ples because in the latter four samples the total surface available for adsorption was too little for nitrogen. The specific surface of each sample was measured twice: once after degassing for 8 hours at 25.0° ± 1.0°C and again after degassing for 4 hours at 200°C. In all instances the specific surface available for adsorption was higher in the second measurement (Table 1). The amount of material vaporized was estimated to be about 10 percent by weight.

The bulk density of samples was determined by placing a small amount of each sample in a capillary column (inner diameter, 2 mm) of known weight and volume and closed at one end. The tube was tapped gently on a hard surface during and after introduction of the sample, until gentle tapping did not increase the height of the sample above a predetermined level in the tube. Duplicate determinations were made for each sample (Table 1).

The densities of the particulate samples were determined by placing a weighed sample in a weighed micropycnometer of about 1-cm³ volume (*9*). While the sample was under vacuum, ethylene glycol was introduced by syringe until it was covered. The weight per unit volume of dust was calculated from glycol density and by assumption that the volumes of glycol and particulate sample were additive (Table 1).

The results for specific surface of suspended particulates are lower than those reported (*10*) for activated carbons (560 to 1397 m^2/g) and silica gel (669 m^2/g), but are higher than that of diatomaceous earth (4.2 m^2/g). Values are lower than those determined for particulates emitted in the exhaust of a diesel engine—28 to 50 m^2/g (*11*).

Little is known about the bulk density or specific density of particulates in urban atmospheres, so it is difficult to compare our results with those of others. Whitby *et al.* (*12*) found that the specific density of acetone-insoluble particles in urban air varied from 1.5 to 3.0 g/cm^3; bulk density varied from 0.2 to 1.5 g/cm^3. Junge, on the basis of chemical composition, suggested values of 1 to 2 g/cm^3 for most natural aerosols, depending on the relative humidity (*13*).

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Enzyme-Catalyzed Reactions of the Carcinogen N-Hydroxy-2-fluorenylacetamide with Nucleic Acid

Abstract. *A protein fraction obtained by gel filtration of a 105,000g supernatant of rat liver catalyzes three reactions of the hepatocarcinogen* N-*hydroxy-2-fluorenylacetamide with nucleic acid. Cofactor requirements and isotopic studies suggest that the reactive intermediates involved may be* N-*2-fluorenylhydroxylamine, and phosphate and sulfate esters of* N-*hydroxy-2-fluorenylacetamide.*

Interactions of chemical carcinogens with nucleic acids and proteins may play an important role in the induction of tumors (*1*). The aromatic amides constitute a class of carcinogens for which metabolic activation is a prerequisite to combination with macromolecules. A primary step in this activation appears to be *N*-hydroxylation (*2*). *N*-Hydroxy derivatives are generally more carcinogenic than the parent amides (*3*) and are thought to be more closely related to the metabolites which ultimately react with protein and nucleic acid (*1*). Further metabolic transformation of the hydroxamic acids is necessary for combination to occur, however, as in the

