculated: C 44.71, H 6.88, N 8.69

Found: C 44.55, H 7.16, N 8.35

Both compounds were prepared by the acetylation, with acetic anhydride, of \(\text{L}\)- or \(\text{D}\)-threonine in alkali in a manner similar to that described for the \(\text{N}\)-acetylation of \(\text{L}\)- or \(\text{D}\)-serine (18, 19).

N-\(\text{Acetyl-L}\)- and N-\(\text{Acetyl-D}\)-threonine were hydrolyzed with 4 \(\text{N}\) HCl to L-threonine, \([\alpha]\)\(^{5\circ}\) = -27.8\(^{\circ}\), and D-threonine, \([\alpha]\)\(^{5\circ}\) = +27.8\(^{\circ}\), respectively. The conditions for the hydrolysis, isolation, and purification of the amino acids were those described for the preparation of \(\text{O-methyl-D}\)- or L-threonine from \(\text{O-methyl-N}\)-chloroacetyl-D- or L-threonine (20).

**Conditions for Modification of RNase by N-Acetoxy-2-FAA**—Ribonuclease A (XII-A, low phosphate, Sigma) was purified either by gel filtration on Sephadex G-25 or by ion exchange chromatography on Bio-Gel CM-30 (Bio-Rad) (Table I). The eluted protein was reacted with N-acetoxy-2-FAA in the media listed in Table I. The ester dissolved in acetone (20 ml) was added to RNase (80 mg) in Tris-HCl buffer (pH 7.4) (20 ml). In one experiment, the ester was dissolved in 0.5 ml of ethanol and added at room temperature to 20 ml of the magnetically stirred protein solution (Experiment 8, Table I). The reactions were carried out at 22\(^\circ\) for 24 hr. The modified proteins were precipitated by cooling the solution in ice and adding 2 to 3 volumes of cold acetone. The precipitate was collected by centrifugation and redissolved in a minimum amount of water. After a second acetone precipitation, the modified proteins were collected and examined by disc electrophoresis or ion exchange chromatography.

**Disc Electrophoresis**—The methods for the electrophoretic resolution of the modified proteins were modifications of the techniques of Reisfeld et al. (21) and of Shepherd and Gurley (22). Solutions A to F were used to prepare the stacking and the resolving gels: Solution A—40\% Prep/Cryst (Canalco) and 0.4\% ethylene diacrylamide; Solution B—4.75 ml of glacial acetic acid and 1.15 ml of TEMED in 400 ml of water titrated to pH 4.0 with KOH and diluted to 500 ml with water; Solution C—0.56\% ammonium persulfate; Solution D—14.0\% Prep/Cry1 and 0.8\% ethylene diacrylamide; Solution E—4.75 ml of glacial acetic acid and 2.3 ml of TEMED in 400 ml of water titrated to pH 6.0 with KOH and diluted to 500 ml with water; Solution F—saturated aqueous riboflavin. The resolving gel (16.6 ml) consisted of 7.0 g of urea dissolved in Solutions A, B, and C (6.0, 2.0, and 3.4 ml, respectively). The solution was degassed and cast in silanized Pyrex tubes (6.0 mm, inner diameter) to a depth of 6.0 to 8.0 cm. After polymerization (2 hours), excess persulfate was removed by electrophoresis of the gels in 0.02 M Tris buffer (pH 4.0) at 1 ma per tube for 2 hours. The stacking gel (16.6 ml) consisted of 7.0 g of urine dissolved in Solutions D, E, and F (8.0, 2.0, and 3.4 ml, respectively). The solution was degassed and cast in silanized Pyrex tubes (6.0 mm, inner diameter) to a depth of 6.0 to 8.0 cm. After polymerization (2 hours), excess persulfate was removed by electrophoresis of the gels in 0.02 M Tris buffer (pH 4.0) at 1 ma per tube for 2 hours. The stacking gel (16.6 ml) consisted of 7.0 g of urine dissolved in Solutions D, E, and F (8.0, 2.0, and 3.4 ml, respectively). The solution was degassed and cast in silanized Pyrex tubes (6.0 mm, inner diameter) to a depth of 6.0 to 8.0 cm. After polymerization (2 hours), excess persulfate was removed by electrophoresis of the gels in 0.02 M Tris buffer (pH 4.0) at 1 ma per tube for 2 hours. The stacking gel (16.6 ml) consisted of 7.0 g of urine dissolved in Solutions D, E, and F (8.0, 2.0, and 3.4 ml, respectively). The solution was degassed and cast in silanized Pyrex tubes (6.0 mm, inner diameter) to a depth of 6.0 to 8.0 cm.
carried out at 1.5 m per tube for 1.5 hours. The proteins were stained with Amido Black (0.25\%) in 7.5\% acetic acid and de-stained electrophoretically in the same acid. The absorption profile of the proteins was measured with the use of a micro-densitometer (Canalco, model F).

