Acylation of Carcinogenic Hydroxamic Acids by Carbamoyl Phosphate to Form Reactive Esters

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1. Acylation of 2-(*N*-hydroxyacetamido)fluorene and several other aromatic hydroxamic acids by carbamoyl phosphate was studied and compared with the acylating activity of acetyl-CoA. Acetyl phosphate was also studied. 2. The carbamoylation reaction had a pH optimum of 4.5. The reaction had a lag period of 1 h and was then linear for 4 h. This linearity ranged between 0.5 mM- and 8 mM-carbamoyl phosphate concentration. 3. At pH 7.5, acetyl-CoA was the most powerful acylating agent. Acetyl phosphate was a weaker acylating agent than either of the others. 4. Among the various hydroxamic acids tested with acetyl-CoA and carbamoyl phosphate at pH 7.5, 2-(*N*-hydroxyacetamido)fluorene was the most reactive. On the other hand the less reactive *N*-hydroxy derivatives of 2-acetamidonaphthalene, 2-acetamidophenanthrene and 4-acetamidostilbene reacted severalfold more with carbamoyl phosphate than with acetyl-CoA. 5. It is suggested that carbamoylation of aromatic hydroxamic acids might be one of the final activation steps in carcinogenesis by these compounds.

N-Hydroxylation is the first step of activation in carcinogenesis by aromatic amines and amides (Miller & Miller, 1969*b*; Miller, 1970). Studies with synthetic acetate esters of aromatic hydroxamic acids suggested that esterification of hydroxamic acids might be the second and final step of activation in the carcinogenic process (Miller & Miller, 1969*a,b*). Recent studies have demonstrated that carcinogenic aromatic hydroxamic acids are converted enzymically into their sulphate and phosphate esters by rat liver cytosol preparations (DeBaun, Rowley, Miller & Miller, 1968; King & Phillips, 1968, 1969; DeBaun, Miller & Miller, 1970; Lotlikar, 1970). Even though there is no enzymic evidence as yet, these hydroxamic acids are also acetylated by acetyl-CoA non-enzymically (Lotlikar & Luha, 1970).

Carbamoyl phosphate is formed in very large quantities in mammalian livers (Ramponi & Grisolia, 1970). Some proteins are readily carbamoylated non-enzymically but others are not (Grisolia, 1968; Ramponi & Grisolia, 1970). It was decided to investigate whether hydroxamic acids could be carbamoylated by carbamoyl phosphate. This paper presents results of carbamoylation of *N*-hydroxy-AAF* and several other aromatic hydroxamic acids. In these studies, acetyl-CoA and acetyl phosphate are compared with carbamoyl phosphate for their acylating activities.

* Abbreviation: AAF, 2-acetamidofluorene.

MATERIALS AND METHODS

Chemicals. *N*-Hydroxy-AAF was prepared by the method of Poirier, Miller & Miller (1963). Other aromatic hydroxamic acids used in the present study, 3-methylthio-AAF and 2-amino-3-methylthiofluorene, were kindly provided by Dr J. A. Miller of the McArdle Laboratory, University of Wisconsin, Madison, Wis., U.S.A. Acetyl-CoA as the lithium salt was obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. Carbamoyl phosphate as the dilithium salt, acetyl phosphate as the dilithium salt, acid phosphatase type I from wheat germ and L-methionine were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Guanosine dihydrate was purchased from Calbiochem, Los Angeles, Calif., U.S.A. L-[³⁵S]-Methionine (25–30 mCi/mmol) and [8-¹⁴C]guanosine (34.1 mCi/mmol) were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. All other chemicals were of reagent grade.

Acylation reaction. The acylation of aromatic hydroxamic acids by carbamoyl phosphate, acetyl-CoA or acetyl phosphate was studied in the presence of either methionine or guanosine. Reaction with these two nucleophiles was used as a measure of acylation reaction.

