Reactions of Esters of \( N \)-Hydroxy-2-acetamidophenanthrene with Cellular Nucleophiles and the Formation of Free Radicals upon Decomposition of \( N \)-Acetoxy-\( N \)-arylacetamides

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SUMMARY

The sulfate ester of \( N \)-hydroxy-2-acetamidophenanthrene was prepared and was found to react significantly with methionine, adenosine, and guanosine. The adduct from the methionine reaction was characterized as 1-methymercapto-2-acetamidophenanthrene. The levels of reaction with adenosine and guanosine were comparable, in contrast with the observation that \( N \)-acetoxy-2-acetamidofluorene shows a marked preference for guanosine as a substrate among the nucleosides. Although the level of reaction was lower, \( N \)-acetoxy-2-acetamidophenanthrene also reacted equally well with adenosine and guanosine. Molecular orbital calculations suggested that the previously studied adduct-forming abilities of \( N \)-acetoxy-\( N \)-arylacetamides might be related to their ability to form radical cations. On this basis, the abilities of these compounds to decolorize the stable free radical 2,2-diphenyl-1-picrylhydrazyl were tested. \( N \)-Acetoxy-2-acetamidofluorene rapidly decolorized 2,2-diphenyl-1-picrylhydrazyl in 20% ethanol at 45°, \( N \)-acetoxy-2-acetamidophenanthrene acted much more slowly, while \( N \)-acetoxy-4-acetamidobiphenyl and \( N \)-acetoxy-4-acetamidostilbene had almost negligible effects. These activities are closely related to the adduct-forming abilities of these compounds and may affect their carcinogenicities. We suggest that the chemistry of esters of \( N \)-hydroxy-2-acetamidophenanthrene shown here is typical of nonradical reactions of \( N \)-aryl-\( N \)-acetylaminetirnium ions, while the reactivity of \( N \)-acetoxy-2-acetamidofluorene reflects a high percentage of radical cations among the ions formed upon decomposition of this ester.

INTRODUCTION

It is now accepted by many that the ultimate reactive form of carcinogenic \( N \)-arylacetamides is an ester of the corresponding hydroxamic acid (21). \( N \)-AcO-AAF, in particular, reacts efficiently with methionine (15) and guanosine (10) in vitro, yielding products identical to those obtained from rat liver after administration of \( N \)-OH-AAF (2, 10). The latter compound can be esterified by several “acylating” systems present in tissue (3, 7, 13, 14), and \( N \)-AcO-AAF is a more powerful carcinogen locally than \( N \)-OH-AAF (21). Thus, there seems to be little doubt of the importance of the ester-forming and -reacting sequence in the mechanism of carcinogenesis by \( N \)-OH-AAF and 2-acetamidofluorene. However, \( N \)-AcO-AAP, which is much more carcinogenic locally than \( N \)-AcO-AAF (21), reacts very poorly with guanosine in comparison with \( N \)-AcO-AAF (26), indicating that an important aspect of the chemistry of esters of \( N \)-aryhydroxamic acids remained undiscovered. Two approaches were taken to discover any unusual chemistry not yet noted. A more reactive ester than the acetate of \( N \)-OH-AAF was synthesized to elicit higher yields of reaction products and correspondingly more accurate comparisons of these yields, and the possibility of free radical formation during the decomposition of the acetate esters was reinvestigated.