Identification and Measurement of o-Methylthio-2-FAA—The amounts of the o-methylmercaptoamide released spontaneously from the protein during the reaction of RNase with N-acetoxy-[9-\(^{14}\)C]2-FAA were measured by inverse isotope dilution (23). Carrier 3-CH\(_3\)-S-2-FAA (0.4 g) was added to the reaction mixture, and the proteins were precipitated with acetone and renatured by centrifugation. The supernatant liquid was concentrated at reduced pressure, and the residue was extracted with a mixture of benzene-n-hexane (15:85). The solvent was evaporated at reduced pressure, and the o-CH\(_2\)-S-[9-\(^{14}\)C]2-FAA was purified by thin layer chromatography.

Enzymatic Activity and Amino Acid Composition of Modified RNase—The enzymatic activity of the modified protein was assayed by the method of Roth with ribonuclease A (type XI, Sigma) as the substrate (24). The hydrolysis of RNA by ribonuclease A (type XII, Sigma) served as a control.

The amino acid analyses of the proteins were carried out on duplicate samples with a Beckman/Spinco amino acid analyzer model 120B (25, 26). The proteins were hydrolyzed in sealed tubes with 6 \(\times\) HCl (3 mg of protein per ml) at 110\(^\circ\) for 22 hours. The NH\(_2\)terminal amino acids of the modified proteins were identified by reacting the proteins with FDNB (27). The proteins were hydrolyzed in 6 \(\times\) HCl (2 ml) at 110\(^\circ\) for 16 hours. The DNP amino acids were extracted with ether and chromatographed on Silica Gel GF254 with four different solvents (28).

Estimation of \([\text{H}]\)Acetyl Groups Released from Modified Proteins by Alkaline or Acid Hydrolysis—The proteins that had been modified by the reaction of RNase with N-[\text{H}]acetoxy-2-FAA were hydrolyzed in alkali in order to release the labeled acetyl group that was bound to the proteins in ester linkage. The protein solution (0.5 ml) was added to 2 \(\times\) NaOH (5 ml), and the mixture stood at 22\(^\circ\) for 2 to 18 hours (Table III). Sodium acetate (4 minutes) was added, and the pH was adjusted to 3.0 with H\(_2\)PO\(_4\). The solution was steam-distilled, the distillate (50 ml) was tiritated with 0.1 \(\times\) NaOH, and its radioactivity was measured. The amounts of [\text{H}]acetyl groups released from the protein were calculated from the specific radioactivity of the distilled [\text{H}]acetic acid (29).

The amounts of [\text{H}]acetyl groups bound to the RNase in amide linkage were determined by acid hydrolysis of the modified proteins at 100\(^\circ\) for 6 hours. The protein solution (0.5 ml) was added to 8.5 ml of 6 \(\times\) H\(_2\)SO\(_4\), and the solution was heated in stoppered tubes in a boiling water bath. After cooling, 4.0 mmoles of sodium acetate were added, the mixture was subjected to steam distillation, and the specific radioactivity of the [\text{H}]acetic acid in the distillate was measured.