Assay for products of reaction with methionine. These reaction products were extracted in benzene and chromatographed on silicic acid paper in benzene–chloroform (2:3, v/v) as described by Lotlikar (1970). The *R_F* values of 0.35–0.44 and 0.67–0.77 respectively were obtained for the synthetic 3-methylthio-AAF and 2-amino-3-methylthiofluorene in the above solvent system. When *N*-hydroxy-AAF was the substrate, the radioactivity corresponding to these zones was used for calculation of

the amounts of products formed. When other hydroxamic acids were used as substrates, the radioactivity zones with R_F values of about 0.35–0.44 and 0.67–0.77 were used for calculation of the amounts of formation of the respective *o*-methylthio amide and *o*-methylthio amine. In some studies, the amount of 3-methylthio-AAF formation was also determined by g.l.c. as described previously (Lotlikar, 1970).

Separation and determination of AAF. Alkali was added after incubation and the reaction mixture was extracted with benzene. The benzene extract was chromatographed on silicic acid paper in benzene–chloroform (2:3, v/v). The zone with R_F 0.10–0.22 was eluted in ethanol. Ethanol was removed under N_2 and the residue was dissolved in diethyl ether. Any contaminating *N*-hydroxy-AAF was removed from the ether solution by extraction with alkali. Finally ether was removed under N_2 , and the residue was dissolved in ethanol. The u.v.-absorption spectrum was recorded between 350 and 270 nm with a Coleman–Hitachi model 124 spectrophotometer. ϵ 28 800 at 287–288 nm was used for calculation of the amount of AAF formation. G.l.c. (Lotlikar, 1970) was also used for determination of AAF.

Assay for product of reaction with guanosine. The reaction product was chromatographed on a cellulose (Brinkman MN300 UV254) t.l.c. plate (250 μ m thick) in butan-1-ol–acetic acid–water (50:11:25, by vol.) as described by Miller *et al.* (1968). After the solvent front had travelled about 15 cm from the origin, the chromatography was terminated. Zones (1 cm long) were quantitatively scraped into vials containing 10 ml of Bray's (1960) solution for radioactivity measurements. Guanosine had R_F 0.23–0.45 in the above solvent system. The radioactive peak with R_F 0.73–0.93 was used for the assay of the formation of the guanosine reaction product.

RESULTS

Incubation of *N*-hydroxy-AAF in the presence of carbamoyl phosphate in tris–HCl buffer, pH 7.5, gave appreciable amounts of methionine and guanosine reaction products (Table 1). However, in the absence of carbamoyl phosphate, *N*-hydroxy-AAF showed very little activity with these two nucleophiles. It was found that the methionine reaction product had the same R_F values (0.35–0.44) as the synthetic 3-methylthio-AAF. Acetate, phosphate and sulphate esters of *N*-hydroxy-AAF also react with methionine to form such a product (Lotlikar, Scribner, Miller & Miller, 1966; DeBaun *et al.* 1968; Miller & Miller, 1969a; Lotlikar & Wasserman, 1970). Similarly, in the presence of carbamoyl phosphate, *N*-hydroxy-AAF reacted with guanosine to a large extent to yield a product with a high R_F value (Table 1). A similar product obtained with acetate and sulphate esters of *N*-hydroxy-AAF has been characterized as *N*-(guanosin-8-yl)-AAF (Kriek, Miller, Juhl & Miller, 1967; King & Phillips, 1969; Miller & Miller, 1969a). These results suggest that the reactive derivative in our present studies was a carbamate ester of *N*-hydroxy-AAF.

Table 1. *Reaction of N-hydroxy-AAF with methionine and guanosine in the presence of carbamoyl phosphate*

Unless otherwise indicated, the complete incubation medium consisted of: 100 mM–tris–HCl buffer, pH 7.5; 4 mM–*N*-hydroxy-AAF; 1.41 M–dimethyl sulphoxide used for dissolving *N*-hydroxy-AAF; 20 mM–carbamoyl phosphate; 10 mM–L-methionine containing [35 S]methionine (4×10^6 d.p.m.) or 2 mM–guanosine containing [14 C]–guanosine (2×10^6 d.p.m.); water to a final volume of 1.0 ml. After incubation in air for 18 h at 37°C, analyses of the reaction products of methionine and guanosine were made as described in the Materials and Methods section.