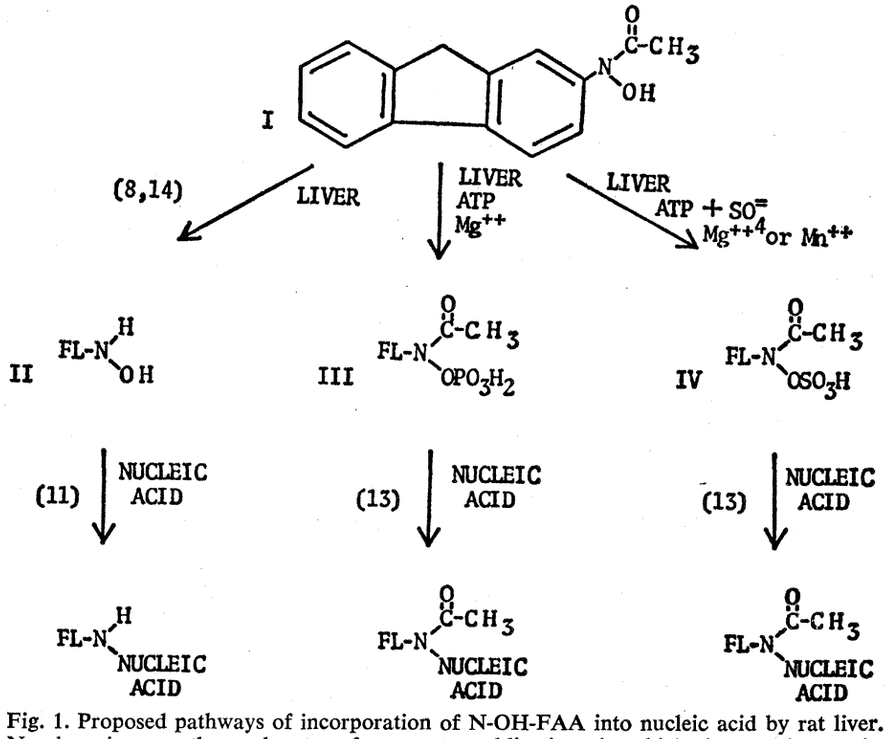

Fig. 1. Proposed pathways of incorporation of N-OH-FAA into nucleic acid by rat liver. Numbers in parentheses denote references to publications in which the reaction or its potential has been demonstrated; *FL* = 2-fluorenyl.

case of *N*-hydroxy-2-fluorenylacetamide (N-OH-FAA, Fig. 1, I). While N-OH-FAA reacts in vivo with both nucleic acid and protein (*4*), studies in vitro have failed to reveal nonenzymatic chemical attachment (*5*, *6*). Certain derivatives of N-OH-FAA combine non-enzymatically with protein or nucleic acid or both. 1,2-Fluorenoquinone-2-acetimide (*7*), 1,2-fluorenoquinone-2-imine (*5*), and 2-nitrosofluorene (*8*) react with protein but not with nucleic acid (*9–11*). *N*-2-Fluorenylhydroxylamine (*8*, *11*) and esters of N-OH-FAA (*6*, *12*, *13*) react with protein and nucleic acid in model systems in vitro. While the reactions of these derivatives are suggestive of possible pathways by which combination in vivo might occur, little is known of the actual metabolites involved.

Identification of such pathways was approached by incubation of liver preparations and the hepatocarcinogen N-OH-FAA labeled with carbon-14 in either the ring (N-OH-FAA-9-^{14}C) or the acetyl group (N-OH-FAA-1′-^{14}C). Soluble ribonucleic acid (sRNA) was added as a trapping agent for the reactive intermediates; preliminary experiments with other nucleic acids indicated that they also react with the intermediates described in this report.

Two to three nanomoles of fluorene per milligram of sRNA was associated with nucleic acid isolated following incubation with ring-labeled N-OH-FAA and a 1000*g* supernatant of liver (Table 1, A). Control incubations with liver preparations which had been heated in a boiling water bath for 1 minute yielded unlabeled nucleic acid. The possibility that radioactive substances other than labeled nucleic acid were being carried through the purification procedure was examined by adding sRNA after incubation and just prior to extraction with phenol. This procedure reduced the incorporation of radioactivity by 98 percent (Table 1, A). Extraction of rat liver nucleic acid labeled during the incubation may account for the residual incorporation.

Incubation of acetyl-labeled N-OH-FAA with the 1000*g* supernatant and sRNA did not result in labeling of sRNA (Table 1, A). Failure of the acetyl group to be incorporated indicated that only the fluorene nucleus was bound to sRNA. Deacetylation of N-OH-FAA by rat liver has been demonstrated (*8*, *14*), and the product of this reaction, *N*-2-fluorenylhydroxylamine (Fig. 1, II), has been shown to react with nucleic acids (*11*). These considerations suggest that the incorporation of the fluorene nucleus reported here results from deacetylation of N-OH-FAA followed by combination of the hydroxylamine with nucleic acid. The *p*H utilized in the present experiments, 7.4, is higher than that at which the hydroxylamine had been shown by an ultraviolet method (*11*) to react with nucleic acids. This method would not have revealed the reactions described here, since no changes in the ultraviolet properties of sRNA were detected at the levels of combination observed in the present study.

In attempts to detect other pathways by which N-OH-FAA might combine with nucleic acid, incubation conditions conducive to phosphorylation were employed. Inclusion of Mg^{++} ($MgCl_2$) and adenosine triphosphate (ATP) in the incubation system resulted in significant incorporation of both acetyl-labeled and ring-labeled N-OH-FAA into nucleic acid (Table 1, A). Incubation of Mg^{++} and ATP with boiled liver preparations yielded unlabeled sRNA. Addition of sRNA after incubation but prior to extraction with phenol reduced the labeling by approximately 97 percent (Table 1, A). Only binding of N-OH-FAA-9-^{14}C was observed in those incubations to which Mg^{++}, but not ATP, had been added (Table 1, A). Incubation of ATP, 1000*g* supernatant, and sRNA in the absence of Mg^{++} did not result in labeling of nucleic acid by N-OH-FAA-1′-^{14}C (Table 1, A). The dependence of this pathway on both ATP and Mg^{++} suggests the involvement of a phosphokinase (*15*).