Isolation and Characterization of Acetylated Amino Acids—The modified proteins from the reaction of RNase with N-[\text{H}]acetoxy-2-FAA were hydrolyzed with Pronase B (Calbiochem) and trypsin (30, 31). The hydrolysate was chromatographed on a column (1 \(\times\) 7 cm) of AG 50W-X4 (Bio-Rad) with 0.2 \(\times\) ammonium acetate (pH 5.5) as the eluent (32). The compounds eluted with the buffer were subjected to thin layer chromatography on Silica Gel GF254 with ethanol-water (63:37) as solvent (28). The acetylated amino acids were located by a radioisotope of the chromatogram and eluted with water. The eluate was lyophilized, the residue was dissolved in citrate buffer, and \([\text{P}]\)aspartic acid, \([\text{P}]\)glycine, and \([\text{P}]\)leucine were added as markers. The acetylated amino acids were then resolved by chromatography on a Beckman-Spinco 120B amino acid analyzer. The effluent was split into two equal portions. One of these was used for amino acid analysis. The other was analyzed for \(\text{H}\) and \(^{13}\)C by liquid scintillation spectrometry. The [\text{H}]amino acid in the elution profile was tentatively identified by its position relative to the positions of the markers.

Estimation of e-N-Acetyl-L-lysine—The amount of e-N-[\text{H}]acetyl-L-lysine in the enzymatic hydrolysates of the modified proteins was measured by inverse isotope dilution (29). e-N-Acetyl-L-lysine (30 mg) was added to a portion of the radioactive eluate that had been obtained by chromatography of the hydrolysates on AG 50W-X4. The solution was brought to a boil, and basic enpic carbonate was added. The copper complex was collected and decomposed with H\(_2\)S. The e-N-[\text{H}]acetyl-L-lysine was isolated by thin layer chromatography on silic gel and purified to constant specific radioactivity by successive thin layer chromatographies with three different solvents (Table IV).

Protein and Radioactivity Measurements—Protein contents were estimated by the modified Folin method with RNase A (type XII, Sigma) as the standard (33).

Radioactivity was determined using a Packard liquid scintillation spectrometer (model 3875). The solutions (1 ml) were added to vials containing 15 ml of Scintisol-Complete (Isolab Inc., Akron, Ohio). All samples were counted in duplicate and corrected for quenching by means of an external standard.

Radiochromatograms were scanned with a Berthold thin layer chromatogram scanner (Drinkmann, Westbury, N. Y.).

RESULTS

Modification of RNase by N-Acetoxy-2-FAA—The initial reactions of RNase with N-acetoxy-2-FAA were carried out in media consisting of 0.1 m Tris-HCl (pH 7.4), 7 m urea, and 50 m\% acetone. These conditions were selected so as to unfold the RNase and to expose the amino acid residues of the protein to the ester (34). Furthermore, this concentration of acetone insured the solubility of the ester as well as that of the protein. N-Acetoxy-2-FAA and RNase are soluble in media with an acetone content between 18 and 65\%. The molar ratio of ester to protein in these experiments was 80. At the completion of the incubation the RNase was precipitated by excess acetone, and the precipitate was found to contain 86\% of the initial protein. The precipitated protein was resolved by disc electrophoresis into three components, designated A, B, and C (Fig. 1), whereas native RNase moved largely as a single component. The acetone-precipitable, heterogeneous RNase is subsequently referred to as modified RNase. Protein A had the same mobility as unreacted RNase and accounted for 45\% of the absorbance of the electrophoretic profile. Proteins B and C were less basic than protein A and accounted for 42 and 13\%, respectively, of the total stained protein. The electrophoretic profiles of equal amounts of untreated RNase and of modified RNase (Fig. 1) indicated that proteins B and C were formed at the expense of native RNase.

The possibility that proteins B and C were products of the cleavage of RNase was investigated in four different experiments. First, we compared the hydrolysis of RNase by native and by modified RNase. These measurements showed that modified RNase had retained 96\% of the enzymatic activity of native RNase. Second, we analyzed the supernatant liquid of the deproteinized reaction mixture for the presence of o-Ch\(_2\)-S-[9-\(^{14}\)C]2-FAA. If the mercaptoamide were found, it would indicate that the resulting sulfonium ion had decomposed spontane-
The proteins (100 µg per gel) were resolved in polyacrylamide gels as described in the text. -, RNase purified by gel chromatography on Sephadex G-25; - - -, proteins precipitated by acetone from the reaction of RNase with N-acetoxy-S-FAA.