Addition of		Reaction product (nmol) with	
<i>N</i> -Hydroxy-AAF	Carbamoyl phosphate	Methionine (3-methylthio-AAF)	Guanosine (R_F 0.73–0.93)
+	–	3.6	6.0
–	+	0.3	0.4
+	+	242	217

More direct evidence of reactive ester formation was obtained when *N*-hydroxy-AAF was incubated with carbamoyl phosphate in the absence of methionine (Table 2). Under these conditions a reactive intermediate was extractable in either benzene or diethyl ether and its formation was dependent on carbamoyl phosphate. The non-polar material extracted in benzene reacted with methionine to form a large amount of 3-methylthio-AAF. If, after the first incubation, the medium was treated with 0.5 M–hydrochloric acid, the benzene-extractable material reacted with methionine without loss of activity. However, if, after the first incubation, the medium was treated with 0.5 M–sodium hydroxide, the derivative extractable in benzene after neutralization of the aqueous solution to pH 7 was unable to react with methionine. These results therefore indicate that the reactive intermediate is an ester, probably a carbamate ester of *N*-hydroxy-AAF. Our other results indicate that this carbamoyl derivative is too unstable to be studied readily.

In most of the present work, interaction with methionine has been used as an assay system for studying acylation of *N*-hydroxy-AAF and other hydroxamic acids.

Rate of acylation of *N*-hydroxy-AAF. Acylation of *N*-hydroxy-AAF by carbamoyl phosphate was checked over a period of 22 h (Fig. 1). After a lag period of about 1 h the reaction was found to be linear for about 4 h. There is no suitable explanation to account for this lag period. Results of our other experiments suggest that this lag might probably

Table 2. Reaction of benzene-extractable reactive intermediate of *N*-hydroxy-AAF and carbamoyl phosphate with methionine

Unless otherwise indicated, the first complete incubation medium contained: 100 mM-acetic acid-potassium acetate buffer, pH 4.5; 4 mM-*N*-hydroxy-AAF dissolved in dimethyl sulphoxide; 40 mM-carbamoyl phosphate. The total volume was 1.0 ml. After incubation in air for 1 h at 37°C, samples were extracted with benzene or diethyl ether. After washing of the benzene or ether extract with water, the solvent was removed under N₂ and the residue was dissolved in 0.1 ml of dimethyl sulphoxide. The second incubation medium consisted of 0.1 ml of dimethyl sulphoxide solution containing the benzene-extractable residue from the first incubation, 100 μmol of acetic acid-potassium acetate buffer, pH 4.5, and 10 μmol of L-methionine containing [³⁵S]methionine (4 × 10⁶ d.p.m.) in a total volume of 1.0 ml. After incubation in air for 18 h at 37°C, analyses of the reaction products of methionine were made as described in the Materials and Methods section.

For first incubation, addition of		Methionine reaction products (nmol) formed during second incubation	
<i>N</i> -Hydroxy-AAF	Carbamoyl phosphate	3-Methylthio-AAF	2-Amino-3-methylthio-fluorene
—	+	1.4	4.0
+	—	0.9	2.5
+	+	152	2.6

be due to the inhibition of carbamoylation by the presence of methionine in the incubation medium.

Influence of pH. Acylation of *N*-hydroxy-AAF by carbamoyl phosphate was also dependent on the pH of the incubation medium (Fig. 2). With acetic acid-potassium acetate buffers (pH 3–7), pH 4.5 was found to be the optimum pH for activity. At pH 7 the activity was about the same with either acetic acid-potassium acetate buffer or tris-HCl buffer. However, the activity declined very rapidly when the pH was raised from 7 to 8.5. In all of the present studies, either the optimum pH (4.5) or a pH close to physiological conditions (pH 7.5) has been used.

Influence of carbamoyl phosphate concentration. Acylation of *N*-hydroxy-AAF was also dependent on the concentration of carbamoyl phosphate (Fig. 3). The reaction was linear over the range 0.5–8 mM-carbamoyl phosphate. From 8 mM to 20 mM there was a small decline in activity.