MATERIALS AND METHODS

Unless otherwise noted, all reagents were obtained from J. T. Baker Chemical Co., Phillipsburg, N. J., and were used as received. Methionine and nucleosides were purchased from Sigma Chemical Co., St. Louis, Mo. Ammonia (anhydrous) and hydrogen sulfide (chemically pure) were from Matheson Gas Products, Newark, Calif. Radioactive nucleosides were obtained from Schwarz/Mann, Van Nuys, Calif. DPPH and pyridine-SO\(_3\) were obtained from Aldrich Chemical Co., Milwaukee, Wis. Elemental analyses were performed by Huffman Laboratories, Wheatridge, Colo. UV spectra were obtained on a Beckman ACTA III spectrometer; IR spectra were obtained on a Perkin-Elmer 257 spectrometer. NMR spectra were obtained on a Beckman ACTA III spectrometer; IR spectra were obtained on a Perkin-Elmer 257 spectrometer. NMR spectra were obtained on a Beckman ACTA III spectrometer; IR spectra were obtained on a Perkin-Elmer 257 spectrometer. NMR spectra were obtained on a Beckman ACTA III spectrometer; IR spectra were obtained on a Perkin-Elmer 257 spectrometer. NMR spectra were obtained on a Beckman ACTA III spectrometer; IR spectra were obtained on a Perkin-Elmer 257 spectrometer. NMR spectra were obtained on a Beckman ACTA III spectrometer; IR spectra were obtained on a Perkin-Elmer 257 spectrometer. NMR spectra were obtained on a Beckman ACTA III spectrometer; IR spectra were obtained on a Perkin-Elmer 257 spectrometer.

Preparation of \( N \)-OSO\(_3\)K-AAP. A solution of 2-nitrophenanthrene (prepared according to the method of Miller et al. (19)), m.p. 118–119°, 8 g, in 200 ml of dimethylformamide and 200 ml of 95% ethanol at 10° was saturated with hydrogen sulfide (chemically pure), 200 ml of 95% ethanol and 200 ml of 95% ethanol at 10° was saturated; NMR, nuclear magnetic resonance; \( N \)-OSO\(_3\)K-AAP, the sulfate ester (as its potassium salt) of \( N \)-OH-AAF; 1-Ch\(_3\)S-AAP, 1-methymercapto-2-acetamidophenanthrene; AAA, 2-acetamidophenanthrene; \( N \)-AcO-AAS, \( N \)-acetoxy-4-acetamidostilbene; \( N \)-OSO\(_3\)K-AABP, the sulfate ester (as its potassium salt) of 4-acetamidobiphenyl.
first with ammonia gas and then with hydrogen sulfide. The mixture was stirred overnight, diluted to 4 liters with water, and centrifuged in four 1-liter bottles for 15 min at 1800 rpm. The precipitate was washed with 2 liters of nitrogen-flushed water and centrifuged. After a decanting, the wet precipitate was extracted with 1200 ml of ether, and the ether solution was held under a gentle stream of nitrogen while being cooled to 0°. Sodium bicarbonate (5.2 g) and water (4 ml) were added; then a solution of 2.2 ml of acetyl chloride in ether was added slowly with rapid stirring. The mixture was stirred for 15 min, and the ether was then evaporated under reduced pressure. The residue was stirred for 30 min with 1600 ml of saturated sodium bicarbonate, filtered, washed with water, and recrystallized from benzene (Darco), to give 6.4 g (71% based on nitrophenanthrene) of white N-OH-AAP, m.p. 188—190°, \( \lambda_{\text{max}} \) 268 nm (e, 6.12 \( \times \) 10^4). This compound was treated with pyridine-SO₃ in pyridine and worked up according to the procedure of Mäher et al. (16). Yield, 86%. Analysis of this product (presumed to be N-OSO₃-K-AAP): C 50.37, H 3.81, ash 22.49. Prominent IR peaks (cm⁻¹): 1670, 1466, 1296, 1270, 1071, 1050, 970, 911, 818, 760, 720, 692, 660.

Preparation of 1-CH₃S-AAP. N-OSO₃-K-AAP (0.74 g) was mixed with methionine (10 g) in 300 ml water, incubated overnight at room temperature, treated with alkali, and extracted with benzene:pentane ether according to an earlier procedure (3). The residue from the extract (120 mg, 22% yield) was recrystallized from CHCl₃ to a melting point of 200.5—201.5° (corrected). The IR spectrum of this compound was published incorrectly (19). Miller and Miller have informed us that our data are in agreement with the data actually obtained by them.