**FIG. 1.** Disc electrophoresis of RNase and of modified RNase.

**FIG. 2.** Examination of the supernatant liquid of the deproteinized reaction mixture of modified RNase for the presence of o-CH₃-S-[9-14C]2-FAA. The isolation of the compound and its purification was carried out as described in the text. The figure shows the migration of the radioactivity in comparison to that of authentic 3-CH₃-S-2-FAA during the first chromatography on Silica Gel GF₃₄ with petroleum ether:chloroform:methanol (50:45:5) as the solvent (23). o-CH₃-S-2-FAA moves on thin layer chromatography at the same rate as 3-CH₃-S-2-FAA (6).

Third, modified RNase was analyzed for new NH₂-terminal amino acids by the DNP-method. Since only di-DNP-lysine was extracted with ether from the acid hydrolysate of the proteins, proteins A, B, and C had the same NH₂-terminal amino acid, l-lysine, as native RNase and no peptide bonds involving other amino acids had been cleaved. Fourth, electrophoresis of modified RNase in gels of different pore size (Fig. 3) showed no appreciable differences in the patterns or in the relative amounts of the three proteins. Since the pore size of the 9.6% gel is greater than that of the 14.6% gel (35), the similarity of the patterns eliminated the possibility that proteins A, B, and C were separated on the basis of differences in molecular size. All of these data led us to conclude that the modification of the RNase that we had observed was not attributable to cleavage of peptide bonds and fragmentation of the native RNase.

As an alternative, we considered the possibility that the interaction of N-acetoxy-2-FAA with RNase yielded three proteins which differed only in their net positive charge. This would account for the electrophoretic separation of modified RNase into three components. If the above explanation were correct, it would also be expected that modified RNase would be resolvable by ion exchange chromatography. Accordingly, we chromatographed modified RNase on Bio-Gel CM-30 with a linear gradient of increasing NaCl concentration (36). As shown in Fig. 4, the modified RNase was fractionated into three components which appeared to be comparable in their relative charge and amounts.
to proteins A, B, and C obtained by electrophoresis. Therefore, we retained the same designation (A, B, and C) for the three proteins resolved by ion exchange chromatography. Protein A was eluted in the same position as native RNase (Fig. 4). Proteins B and C were eluted with lower concentrations of NaCl than protein A and were therefore more acidic than protein A or RNase. It appeared very plausible from disc electrophoresis and ion exchange chromatography that protein B differed from protein A by 1 charge unit per molecule, and protein C differed from protein A by at least 2 charge units per molecule.

The conditions for the formation of proteins A, B, and C from N-acetoxy-2-FAA and RNase are summarized in Table I. The reaction of N-acetoxy-2-FAA with RNase in 0.005 M Tris-HCl + 7 M urea yielded progressively larger amounts of protein B, as the molar ratio of ester to protein was increased from 1 to 5. Protein C was formed only at the higher concentration of the ester. However, these experiments were complicated by the fact that native RNase, even after purification by gel chromatography, still contained a second protein (<5%) that was eluted in the same position as protein B and that appeared to be increased in amount by exposure of the RNase to 7 M urea (Table I). This protein was largely eliminated by chromatography of native RNase on Bio-Gel CM-30 and by omission of urea from the medium. Under these conditions, the formation of proteins B and C was demonstrated unequivocally (Experiments 2 and 3, Table I).

The separation of modified RNase into three components on the basis of charge would require that a reactive species derived from N-acetoxy-2-FAA had reacted with functional groups of those amino acids in RNase that confer a net positive charge on the protein. However, amino acid analyses indicated that the composition of proteins A, B, and C was virtually identical and the same as that of RNase A. It appeared as if the molecular species that had interacted with proteins B and C was removed during acid hydrolysis. Based on the known reactivities of N-acetoxy-2-FAA (6, 7) we considered two mechanisms as being consistent with this interpretation of the data. First, an amionic ion arising from the spontaneous decomposition of N-acetoxy-2-FAA might react with a lysine residue that formed a hydrogen bond with a carboxyl group (34). The arylation would abolish the hydrogen bond, thereby liberating an additional carboxylate ion and decreasing the net positive charge of the protein. Alternatively, N-acetoxy-2-FAA might acylate the ε-amino group of lysine, which would also have the effect of decreasing the positive charge on the protein. There is precedent for the nonenzymatic transfer of acetyl groups from N,N-diacetylarylhydroxylamines to cellular acceptors. Thus, Miller et al. reported the acetylation of guanosine by N-acetoxy-4-acetylaminobiphenyl and by N-acetoxy-4-acetylaminoantranilic acid (6).