Because of the influence of concentration of carbamoyl phosphate on the rate of conversion of *N*-hydroxy-AAF into a reactive intermediate, it was necessary to investigate the effect of phosphatase in this system. Results of such an experiment

Fig. 1. Rate of acylation of *N*-hydroxy-AAF with carbamoyl phosphate. The incubation medium was the same as described in Table 1 except that the acetic acid-potassium acetate buffer, pH 4.5, was used instead of tris-HCl buffer, pH 7.5, and the total volume of the incubation medium was 10 ml. At various time-intervals, a 1.0 ml sample from the incubated sample was withdrawn. 3-Methylthio-AAF was measured as described in the Materials and Methods section.

Fig. 2. Effect of pH on acylation of *N*-hydroxy-AAF by carbamoyl phosphate. The incubation medium was the same as described in Table 1 except the type of buffer and pH used: ●, acetic acid-potassium acetate; ▲, tris-HCl buffer. Samples were incubated for 4 h at 37°C. 3-Methylthio-AAF was measured as described in the Materials and Methods section.

are summarized in Table 3. In the presence of carbamoyl phosphate, *N*-hydroxy-AAF reacted with methionine to form an appreciable amount of

3-methylthio-AAF. Under these conditions only a small amount (<1 nmol) of 2-amino-3-methylthio-fluorene was formed. Addition of acid phosphatase to the incubation system decreased the yield of 3-methylthio-AAF to a large extent. It was also notable that the interaction of carbamoyl phosphate and *N*-hydroxy-AAF gave a large amount of AAF in the absence of methionine. However, the yield of AAF was decreased considerably in the presence of methionine. The formation of AAF was decreased even further in the presence of phosphatase.

Fig. 3. Effect of carbamoyl phosphate concentration on acylation of *N*-hydroxy-AAF. The incubation medium was the same as described in Table 1 except for the concentration of carbamoyl phosphate and the type of buffer. In these studies samples were incubated in 0.1 M-acetic acid-potassium acetate buffer, pH 4.5, for 4 h at 37°C. 3-Methylthio-AAF was measured as described in the Materials and Methods section.

We have shown that acetyl-CoA was able to acylate *N*-hydroxy-AAF and other hydroxamic acids non-enzymically (Lotlikar & Luha, 1970). It was decided to compare three physiological agents with regard to their ability to acylate *N*-hydroxy-AAF (Table 4). In these studies, reactions with methionine and guanosine were used for assay. It was found that at pH 7.5, acetyl-CoA was a much better acylating agent than carbamoyl phosphate and acetyl phosphate.

Since acetyl-CoA and carbamoyl phosphate were the most active acylating agents, they were used in studies with other hydroxamic acids (Table 5). In these experiments, *N*-hydroxy-AAF was found to be the most reactive hydroxamic acid. 7-Fluoro-*N*-hydroxy-AAF was the second most active compound tested. Like *N*-hydroxy-AAF, acetyl-CoA was found to be better than carbamoyl phosphate in acylating the 7-fluoro-derivative. Carbamoyl phosphate was severalfold better than acetyl-CoA in acylating derivatives of stilbene, phenanthrene and naphthalene. However, there was not any significant difference in activity between these two acylating agents for the biphenyl derivative.

The ability of acetyl-CoA to acylate *N*-hydroxy-AAF is completely abolished in acetate buffer, pH 4.5 (Table 6). This was not due to the presence of acetate ions because, in a separate experiment, similar results were also obtained with potassium citrate buffer, pH 4.5. However, carbamoyl phosphate shows the optimum activity at that pH (see also Fig. 2).

DISCUSSION

Synthetic acetate esters of aromatic hydroxamic acids are more carcinogenic than their parent compounds (Miller & Miller, 1969a,b; Miller, 1970). The acetate ester of *N*-hydroxy-AAF is very reactive

Table 3. Effect of phosphatase on acylation of *N*-hydroxy-AAF by carbamoyl phosphate

Unless otherwise indicated, the complete incubation medium contained 100 mM-acetic acid-potassium acetate buffer, pH 4.5; 4 mM-*N*-hydroxy-AAF; 1.41 M-dimethyl sulphoxide, used for dissolving *N*-hydroxy-AAF; 20 mM-carbamoyl phosphate; 10 mM-L-methionine containing [³⁵S]methionine (4 × 10⁶ d.p.m.); 10 mg of phosphatase. The total volume was 2.0 ml. After incubation in air for 3 h at 37°C, analyses of AAF and the methionine reaction product were made as described in the Materials and Methods section.