Preparation of 2-Acetylphenanthryl-1-acetate. The product itself was predicted by molecular orbital calculations to be 1-CH₃S-AAP (24, 26). The structural characterization of a methylmercapto-2-aminophenanthrene from the reaction of this compound with methionine is evidence that it is indeed N-OSO₃-K-AAP. The isolation and characterization of a methylmercapto-2-aminophenylacetamide from the reaction of this compound with methionine is evidence that it is indeed N-OSO₃-K-AAP. The isolation and characterization of a methylmercapto-2-aminophenylacetamide from the reaction of this compound with methionine is evidence that it is indeed N-OSO₃-K-AAP.
Adducts of AAP

The aromatic substitution pattern (C—H bending) of the product is much like that of 2-acetylphenanthryl-1-acetate (Chart 1). The NMR spectrum shows the addition of a 2nd methyl group to the aromatic amide structure, and the decoupling experiments indicate the probable presence of a single AB pair of protons in the aromatic nucleus, a condition satisfied only by substitution at position 1 or by substitution in the C-ring. The latter possibility would appear to be excluded by the aromatic substitution pattern (IR) and by the shift in the N—H stretching band (IR) from 3430 cm⁻¹ in AAP to 3340 cm⁻¹ in the new compound (CH₂Cl₂, 2 mg/ml), an indication of intramolecular hydrogen bonding. The existence of intramolecular hydrogen bonding to sulfide sulfur in α-methylmercapto arylamines has previously been demonstrated (25). Recovery of a single product from hydrolysis of this compound is further evidence that the purified starting material was indeed a single compound.

The radiochromatogram scans from the nucleoside reaction mixtures are shown in Chart 2. Clearly, N-OSO₃K-AAP reacts with adenosine as well as it reacts with guanosine. N-AcO-AAF displays its reported high reactivity toward guanosine and its relatively low reactivity toward adenosine. N-AcO-AAP shows significant reactivity towards adenosine and guanosine, although not as great as that of the sulfate ester. The sharp peak running just ahead of starting material in the N-AcO-AAP reaction mixtures has previously been identified as acetylated nucleoside (21). Table 1 gives quantitative values for the peaks shown in the chart.

Chart 3 shows the rate of decolorization of DPPH by N-acetoxy-N'-arylacetamides. The rate of destruction of DPPH by N-AcO-AAF is essentially identical with the rate of ionization of this ester reported earlier (26), but the rate of ionization of N-AcO-AAS is much faster than the rate of decolorization seen here (26). N-AcO-AAP appears to decolorize DPPH significantly faster than it decomposes in 20% acetone alone (26). N-OH-AAF at the same concentration, at 54°, produced no decolorization within 30 min. Chart 4 shows the effect of increasing DPPH concentration on the rate of reaction with N-acetoxy-N'-arylacetamide. There is little concentration effect on the reaction with N-AcO-AAF, but the reaction with N-AcO-AAP is proportional to DPPH concentration.

DISCUSSION

Previous work has emphasized the ionic character of reactions of N-acetoxy-N'-arylacetamides in aqueous media (10, 15, 21, 26), with considerable emphasis placed on the reaction of N-AcO-AAF with guanine derivatives (6, 9, 10, 26).