In order to decide whether N-acetoxy-2-FAA modified RNase by arylation or by acetylation we reacted the protein with N-[3H]acetoxy-2-FAA. The incorporation of 3H and of 14C into proteins A, B, and C was measured as a measure of arylation and of acetylation, respectively. As indicated by the specific radioactivities of the three proteins (Table II), acetylation of proteins B and C was approximately 5- to 10-fold greater than arylation in media containing acetone + 7 M urea or acetone alone. Since the dissociation of N,N-diacetylarylhydroxylamines

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tr>
<td>Formation of proteins B and C by reaction of N-acetoxy-2-FAA with RNase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Reaction medium</th>
<th>Percent of total protein eluted from Bio-Gel CM-30 in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tris-HCl, urea, acetone</td>
<td>95.7</td>
</tr>
<tr>
<td>2</td>
<td>Tris-HCl, urea, acetone</td>
<td>88.7</td>
</tr>
<tr>
<td>3</td>
<td>Tris-HCl, urea, acetone</td>
<td>55.0</td>
</tr>
<tr>
<td>4</td>
<td>Tris-HCl, acetone</td>
<td>97.7</td>
</tr>
<tr>
<td>5</td>
<td>Tris-HCl, acetone</td>
<td>97.6</td>
</tr>
<tr>
<td>6</td>
<td>Tris-HCl, acetone</td>
<td>47.5</td>
</tr>
<tr>
<td>7</td>
<td>Tris-HCl, acetone</td>
<td>46.4</td>
</tr>
<tr>
<td>8</td>
<td>Tris-HCl, 2.4% EtOH</td>
<td>47.5</td>
</tr>
</tbody>
</table>

* In Experiments 1 to 4, commercial RNase was purified by gel filtration on Sephadex G-25 with 0.10 M Tris-HCl (pH 7.4) as the eluent. In Experiment 1, the purified RNase was rechromatographed on Bio-Gel CM-30 without incubation. In Experiments 2 to 4, the eluate containing the purified RNase was made 7 M with respect to urea, an equal volume of acetone was added, and the mixtures were incubated as described in the text. In Experiments 3 and 4, the acetone contained the appropriate amount of N-acetoxy-2-FAA. In Experiments 5 to 8, commercial RNase was purified by chromatography on Bio-Gel CM-30 as described in the text. In Experiment 5, the purified protein was rechromatographed on Bio-Gel CM-30 without incubation. In Experiments 6 and 7, the purified protein was titrated to pH 7.4 and diluted with an equal volume of acetone, and the mixtures were incubated as described in the text. In Experiment 7, the acetone contained the appropriate amount of N-acetoxy-2-FAA. In Experiment 8, the N-acetoxy-2-FAA in 0.5 ml of ethanol was added over a period of 3 hours to 20.0 ml of a magnetically stirred solution of RNase purified by chromatography on Bio-Gel CM-30 and titrated to pH 7.4. The reaction mixture was then incubated as described in the text. At the completion of the incubations, the proteins were precipitated with acetone, and the modified RNase was chromatographed on Bio-Gel CM-30.  

The designation of the isolated proteins is that shown in Fig. 4 and described in the text.  

The values are the means and the average deviations from the means obtained in two separate incubations.