The addition of Mg^{++} and ATP to the incubation system thus induces binding through a pathway in which the acetyl group of N-OH-FAA is retained. In model systems in vitro, *N*-acetoxy-2-fluorenylacetamide (*N*-acetoxy-FAA) reacts with nucleosides without loss of the *N*-acetyl group (*16*). The phosphate ester of N-OH-FAA has been shown to be more reactive toward nucleosides (*13*) and would by analogy be expected to retain the acetyl group in such reactions. These considerations support the proposal that in the presence of ATP and Mg^{++}, a phosphokinase of rat liver

Table 1. Incorporation of N-OH-FAA into sRNA (nanomoles per milligram) by rat liver preparations. Liver from male Sprague-Dawley rats was homogenized in the cold with 0.055*M* tris-HCl buffer (*p*H 7.4, 4 ml per gram of liver). The 1000*g* supernatant was prepared by centrifugation of the homogenate at 1000*g* for 10 minutes at 4°C. The soluble protein fraction was obtained by gel filtration on Sephadex G-10 (Pharmacia) of the 105,000*g* supernatant (75 minutes at 4°C). The equivalent of 50 mg of tissue, 5 mg of soluble RNA from yeast (sRNA, Calbiochem.), 8.4 × 10^{-2} μmole of N-OH-FAA-^{14}C (0.51 μc/μmole) in 0.1 ml of 2-methoxyethanol, and 110 μmole of tris buffer (*p*H 7.4) in a final volume of 2.1 ml were incubated in open flasks at 37°C, with shaking for 1 hour. ATP (5 μmole, Sigma), Na_2SO_4 (20 μmole), $MgCl_2$ (8 μmole), or $MnCl_2$ (8 μmole) were added as indicated. RNA was recovered and purified by extraction with phenol (2 times) and ether (4 times), precipitation with ethanol–potassium acetate, and solution in tris buffer. The nucleic acid was converted to a cetyltrimethylammonium salt by the method of Jones (*20*), washed twice with water, dried *in vacuo*, dissolved in methanol, and counted in a liquid scintillation spectrometer (*18*).

Additions to basic incubation system	(A) Fluorene derivatives bound to sRNA on incubation with 1000*g* supernatant		(B) Fluorene derivatives bound to sRNA on incubation with a soluble protein fraction	
	N-OH-FAA-9-^{14}C	N-OH-FAA-1′-^{14}C	N-OH-FAA-9-^{14}C	N-OH-FAA-1′-^{14}C
None	2.44	<0.01	5.94	<0.01
None*	0.04			
$MgCl_2$+ATP	1.32	0.23	3.84	1.11
$MgCl_2$+ATP*	0.03	<0.01		
$MgCl_2$	0.84	<0.01	2.45	<0.01
ATP		<0.01		<0.01
$MgCl_2$+ATP+ Na_2SO_4	2.11	1.54	6.30	4.83
$MgCl_2$+ATP+ Na_2SO_4*	0.05	0.02		
$MgCl_2$+Na_2SO_4	0.76	<0.01	2.26	<0.01
ATP+Na_2SO_4		0.25		0.15
$MnCl_2$+ATP				0.02
$MnCl_2$+ATP+ Na_2SO_4				2.17
$MnCl_2$+Na_2SO_4				<0.01

* Incubations in these experiments were carried out prior to addition of the sRNA.

catalyzes the formation of a phosphate ester of N-OH-FAA (Fig. 1, III), which then reacts spontaneously with nucleic acid.

Indication of a third pathway by which N-OH-FAA reacts with nucleic acids was obtained from experiments in which conditions were favorable for sulfate conjugation (*17*). Addition of sulfate to an incubation system containing ATP and Mg^{++} increased the incorporation of label into RNA from both ring-labeled and acetyl-labeled N-OH-FAA (Table 1, A). Combination was prevented by boiling the 1000*g* supernatant, and addition of sRNA after the incubation reduced the labeling by 97 percent (Table 1, A). This pathway was completely dependent on the addition of ATP, while incubation of ATP and sulfate without Mg^{++} permitted a low level of binding of the acetyl-labeled N-OH-FAA (Table 1, A). The increase in labeling on addition of sulfate and the less rigorous dependence on added Mg^{++} were indications that this mechanism was distinct from the pathway in which a phosphate ester appears to be the intermediate.