Addition of				Reaction product (nmol)	
<i>N</i> -Hydroxy-AAF	Carbamoyl phosphate	Acid phosphatase	Methionine	With methionine (3-methylthio-AAF)	AAF
+	—	—	+	1.8	9.0
+	+	—	+	268	414
+	+	+	+	45	121
+	—	—	—		9.0
+	+	—	—		1085

with various tissue nucleophiles (Lotlikar *et al.* 1966; Miller, Juhl & Miller, 1966; Kriek *et al.* 1967; Maher, Miller, Miller & Szybalski, 1968; Fink, Nishimura & Weinstein, 1970; Scribner, Miller & Miller, 1970), whereas *N*-hydroxy-AAF is unreactive (Bahl & Gutmann, 1964; King, Gutmann & Chang, 1963; Miller *et al.* 1966, 1968). We have demonstrated that *N*-hydroxy-AAF could be acetylated non-enzymically by acetyl-CoA at physiological pH values *in vitro* and that the acetylated product was able to react with methionine and guanosine (Lotlikar & Luha, 1970; and the present paper, Table 3).

In this report, we have shown that carbamoyl phosphate was able to react with *N*-hydroxy-AAF at physiological pH values to form a product that

reacted with methionine and guanosine. On the basis of studies with acetate (Lotlikar *et al.* 1966; Miller *et al.* 1966, 1968; Miller & Miller, 1969*a,b*; Kriek *et al.* 1967; Maher *et al.* 1968; Fink *et al.* 1970; Scribner *et al.* 1970), sulphate (Miller & Miller, 1969*a,b*; Miller, 1970; DeBaun *et al.* 1968, 1970; King & Phillips, 1968, 1969; Maher *et al.* 1968; Maher, Miller, Miller & Summers, 1970) and phosphate (Lotlikar, Irving, Miller & Miller, 1967; DeBaun *et al.* 1968; King & Phillips, 1969; Lotlikar & Wasserman, 1970) esters of *N*-hydroxy-AAF and other hydroxamic acids, and their interaction with nucleic acids and proteins and their constituents guanosine and methionine, our present studies suggest that carbamoyl phosphate is acylating *N*-hydroxy-AAF to form an ester, AAF-*N*-carbamate, that reacts non-enzymically with tissue nucleophiles such as methionine and guanosine.

Carbamoyl phosphate acylates non-enzymically lysine residues of glutamate dehydrogenase, thereby causing a rapid inactivation (Grisolia, 1968). Lysine residues of histones are also acylated by carbamoyl phosphate; this acylation is prevented by acyl phosphatase (Ramponi & Grisolia, 1970). In the present studies also, acylation of *N*-hydroxy-AAF measured by the methionine reaction was decreased to a large extent by acid phosphatase.

For acetylation of *N*-hydroxy-AAF by acetyl-CoA the optimum pH was found to be about 10–11 (Lotlikar & Luha, 1970) in contrast with the present studies, where the optimum pH for carbamoylation of *N*-hydroxy-AAF was about pH 4.5. At about neutral pH, acetyl-CoA was severalfold better than carbamoyl phosphate and acetyl phosphate in forming an ester of *N*-hydroxy-AAF that reacted with methionine and guanosine (Table 4). However, at pH 4.5, acetyl-CoA had

Table 4. *Reaction of N-hydroxy-AAF with methionine and guanosine in the presence of various acylating agents*

Unless otherwise indicated, the complete incubation medium was the same as described in Table 1 except that the concentration of carbamoyl phosphate or other acylating agents was 4 mM. The total volume of the sample was 1.0 ml. After incubation for 18 h at 37°C, reaction products of methionine and guanosine were assayed as described in the Materials and Methods section.

