Reactive Phosphate Ester of the Carcinogen 2-(N-Hydroxy)acetamidofluorene

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1. Reaction of 2-(N-acetoxy)-acetamidofluorene with orthophosphate buffer at pH 7 yielded a large quantity of water-soluble fluorene derivatives, which showed absorption peaks at 303, 290 and 280 nm. Tris buffer under similar conditions gave negligible reaction. 2. Hydrolysis of polar material with acid or alkaline phosphatases liberated equimolar amounts of inorganic phosphate and an ether-extractable fluorene derivative. On the basis of its u.v. spectrum, $R_f$ values after paper chromatography, solubility in alkali and colour with spray reagents, the derivative was characterized tentatively as 2-acetamido-5-hydroxyfluorene. 3. Polar material also contained a reactive fluorene derivative which gave characteristic reaction products with methionine and guanosine. The reactive derivative was characterized as a phosphate ester of 2-(N-hydroxy)-acetamidofluorene. 4. It is suggested that such reactive phosphate esters may also be some of the ultimate carcinogenic metabolites of carcinogenic aromatic hydroxamic acids.

Most drugs and foreign compounds are excreted by various animals in the form of glucuronides, acetates and sulphates (Williams, 1967). There is no evidence that any foreign compound is excreted in the urine of rats as a phosphate conjugate even though bis-(2-amino-1-naphthyl) phosphate was excreted in the urine of dogs and man dosed with 2-naphthylamine (Troll, Belman & Nelson, 1959; Boyland, Kinder & Manson, 1961; Troll, Tessler & Nelson, 1963; Troll, Belman & Mukai, 1969).

N-Hydroxylation has been considered to be the first step of activation in carcinogenesis by aromatic amines and amides (Miller, 1970). Even though the degree of carcinogenicity of these compounds and their degree of binding to tissue proteins, RNA and DNA in vivo have been correlated to a great extent (Marroquin & Farber, 1965; Irving, Veazey & Williard, 1967; Irving & Veazey, 1969; DeBaun, Miller & Miller, 1970a), these carcinogenic $N$-hydroxylamides do not react with tissue macromolecules or their constituents in vitro (King, Gutmann & Chang, 1963; Bahl & Gutmann, 1964; Miller, Juhl & Miller, 1966; Lotlikar, Scribner, Miller & Miller, 1966; Miller et al. 1968). Since synthetic $N$-acetoxyamides have stronger carcinogenicity than $N$-hydroxy derivatives and interact with proteins, nucleic acids and their constituents non-enzymically (Miller et al. 1966; Lotlikar et al. 1966; Kriek, Miller, Juhl & Miller, 1967; Maher, Miller, Miller & Szybalski, 1968; Miller & Miller, 1969b; Fink, Nishimura & Weinstein, 1970) it was suggested that esterification of the $N$-hydroxyamides might be a second and final step of activation of these compounds (Miller & Miller, 1969b; Miller, 1970). It has been shown (DeBaun, Rowley, Miller & Miller, 1968; King & Phillips, 1968, 1969) that $N$-hydroxy-AAF* in the presence of ATP and Mg$^{2+}$ ions was converted by rat liver enzyme into AAF N-phosphate. $N$-Hydroxyamides were also found to be acetylated non-enzymically by acetyl-CoA to form reactive esters (Lotlikar & Luh, 1970).

The present paper describes the interaction of $N$-acetoxy-AAF with P$_1$ to form AAF N-phosphate and AAF ring-phosphate. Like acetate and sulphate esters (Lotlikar et al. 1966; Kriek et al. 1967; King & Phillips, 1969; DeBaun et al. 1970a), AAF N-phosphate reacts with methionine and guanosine in vitro. Results of some of these studies have been published in a preliminary report (Lotlikar, Irving, Miller & Miller, 1967b).