<table>
<thead>
<tr>
<th>TABLE II</th>
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<tbody>
<tr>
<td>Specific radioactivities of proteins isolated from reaction of RNase with N-[3H]acetoxy-[9-14C]-2-FAA</td>
</tr>
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</table>

The specific radioactivities were determined on the proteins isolated in Experiments 4, 7, and 8 of Table I.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Reaction medium</th>
<th>Protein isolated</th>
<th>(atoms H)/100 pmol RNase</th>
<th>(atoms 14C)/100 pmol RNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tris-HCl, urea, acetone</td>
<td>A</td>
<td>1.2</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>Tris-HCl, acetone</td>
<td>B</td>
<td>61.5</td>
<td>4.7</td>
</tr>
<tr>
<td>3</td>
<td>Tris-HCl, 2.4% EtOH</td>
<td>C</td>
<td>108.5</td>
<td>12.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Reaction medium</th>
<th>Protein isolated</th>
<th>(atoms H)/100 pmol RNase</th>
<th>(atoms 14C)/100 pmol RNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Tris-HCl, acetone</td>
<td>A</td>
<td>4.4 ± 1.2</td>
<td>6.9 ± 2.9</td>
</tr>
<tr>
<td>3</td>
<td>Tris-HCl, 2.4% EtOH</td>
<td>B</td>
<td>67.2 ± 20.0</td>
<td>10.7 ± 4.6</td>
</tr>
<tr>
<td>4</td>
<td>Tris-HCl, 2.4% EtOH</td>
<td>C</td>
<td>55.6 ± 10.1</td>
<td>11.5 ± 1.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Reaction medium</th>
<th>Protein isolated</th>
<th>(atoms H)/100 pmol RNase</th>
<th>(atoms 14C)/100 pmol RNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Tris-HCl, acetone</td>
<td>A</td>
<td>59.0 ± 15.0</td>
<td>16.3 ± 3.0</td>
</tr>
<tr>
<td>6</td>
<td>Tris-HCl, acetone</td>
<td>B</td>
<td>97.7 ± 17.7</td>
<td>31.9 ± 9.5</td>
</tr>
</tbody>
</table>
RNase (80 mg, 5.8 pmol) purified by chromatography on Bio-Gel AM-50 was reacted with N-[3H]acetoxy-2-FAA (8.2 mg, 26 pmol, 7.2 × 10^4 dpm) as described in the text and in Experiment 7 of Table I. The modified RNase was precipitated and hydrolyzed as indicated in the text. The [3H]acetic acid in the hydrolysate was determined by inverse isotope dilution as described in the text.

### Table III

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time</th>
<th>Temperature</th>
<th>Protein-bound [3H]</th>
<th>Steam distillate [3H]</th>
<th>Fraction of bound [3H] recovered as [3H]-acetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 N NaOH</td>
<td>2</td>
<td>25°</td>
<td>50</td>
<td>1.3 ± 0.3</td>
<td>2.5</td>
</tr>
<tr>
<td>2 N NaOH</td>
<td>18</td>
<td>25°</td>
<td>50</td>
<td>5.7 ± 0.8</td>
<td>11.4</td>
</tr>
<tr>
<td>6 N H2SO4</td>
<td>6</td>
<td>100</td>
<td>50</td>
<td>39.2 ± 0.8</td>
<td>78.4</td>
</tr>
</tbody>
</table>

The acetylation of RNase by N-[3H]acetoxy-2-FAA was confirmed by the recovery of [3H]acetic acid from acid or alkaline hydrolysates of modified RNase. The identity of the [3H] in the hydrolysate with [3H]acetic acid was proved by redistillation of the acid without change of the specific radioactivity. Minor amounts of [3H]acetic acid, equivalent to 8.3% of bound [3H], were detected without subjecting modified RNase to acid or alkaline hydrolysis (Table III). This [3H]acetic acid came either from acetate that was adsorbed to the modified protein or from acetyl groups that were cleaved off acetylated amino acids under the conditions of the analysis. Since this quantity of [3H]acetic acid could not be unequivocally assigned to acetyl groups released from an ester or amide linkage, it was subtracted from the [3H]acetic acid obtained after acid or alkaline hydrolysis of modified RNase. Alkaline hydrolysis for 2 and 18 hours yielded 3 and 12% of the bound [3H], respectively, as [3H]acetic acid (Table III). These alkali-labile acetyl groups were presumably bound in ester linkage to tyrosine, serine and/or threonine (34). Acid hydrolysis liberated 78% of the bound [3H]acetyl groups in modified RNase. Since the ester linkage of certain N-,O-diacylhydroxyamino acids appears to be resistant to mild acid hydrolysis (38), the contribution to the above value by acetyl groups bound to RNase in ester linkage is uncertain. A minimum value for acetyl groups in amide linkage (67%) may be calculated by subtracting the acetyl groups released from modified RNase by alkali (11%) from those liberated by acid (78%). In any event, it seems clear that the major share of the hydrolyzable acetyl groups in modified RNase was derived from amide linkages.