The cofactor requirements of this third pathway suggest the participation of a sulfokinase. The sulfate ester of N-OH-FAA has been shown to combine with nucleosides (*13*) and would be expected to react with retention of the acetyl group. It is thus likely that in the presence of ATP, Mg^{++}, and sulfate, N-OH-FAA is converted to a sulfate ester (Fig. 1, IV), which then reacts with nucleic acid.

The enzyme systems for all three pathways described above appear to be located in the supernatant obtained by centrifugation of the homogenate at 105,000*g* for 75 minutes at 4°C, since this supernatant gave results comparable to those observed with the 1000*g* supernatant.

Incubation of N-OH-FAA-9-^{14}C with the pigmented protein fraction obtained by gel filtration of the 105,000*g* supernatant on Sephadex G-10 (Pharmacia) yielded more highly labeled nucleic acid than did incubations employing the 1000*g* or 105,000*g* supernatants (Table 1, B). Addition of $MgCl_2$ inhibited binding of N-OH-FAA-9-^{14}C by 58 percent (Table 1, B). Combination of both the ring-labeled and acetyl-labeled N-OH-FAA was stimulated when ATP was included in the incubation, and a greater increase in binding resulted from the further addition of sulfate (Table 1, B). Magnesium ion was an absolute requirement for the ATP-induced stimulation, while incubation of ATP and sulfate in the absence of Mg^{++} reduced binding of N-OH-FAA-1′-^{14}C by 97 percent. Demonstration of all three pathways with this chromatographic fraction argues against the participation of cofactors other than those added.

Additional evidence that the presence of sulfate gives rise to a reaction distinct from that observed when only Mg^{++} and ATP are added to incubations was obtained from experiments carried out with Mn^{++}. Substitution of $MnCl_2$ for $MgCl_2$ in experiments with the soluble chromatographic fraction reduced by 98 percent the ATP-stimulated incorporation of N-OH-FAA-1′-^{14}C into sRNA (Table 1, B). Replacement of Mg^{++} by Mn^{++} in incubations with sulfate and ATP still permitted considerable binding to occur (Table 1, B).

The requirement for the *N*-hydroxy group in these reactions was borne out by results of experiments with *N*-2-fluorenylacetamide (FAA). Incorporation of ring-labeled FAA was less than 0.5 percent that of N-OH-FAA in incubations with the 105,000*g* supernatant alone or with the system favoring sulfate formation. It is improbable that incorporation of the *N*-acetyl group of N-OH-FAA into sRNA followed exchange with an acetate pool, since incubations of sodium acetate-1-^{14}C (1.8×10^{-2} μmole, 28.4 μc/μmole) with the soluble protein fraction, N-OH-FAA, ATP, $MgCl_2$, and Na_2SO_4 did not yield radioactive sRNA.

Three biochemical pathways by which N-OH-FAA may react with nucleic acids are depicted in Fig. 1. These pathways are proposed on the basis of our results and the significant contributions of the Millers and their collaborators (*13*) and of Kriek (*11*). Non-enzymatic combinations of the sulfate, phosphate, and other esters of N-OH-FAA with nucleosides (*13*), and the binding of *N*-2-fluorenylhydroxylamine to nucleic acid (*11*) have been demonstrated. These findings and the data on the participation of cofactors in the incorporation of isotopically labeled N-OH-FAA into sRNA suggest that enzymes present in liver of rats convert N-OH-FAA into *N*-2-fluorenylhydroxylamine and phosphate and sulfate esters of N-OH-FAA, followed by spontaneous combination of these reactive intermediates with nucleic acids.

The fluorene compounds in Fig. 1 are shown attached to nucleic acids by a bond with the N atom. This type of bond has been demonstrated in the adduct of guanosine and *N*-acetoxy-FAA (*16*), an ester similar in structure and reactivity to the phosphate and sulfate esters. It appears improbable that bonds with the aromatic nucleus are formed, since two related fluorene derivatives which have activated aromatic nuclei, 1,2-fluorenoquinone-2-imine and 1,2-fluorenoquinone-2-acetimide, do not react with nucleic acid (*9*, *10*).

The reactions of the three intermediates described here are not restricted to combinations with sRNA. Combination of N-OH-FAA with protein and DNA by pathways similar to those described in Fig. 1 has been demonstrated (*18*). The formation of phosphate and sulfate esters may be of importance in biological systems in view of the fact that the acetyl group of N-OH-FAA is retained in its combination in vivo with RNA (*19*).

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