MATERIALS AND METHODS

Chemicals. $N$-Hydroxy-AAF and N-acetoxy-AAF were prepared as described previously (Poirier, Miller & Miller, 1963; Lotlikar et al. 1966). The following compounds were kindly supplied by Dr J. A. Miller of the Mc Ardle Laboratory, Madison, Wis., U.S.A.: 3-methylmercapto-AAF; 2-amino-3-methylmercaptofluorene and 1-, 3-, 5- and 7-hydroxy-AAF. Acid phosphatase type I from wheat germ, alkaline phosphatase type I from calf intestinal mucosa, bacterial type $\beta$-glucuronidase and L-methionine were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.; Taka diastase was a product of Parke, Davis and
Co., Detroit, Mich., U.S.A. Guanosine dihydrate, grade A, was bought from Calbiochem, Los Angeles, Calif., U.S.A. [8-14C]Guanosine (34.1 mcCi/mmol) and L-[3H]methionine (25–50 mcCi/mmol) were purchased from Schwarz Bio Research Inc., Orangeburg, N.Y., U.S.A. Amberlite XAD-2, a neutral cross-linked polymer, was obtained from the Rohm and Haas Co., Philadelphia, Pa., U.S.A., in the form of hydrated beads with an effective size of 0.3–0.45 mm. All other chemicals were of reagent grade.

Preparation of polar AAF N-phosphate. (a) Incubation and extraction. N-Acetoxy-AAF (10 µmol dissolved in 0.5 ml of ethanol) was incubated with 50 µmol of either KH₂PO₄-K₂HPO₄ or tris-HCl buffer, pH 7.0, in a total volume of 2.0 ml at 25°C for 2 h. After incubation samples were diluted to 5 ml with water and were extracted five times with 10 ml of diethyl ether to remove unchanged N-acetoxy-AAF or any free fluorene derivative. A Coleman-Hitachi model 124 spectrophotometer with a recorder was used for determination of water-soluble fluorene derivatives in the aqueous phase by reading the u.v. absorption spectrum between 350 and 270 nm. The molar extinction coefficient of N-acetoxy-AAF in ethanol of about 18,000 at 290 nm was used for calculation of the total amount of water-soluble fluorene derivatives formed. The absorption of the aqueous phase at 290 nm was used for these calculations.

(b) Reaction with methionine. To a 2 ml sample of the above-described aqueous phase was added 4 µmol of L-methionine containing about 2 µCi of [35S]methionine. After reaction at 25°C for 18 h the mixture was made 1 x with respect to KOH. After 15 min, the mixture was extracted with benzene. The benzene extract was washed with water, benzene was removed under N₂ and the residue was then chromatographed on silicic acid paper (Whatman SG-81) in benzene-chloroform (2:3, v/v) solvent system. Chromatography was terminated after the solvent front had travelled 16 cm from the origin. From each chromatogram were cut 1 cm zones and each was eluted in 1 ml of methanol in a vial for 30 min. Bray's (1960) solution (10 ml) was added to each vial and radioactivity was measured in a Packard Tri-Carb spectrometer. Synthetic 3-methylmercapto-AAF and 2-amino-3-methylmercaptofluorene showed RF values of 0.35–0.44 and 0.67–0.77 respectively with the above solvent system when the paper chromatogram was viewed under u.v. light. Most of the reaction product of AAF N-phosphate with methionine had RF 0.35–0.44. A small amount of radioactivity (~1%) appeared in the RF 0.67–0.77 region.

(c) Reaction with guanosine. To a 2 ml sample of the aqueous phase was added 1 µmol of guanosine containing 0.5 µCi of [8-14C]guanosine. As a control 1 µmol of [8-14C]guanosine was also incubated with 20 µmol of KH₂PO₄-K₂HPO₄ buffer, pH 7.0, in a total volume of 2.5 ml. Similarly, 1 µmol of [8-14C]guanosine was incubated with 5 µmol of N-acetoxy-AAF in 10 mm-tris-HCl buffer, pH 7.0. Like the methionine reaction, the guanosine reaction mixture was also incubated at 25°C for 18 h. Usually 100–150 µl of the reaction mixture was chromatographed on a 250 µm-thick cellulose thin-layer plate (Brinkman MN390 U2)254) in the solvent system butan-1-ol-acetic acid-water (50:11:25, by vol.) as described by Miller et al. (1968). When chromatograms were viewed under u.v. light guanosine showed RF values 0.23–0.45. The reaction product of N-acetoxy-AAF was visible under u.v. light with RF value 0.78–0.94. The chromatogram was divided into 1 cm zones from the origin to the solvent front at 15 cm. These 1 cm zones were quantitatively scraped into vials containing 10 ml of Bray's (1960) solution for radioactivity measurements in a Packard Tri-Carb spectrometer. Assay of the guanosine reaction product of AAF N-phosphate was based on the radioactivity of the material with RF value 0.78–0.94. The reaction product of N-acetoxy-AAF and [8-14C]guanosine was assayed in the same way.