A functional group of the amino acids of proteins that is available for acetylation to an amide under physiological conditions (39) is the ε-amino group of lysine. Therefore, we attempted the isolation and identification of ε-N-acetyl-L-lysine in modified RNase by two different methods. In the first experiment, protein B was hydrolyzed with Pronase and trypsin and the hydrolysate was chromatographed on AG 50W-X4. Nearly 80% of the radioactivity was eluted with 0.20 M ammonium acetate (pH 5.5) (92).
Fig. 7. Identification of e-N-[3H]acetyl-L-lysine in an enzymatic hydrolysate of modified RNase. The N-acetylated amino acid was isolated and purified by successive chromatographies on AG 50W-X4 (Fig. 5) and thin layer chromatography (Fig. 6) prior to resolution on a Beckman-Spinco amino acid analyzer.

**TABLE IV**

Fraction of [3H]labeled amino acids in enzymatic hydrolysate of modified RNase associated with e-N-acetyl-L-lysine

| Purified RNase (4.6 μmoles) in 50% acetone (15.0 ml) was reacted with N-[3H]acetoxy-2-FAA (22.8 μmoles, 7.4 × 10⁸ dpm) as described in the text. The modified RNase was hydrolyzed with trypsin (1.1 mg) and Pronase (30 mg) at 37° for 4 days (30, 31). The e-N-[3H]acetyl-L-lysine was isolated from the hydrolysate as described in the text.

<table>
<thead>
<tr>
<th>Specific radioactivity of modified e-N-[3H]acetyl-L-lysine</th>
<th>Specific radioactivity of modified e-N-[3H]acetyl-L-lysine</th>
<th>Specific radioactivity of modified e-N-[3H]acetyl-L-lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H] associated with free amino acids</td>
<td>[3H] associated with free amino acids</td>
<td>[3H] associated with free amino acids</td>
</tr>
<tr>
<td>dpm</td>
<td>dpm</td>
<td>dpm</td>
</tr>
<tr>
<td>1.12 × 10⁷</td>
<td>1.91 × 10⁷</td>
<td>6.49 × 10⁷</td>
</tr>
</tbody>
</table>

a The e-N-[3H]acetyl-L-lysine was purified to constant specific radioactivity by successive thin layer chromatographies on silica gel with ethanol-water (63:37), chloroform-methanol-2 N NH₄OH (2:2:1) and 1-butanol-acetic acid-water (6:2:2).

The release of [3H]acetyl groups from modified RNase by alkaline hydrolysis indicates that N-acetoxy-2-FAA esterified hydroxyamino acids in RNase, although the particular amino acids have not been identified. O-Acetyl-L-tyrosine can be eliminated from consideration since it is deacetylated in 1 hour by 2 N NaOH at 25° (40). In our experiments, only trace amounts of [3H]acetyl groups were obtained from modified RNase under these conditions (Table III). t-Serine and t-threonine would also be available for esterification to O-acetyl-L-serine and O-acetyl-L-threonine (34). These acetylated amino acids were not found in enzymatic hydrolysates of modified RNase despite the fact that 11% of bound [3H]acetyl groups were liberated from the modified protein by alkaline hydrolysis (Table III). The absence of the O-acetylated amino acids might have been due to their rearrangement to the N-acetylated derivatives at the pH maintained during enzymatic hydrolysis (16). If rearrangement had occurred, the N-acetylated compounds would have been washed through AG 50W-X4 and would have appeared in the minor radioactive fraction eluted from the resin with water (Fig. 5). Because O-acetylation of serine and threonine by N-acetoxy-2-FAA represents a relatively minor reaction in comparison to N-acetylation of lysine and because the charge properties of RNase would not be affected by O-acetylation, the identification of the products of the rearrangement, i.e. N-acetyl-L-serine and N-acetyl-L-threonine, was not further pursued.