![Chart 1. Formulas.](image)

![Chart 2. Reactions of esters of N-arylacetohydroxamic acids with adenosine and guanosine. Radiochromatograms were done on cellulose strips in 1-butanol:glacial acetic acid:water (50:11:25) (10). A, scans from reaction mixtures containing nucleotide only. A, adenosine + N-OSO₃K-AAP; B, adenosine + N-AcO-AAP; C, adenosine + N-AcO-AAF; D, guanosine + N-OSO₃K-AAP; E, guanosine + N-AcO-AAP; F, guanosine + N-AcO-AAF.](image)

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![2-Acetyl-1-phenanthryl acetate](image)
In this report, we present evidence that the reactivity of N-AcO-AAF is a special case, untypical of the chemistry of N-acetoxy-N-arylacetamides, and that this special position arises from the rapid transition of the N-2-fluorenyl-N-acetyl-nitrenium ion from a relatively high-energy singlet (nonradical) state to a triplet (radical) ground state. We also present evidence from a new viewpoint suggesting that reaction of N-aryl-N-acetylnitrenium ions with adenine in nucleic acids may be more closely related to the carcinogenicity of the parent compounds than is reaction with guanine. Kriek and Reitsma (11) have already investigated the chemistry of N-AcO-AAF with AMP and polyadenylic acid and have examined, as did Miller and Miller, the reactivity of N-OSO$_2$K-AABP with guanosine (8, 21). However, these investigations could not be adequately interpreted due to a lack of appropriate comparison studies. Earlier studies by the Millers and a recent report by Kriek had failed to show any binding of 2-acetamido-fluorene residues to adenine after treatment of DNA with N-AcO-AAF (9, 18), and the only significant suggestion that carcinogenicity of aromatic amines might be associated with attack on adenine has come from mutagenesis studies by Corbett et al. (1). Similarly, previous studies with N-AcO-AABP had shown the same qualitative pattern of reactivity with guanosine and adenosine, with the only difference from that of N-AcO-AAF being a lower reaction rate (20). Thus, although a reaction between N-AcO-AAF and polyadenylic acid can be easily carried out (6, 11, 17), there was no compelling reason to pursue this line of investigation. With the demonstration that esters of N-OH-AAF, a carcinogen more potent than N-OH-AAF (21), react equally well with adenosine and guanosine, we have provided this compelling reason. We consider it possible that the reaction with nucleic acid guanine represents a competition with adenine for available electrophile (nitrenium ion), which may actually reduce the efficiency of the N-acetoxy-N-arylacetamide as a carcinogen. An alternative possibility is that this high level of attack on guanine may be lethal to many cells that would otherwise be transformed by N-AcO-AAF.

An explanation for this variability in chemistry of ostensibly similar compounds may lie in the varying propensities of these compounds to form radical cations following ionization. Lee and Morokuma (12) have shown by theoretical methods that the ground state of the unsubstituted nitrenium ion (NH$_2^+$) is a radical cation in which 1 of the 2 electrons possibly otherwise present in the nonbonding sp$^2$ orbital is found in the p-orbital of the nitrogen. Gassman and Hartman (5) have shown, in a paper published since we completed these studies, that N-benzoyloxy-piperidines can solvolyze in methanol via either N—O cleavage or C—O cleavage, with the major reaction pathway determined by the nature of the substituent on the aromatic ring. They showed that essentially all of the nitrenium ion formed in these reaction mixtures appears as piperidine (hydrogen abstraction), a finding easily explained only by assuming a transition from the singlet state to a lower-energy triplet state. Comparable hydrogen abstraction has already been reported by Lotlikar and and Luha (13, 14) from studies with various esters of N-OH-AAF. If such a transition is possible in the N-aryl-N-acylnitrenium ions postulated to arise by decomposition of N-acetoxy-N-arylacetamides (26) (Chart 5), the degree of stabilization of the p-orbital by the aryl residue could affect the energy required for such a transition. Although simple molecular orbital calculations (24) suggest that different N-acyl-N-arylnitrenium ions may indeed have varying tendencies to make the transition to a triplet species, meaningful predictions can be obtained only from higher-order calculations requiring excessive amounts of computer time (C. A. Coulson, personal communication). The data in Charts 3 and 4 substantiate this suggestion, however. Although N-AcO-AAS and N-AcO-AAF ionize at very nearly the same rate (26), only the latter effects
decolorization of DPPH. N-AcO-AAP and N-AcO-AABP also react at essentially the same rate (much more slowly), and primarily by bimolecular substitution (25), either by water or by nucleosides to give acetylated nucleosides (20). Yet, as seen in Chart 3, it appears that the tendency of N-AcO-AAP to give eventual rise to a triplet ion is great enough that it will react directly with DPPH in a bimolecular fashion. The experiment shown in Chart 4 substantiates this suggestion by demonstrating that the rate of decolorization of DPPH by N-AcO-AAP is proportional to the concentration of DPPH. In the absence of an attacking free radical, however, N-AcO-AAP will react as a singlet with any nucleophile, since the nucleophile will be attacking the acetoxy compound in a nonradical configuration. This choice is not open to nucleophiles reacting with N-AcO-AAF, since it rapidly ionizes unimolecularly and immediately makes the transition to a radical as shown by the observation that the concentration of DPPH has little effect on the rate at which the DPPH is attacked by N-AcO-AAF (Chart 4).