Preparation of polar fluorene derivatives. (a) Incubation and extraction. A large quantity of the ring-phosphate of AAF was prepared by incubating four batches of 100 µmol of N-acetoxy-AAF dissolved in 10 ml of ethanol with 2000 µmol of KH₂PO₄-K₂HPO₄ buffer, pH 7.0, in a total volume of 40 ml. After 24 h of incubation at 37°C, the contents were diluted to 100 ml with water and were then extracted five times each with 100 ml of diethyl ether. The aqueous phase showed u.v.-absorption peaks at 303, 290 and 280 nm. The total u.v. absorption of 100 ml of aqueous phase at 290 nm was about 840. The aqueous phases of four batches (400 ml) were concentrated to 100 ml (total E₂₉₀ 3360). Polar material obtained with tris-HCl buffer, pH 7.0, under similar conditions was less than 1% of that obtained with phosphate buffer.

(b) Separation of organic phosphate from P₁. AAF phosphate in 20 ml of aqueous phase (E₂₉₀ 972) was separated from P₁ by adsorption of the fluorene conjugate on an Amberlite XAD-2 column (3 cm × 40 cm) and elution with ethanol as described by Bradlow (1968) for extraction of steroid conjugates from urine. Ethanol was removed with a flash evaporator and the residue was dissolved in water.

(c) Incubation with phosphatases, and extraction. After recording the u.v. absorption of the aqueous solution at 290 nm, samples were taken for treatment with acid or alkaline phosphatase. After incubation with enzyme for 2 h at 37°C, the pH was adjusted to about 6.0 and the contents were extracted with ether. The ether extract was washed with water, ether was removed under N₂ and the residue was dissolved in ethanol for u.v.-absorption (350–270 nm) measurements. The aqueous phase was checked for liberation of P₁, which was determined by a modification of the method of Martin & Doty (1949).

(d) Paper chromatography of the ether-extractable fluorene derivative. The phosphatase-liberated fluorene derivative that was ether-extractable was chromatographed on Whatman no. 1 paper with cyclohexane-2-methylpropan-2-ol-acetic acid-water (16:4:2:1, by vol.) (Weisburger, Weisburger, Morris & Sober, 1956) or (18:2:2:1, by vol.) (Lotlikar, Enomoto, Miller & Miller, 1967a). After chromatography strips were viewed under u.v. light and absorption bands were marked. Duplicate strips were sprayed with Folin–Ciocalteu phenol reagent and 20% (w/v) Na₂CO₃ (Cramer, Miller & Miller, 1960) or with diazotized 2-amino-7-nitrofluorene and 20% Na₂CO₃ (Weisburger et al. 1956).

RESULTS

Incubation of N-acetoxy-AAF in the presence of potassium phosphate buffer, pH 7.0, converted a large amount into water-soluble fluorene derivatives.
Table 1. Formation of AAF-phosphate by interaction of N-acetoxy-AAF with $P_i$

$N$-Acetoxy-AAF (5 $\mu$mol dissolved in 0.5 ml of ethanol) was incubated with 100 $\mu$mol of either KH$_2$PO$_4$--K$_2$HPO$_4$ or tris-HCl buffer, pH 7.0, in a total volume of 2.0 ml for 3 h at 37°C. After incubation samples were diluted to 5 ml and extracted five times with 10 ml of ether. The u.v.-absorption spectrum of the aqueous phase showed peaks at 303, 290 and 280 nm. The molar extinction coefficient of $N$-acetoxy-AAF in ethanol at 290 nm was about 18000. This value was used for approximate calculation of the total amount of water-soluble fluorene derivatives formed.

<table>
<thead>
<tr>
<th>Buffer used</th>
<th>Total $E_{290}$ of aqueous phase</th>
<th>Water-soluble fluorene derivatives formed (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>0.35</td>
<td>19</td>
</tr>
<tr>
<td>Phosphate</td>
<td>34.4</td>
<td>1911</td>
</tr>
</tbody>
</table>

Table 2. Reaction of water-soluble fluorene derivative with methionine and guanosine at pH 7

$N$-Acetoxy-AAF (10 $\mu$mol dissolved in 0.5 ml of ethanol) was incubated with 50 $\mu$mol of either KH$_2$PO$_4$--K$_2$HPO$_4$ or tris-HCl buffer, pH 7.0, in a total volume of 2.0 ml for 2 h at 25°C. After incubation samples were diluted and extracted several times with ether. Samples of the aqueous phase were taken for reaction with methionine, guanosine and for u.v.-absorption measurements. Incubation medium and analyses of their reaction products were as described in the Materials and Methods section.