It has been postulated that N-acetoxy-2-FAA decomposes to an acylamidium ion through an activated ion pair in which the acetoxy group is spatially separated from the remainder of the molecule (37). It has also been shown that the rate of formation of the amidonium ion is a function of the water content of the medium (37). Our data are in agreement with these findings since acetylation of proteins B and C was increased by 50% when the reaction between N-acetoxy-2-FAA and RNase was carried out in 2.4% ethanol instead of in 50% acetone (Table II).

It has been reported that certain N-acetoxy-N-arylamides, such as N-acetoxy-4-acetylaminobiphenyl and N-acetoxy-2-acetaminophenanthrene, acetylate, rather than arylamidate, guanosine (6). It also has been inferred that the acetylation proceeds through an ion pair similar to that proposed for arylamidation (37). However, our data indicate that acetylation of RNase by N-acetoxy-2-FAA was decreased by 11% under conditions which increased the extent of acetylation by 50%. Because of the lack of correspondence between arylamidation and acetylation, it appears unlikely that acetylation proceeds through an activated ion pair resembling that proposed for arylamidation. The possibility remains to be explored whether the mechanism of the acetylation of proteins by N-acetoxy-2-FAA consists instead of a nucleophilic attack of the nitrogen of the e-amino group of lysine on the partially positively charged carbonyl carbon atom of N-acetoxy-2-FAA, as shown in Fig. 8.

**DISCUSSION**

Our experiments show that acetylation is clearly the major reaction in the modification of RNase by N-acetoxy-2-FAA. However, examination of the data indicates that acetylation explains only in part the decrease in the positive charge of RNase and thus the formation of proteins B and C. This conclusion is based on the following considerations. As shown in Experiments 2 and 3 of Table II, 100 μmoles of protein B contained 67 and 60 μmoles of [3H]acetyl groups, respectively. Since a decrease in 1 unit of charge per 100 μmoles RNase would require the incorporation of at least 100 μmoles of acetyl groups, acetylation by N-acetoxy-2-FAA is evidently insufficient to account quantitatively for the observed decrease in the positive charge of RNase, and thus for the formation of protein B. Application of these considerations to the formation of protein C leads to the same conclusion. We are forced to assume that arylamidation and other unknown interactions of N-acetoxy-2-FAA with RNase contribute to the alteration of the charge properties of the protein.
Fig. 8. Hypothetical scheme for the acetylation of lysine in RNAse by nucleophilic attack of the ε-amino group on N-acetoxy-2-FAA. The figure also indicates the possibility of a cyclic acetyl transfer following the release of N-hydroxy-2-FAA from the intermediate adduct and reacetylation of the hydroxamic acid (5).

regenerated and be available for re-esterification as indicated in Fig. 8. By a repetition of this cyclic process, a few donor molecules could modify a large number of receptors. In contrast, in modification by acetylation the hydroxamic acid is not regenerated and a cyclic process is, therefore, not operative. The limiting factor in modification by acetylation would be the amount of activated carcinogen present in the cell.

We have previously presented evidence that N-2-fluorenylacetamide or its activated metabolite(s) acylamidate rat liver histones at a trace level (8). On the basis of our present data, it seems possible that the histones and other nuclear proteins were extensively modified in these earlier experiments by the mechanism suggested above. There is evidence that reversible acetylation or phosphorylation, or both, of histones and acidic nuclear proteins removes the inhibition that these proteins exert on the transcriptional process (9). Further experiments are needed to determine whether N-acetoxy-2-FAA and related carcinogens donate acetyl, phosphoryl, or sulfonyl groups to chromosomal proteins and whether the transfer of these groups affects the control of transcription by these proteins.

REFERENCES

40. Bergmann, M., and Zevras, L. (1928) Biochem. Z. 203, 280
Protein Modifications by Activated Carcinogens: I. THE ACETYLATION OF RIBONUCLEASE BY N-ACETOXY-2-FLUORENYLACETAMIDE

Eugene J. Barry and Helmut R. Gutmann


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