Acylating agent	Reaction product (nmol) with	
	Methionine (3-methylthio-AAF)	Guanosine (<i>R_F</i> 0.73–0.93)
—	5.0	7.1
Acetyl-CoA	332	349
Acetyl phosphate	11	16
Carbamoyl phosphate	70	49

Table 5. *Reaction of hydroxamic acids with methionine in the presence of either carbamoyl phosphate or acetyl-CoA*

The complete incubation medium contained 50 μ mol of tris-HCl buffer, pH 7.5, 2 μ mol of hydroxamic acid dissolved in 0.05 ml of dimethyl sulphoxide, 2 μ mol of either acetyl-CoA or carbamoyl phosphate and 5 μ mol of L-methionine (1 μ Ci) in a total volume of 0.5 ml. After incubation in air for 18 h at 37°C, the reaction products, *o*-methylthio amide and *o*-methylthio amine, were assayed as described in the Materials and Methods section.

Hydroxamic acid	Amount formed (nmol)			
	<i>o</i> -Methylthio amide		<i>o</i> -Methylthio amine	
	From acetyl-CoA	From carbamoyl phosphate	From acetyl-CoA	From carbamoyl phosphate
—	0.5	0.1	0.03	0.3
<i>N</i> -Hydroxy-AAF	247	33	0.8	0.8
7-Fluoro- <i>N</i> -hydroxy-AAF	70	9.0	0.3	0.5
4-(<i>N</i> -Hydroxyacetamido)stilbene	0.3	2.6	0.2	0.5
4-(<i>N</i> -Hydroxyacetamido)biphenyl	2.6	2.5	0.8	0.5
2-(<i>N</i> -Hydroxyacetamido)phenanthrene	0.3	1.6	0.2	3.5
2-(<i>N</i> -Hydroxyacetamido)naphthalene	0.4	5.4	0.2	1.3

Table 6. *Effect of various acylating agents on reaction of N-hydroxy-AAF with methionine and guanosine at pH 4.5*

The incubation medium was the same as described in Table 1 except that the concentration of carbamoyl phosphate or other acylating agent was 2mM and the buffer was acetic acid-potassium acetate buffer, pH 4.5. Samples were incubated for 5h at 37°C. The reaction products of methionine and guanosine were assayed as described in the Materials and Methods section.

Acylating agent	Reaction product (nmol) with	
	Methionine (3-methylthio-AAF)	Guanosine (R_F 0.73-0.93)
—	1.3	1.1
Acetyl-CoA	0.5	1.4
Acetyl phosphate	1.5	1.3
Carbamoyl phosphate	44	55

negligible activity whereas carbamoyl phosphate was very active (Table 6).

When the acylating activity of acetyl-CoA and carbamoyl phosphate was compared with various hydroxamic acids, the two acylating agents showed different activities (see Table 5). In these experiments, formation of acylated product was not measured directly but their interaction with methionine was used as a measure of their formation and reactivity. Like acetate esters (Scribner *et al.* 1970; Maher *et al.* 1970) of various hydroxamic acids the carbamate ester of *N*-hydroxy-AAF was the most reactive.

Interaction of *N*-hydroxy-AAF with carbamoyl phosphate in the absence of methionine yielded a large amount of AAF (Table 2). However, in the presence of methionine, some 3-methylthio-AAF was formed and the amount of AAF was decreased. A tentative mechanism may be proposed to explain these and other results. First, carbamoyl phosphate would react with *N*-hydroxy-AAF to form a carbamate ester. Secondly, the carbamate ester would ionize to form a positively charged amidonium ion and a negatively charged carbamate ion. Thirdly, in the presence of methionine or guanosine, the amidonium ion would react with these tissue nucleophiles to give reaction products that have already been identified for acetate, sulphate and phosphate esters of *N*-hydroxy-AAF (Lotlikar *et al.* 1966; Kriek *et al.* 1967; King & Phillips, 1969; DeBaun *et al.* 1970). Alternatively, as suggested by Gutmann & Erickson (1969), the positively charged nitrogen atom of the amidonium ion may accept hydride ions to give AAF.

Carbamoyl phosphate is made in very large amounts in many mammalian systems, e.g. about 1mol per day in about 1kg of adult human liver

(Ramponi & Grisolia, 1970). Therefore it is conceivable that carbamoylation of *N*-hydroxy-AAF and other hydroxamic acids might occur in biological systems non-enzymically. These carbamate esters of carcinogenic aromatic hydroxamic acids, if formed, would be reactive with tissue nucleophiles. Carbamoylation of hydroxamic acids might be one of the activation steps in their carcinogenic process.

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