A question of concern is whether the decolorization seen might be due to direct homolysis of the N-acetoxy-N-arylacetamidamides or to reaction with solvolysis products of the degradation reaction. The latter possibility was tested by incubating DPPH with N-OH-AAF under more vigorous conditions than those shown for Chart 3. No effect was seen after 0.5 hr at 54°. The possibility of homolysis is most unlikely, based on previous studies combined with those reported here. It was shown earlier that the rate of release of acetyl group (as water-soluble, non-ether-extractable radioactivity = acetate ion) from N-AcO-AAF (AcO.14C) is highly dependent on water concentration in an acetone:water medium (26). This is strong evidence for the formation of ions from an uncharged precursor. In addition, the rates of acetate release from N-AcO-AAF and N-AcO-AAS are comparable (26); the rates of DPPH decolorization are not. The same holds true for the decomposition of N-AcO-AAP and N-AcO-AABP. Finally, the observation that N-AcO-AAP and N-AcO-AABP acetylate guanosine extensively but that N-AcO-AAF and N-AcO-AAS acetylate guanosine very little (21) suggests simply that nucleophilic substitution on the C-O bond is a reaction that proceeds at roughly the same rate for all 4 esters but that cannot compete against the rapid unimolecular ionization of N-AcO-AAF and N-AcO-AAS. If homolysis were indeed the primary reaction, N-AcO-AAF should produce even more acetylated nucleoside than N-AcO-AAP, which it clearly does not.

A final matter of interest is the possible relationship of these radical reactions to carcinogenesis. Chart 3 suggests that the radical cation-forming tendency of these N-acetoxy-N-arylacetamidamides is an explanation of their abilities to form adducts with methionine or guanosine. Since these adduct-forming abilities are not correlated with local carcinogenicity (21, 26), the tendency toward radical cation formation and reactivity with nucleic acid guanine may be a process that competes with a reaction actually necessary to initiate tumorigenesis. This reaction may be bimolecular substitution on the ester by nucleic acid adenine, or it may be formation of an adduct with guanine different in nature from the major product of guanine or adenine with N-AcO-AAF (9–11). Such an adduct has been found by Miller and Miller (21) in the reaction between guanosine and N-AcO-AAS. Since N-AcO-AAS does not generate radical cations upon decomposition and is a stronger carcinogen than N-AcO-AAF (as is N-AcO-AAP) (21), identification of such adducts from reactions of esters of N-OH-AAS and N-OH-AAF is a matter of importance which is now being undertaken. It may be that the less likely an N-acetoxy-N-arylacetamide is to generate a radical cation, the better carcinogen it will be. A clear exception is N-AcO-AABP. This may be due to the high solubility of biphenylamine derivatives relative to comparable derivatives of the other aromatic amines in question, or it may truly indicate that this hypothesis is faulty. Further studies are being undertaken to clarify this point.

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