<table>
<thead>
<tr>
<th>Buffer used</th>
<th>Total $E_{290}$ of aqueous phase</th>
<th>Water-soluble fluorene derivatives formed (nmol)</th>
<th>Methionine (3-methylmercapto-AAF) ($R_f$ 0.78--0.94)</th>
<th>Guanosine ($R_f$ 0.66--0.70)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>0.08</td>
<td>4.4</td>
<td>0.4</td>
<td>140</td>
</tr>
<tr>
<td>Phosphate</td>
<td>5.0</td>
<td>278</td>
<td>1.4</td>
<td>158</td>
</tr>
</tbody>
</table>

that showed absorption peaks at 303, 290 and 280 nm (Table 1). The formation of this water-soluble material was phosphate-dependent; a negligible amount of water-soluble fluorene derivatives was formed on incubation with tris-hydrochloric acid buffer.

In a separate experiment it was noted that on incubation at 25°C the yield of polar material was about 30% of that formed at 37°C. However, the percentage of reaction product formed with methionine (3-methylmercapto-AAF) was much more with the polar material formed at 25°C than at 37°C. Therefore $N$-acetoxy-AAF was incubated with phosphate buffer at 25°C to yield a high percentage of water-soluble fluorene derivative that would react with methionine and guanosine (Table 2). It was found that methionine reacted with the phosphate-dependent polar fluorene derivative to give a product that had the same $R_f$ value (0.35--0.44) as the synthetic 3-methylmercapto-AAF. $N$-Hydroxy-AAF does not react with methionine to form such a product (Lotlikar et al. 1966; Miller et al. 1968; DeBaun et al. 1968). However, $N$-acetoxy-AAF and AAF $N$-sulphate react with methionine to form this product (Lotlikar et al. 1966, 1967b; DeBaun et al. 1968; Miller & Miller, 1969a). Similarly like $N$-acetoxy-AAF and AAF $N$-sulphate (Kriek et al. 1967; Miller & Miller, 1969a; King & Phillips, 1969; DeBaun, Smith, Miller & Miller, 1970b), the phosphate-dependent polar material reacted with guanosine to a large extent to yield a product with a high $R_f$ value (0.78--0.94). This product has been characterized as $N$-(guanosin-8-yl)-AAF (Kriek et al. 1967; King & Phillips, 1969). When tris-hydrochloric acid buffer was used a very small amount of polar material was formed. Hence no appreciable amounts of reaction products with methionine or guanosine were detected. These results suggest that the reactive derivative was a phosphate ester of $N$-hydroxy-AAF, also called AAP $N$-phosphate.

The reactive AAP N-phosphate was very labile, which suggested that it decomposed or rearranged, most probably to a ring-phosphate, after standing for a few hours. When methionine or guanosine was added at that time, no reaction products could be obtained. It was important to demonstrate, however, whether or not the unreactive polar AAF was a phosphate conjugate. A large amount of polar AAF was prepared by interaction of $N$-acetoxy-AAF and $P_i$ (Table 3). Both acid and alkaline phosphatases hydrolysed the polar fluorene derivatives by liberation of $P_i$ and ether-extractable fluorene derivatives, which showed an inflexion point at 315 nm and absorption peaks at 305, 292 and 280--81 nm. Incubation of polar material with Taka diastase or $\beta$-glucuronidase in 0.06 M-sodium acetate buffer, pH 6.0, was not effective. These results suggested that the polar material was a phosphate conjugate. The hydrolysed ether-extractable fluorene derivati-
tive, when chromatographed on Whatman no. 1 paper with two different solvent systems, gave a u.v.-absorption band with an $R_f$ value similar to that of 5-hydroxy-AAF. This zone reduced the Folin–Ciocalteu phenol reagent (Cramer et al. 1960) to give a blue colour and coupled with diazotized 2-amino-7-nitrofluorene (Weisburger et al. 1956) to give a pink colour. The product was alkali-soluble. Even after chromatography its absorption spectrum in ethanol showed an inflexion point at 315 nm and absorption peaks at about 305, 292 and 280–81 nm. Synthetic 5-hydroxy-AAF does not show any inflexion point at 315 nm; it has absorption peaks at 307, 296 and 282–83 nm. Synthetic 3-hydroxy-AAF does have an absorption peak at 315 nm. On the basis of $R_f$ values, u.v.-absorption spectrum, pink colour with diazotized reagent and alkali solubility, the ether-extractable fluorene derivative has been characterized tentatively as 5-hydroxy-AAF.

Attempts to detect phosphate metabolites in rat urine after adult male rats had been injected intra-peritoneally with AAF, 5-hydroxy-AAF, N-acetoxy-AAF or AAF ring-phosphate were unsuccessful.

**DISCUSSION**

The present results indicate that a potent synthetic carcinogen, N-acetoxy-AAF, reacts with $P_1$ to yield a reactive AAF $P_1$-phosphate and an unreactive ring-phosphate of AAF.

Synthetic N-acetoxy-AAF and AAF $N$-sulphate react non-enzymically with proteins, nucleic acids and their constituents methionine and guanosine (Lotlikar et al. 1966; Miller et al. 1966; Kriek et al. 1967; Maher et al. 1968; Miller & Miller, 1969b; Fink et al. 1970), whereas N-hydroxy-AAF does not react with these nucleophiles in vitro (Miller & Miller, 1969b). Owing to its instability, enzymatic formation of AAF $N$-phosphate has been based on its requirements for ATP and Mg$^{2+}$ ions and its reaction with tissue nucleophiles (King & Phillips, 1968, 1969; DeBaun et al. 1968, 1970a). In the present experiments reactivity with methionine and guanosine has also been used as a criterion for suggesting formation of AAF $N$-phosphate.

Like AAF $N$-phosphate, enzymic formation of AAF $N$-sulphate in vitro was shown by its dependence on adenosine 3'-'phosphate 5'-sulphato-phosphate and its reaction with nucleophiles (King & Phillips, 1968, 1969; DeBaun et al. 1968, 1970a). DeBaun et al. (1970b) have shown that injection of SO$_4^{2-}$ ions increased the toxicity of N-hydroxy-AAF in rats and also caused increased binding of fluorene derivatives to hepatic macromolecules. These studies were performed on rats given prior treatment with p-hydroxyacetanilide to deplete the pool of SO$_4^{2-}$ ions in vivo. These results coupled with carcinogenicity studies (Weisburger, Yamamoto, Grantham & Weisburger, 1970) suggest that AAF $N$-sulphate is formed from N-hydroxy-AAF in vivo also. The lack of effect of PO$_4^{3-}$ ions in the above studies (DeBaun et al. 1970b) might be due to the great abundance of these ions in vivo.

There is no evidence yet that N-hydroxy-AAF could be enzymically acetylated to N-acetoxy-AAF. However, there is evidence that such acetylation can occur non-enzymically in the presence of acetyl-CoA (Lotlikar & Luha, 1970). Therefore it appears that AAF $N$-phosphate might be formed in vivo either by interaction of $N$-acetoxy-AAF and $P_1$ or by enzymic conversion of N-hydroxy-AAF in the presence of ATP and Mg$^{2+}$ ions. If such a phosphate ester is formed in vivo, it would be very reactive in attacking cellular macromolecules. Like AAF $N$-sulphate, it also might be an ultimate carcinogenic metabolite of AAF and its $N$-hydroxy derivative.

In these studies it was not possible to demonstrate...
whether the ring-phosphate of AAF, which was tentatively characterized as a phosphate conjugate of 5-hydroxy-AAF, was derived directly or via AAF N-phosphate by rearrangement.

There is no evidence that any foreign compound is excreted in the urine of rats as a phosphate conjugate. There is only an isolated case of one compound, 2-naphthylamine, which was excreted in the urine of dog and man as a phosphate conjugate (Troll et al. 1959, 1963, 1969; Boyland et al. 1961). In our experiments also, it was not possible to detect any phosphate conjugate of fluorene in the rat urine even when these animals were injected intraperitoneally with the ring-phosphate of AAF. Such failures might be due to the presence of large amounts of phosphataes in several tissues, blood and urine (Schmidt, 1961).

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