The Conversion of the Carcinogen N-Hydroxy-2-fluorenylacetamide to o-Amidophenols by Rat Liver in Vitro

AN INDUCIBLE ENZYMATIC REACTION*

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SUMMARY

The enzymatic conversion of the carcinogenic arylhydroxamic acid, N-hydroxy-2-fluorenylacetamide, to the o-amidophenols, N-(1-hydroxy-2-fluorenyl)acetamide and N-(3-hydroxy-2-fluorenyl)acetamide, referred to as the isomerization of N-hydroxy-2-fluorenylacetamide, by cell fractions of rat liver has been studied. Cell fractions prepared from the livers of normal rats were unable to perform the reaction. Isomerization was stimulated by 3-methylcholanthrene and several other polycyclic aromatic hydrocarbons administered 24 hours preceding the preparation of the cell fractions. Since DL-ethionine inhibited the stimulation of the formation of the o-amidophenols by 3-methylcholanthrene and since the inhibition was counteracted by the concurrent administration of DL-methionine, the isomerization appeared to be an induced enzymatic reaction. The isomerization proceeded optimally at a pH near 7 in phosphate buffer, Tris buffer, or deionized water and, in contrast to the microsomal p-hydroxylation of N-2-fluorenylacetamide, did not require a NADPH-generating system. N-2-Fluorenylacetamide, the substrate for the NADPH-dependent formation of the p-amidophenols, N-(7-hydroxy-2-fluorenyl)acetamide and N-(5-hydroxy-2-fluorenyl)acetamide, did not serve as a substrate for the isomerization. The isomerization was dependent on the synergistic action of two components. One of these was inducible and associated with the microsomal fraction of rat liver. The second component, localized in the soluble fraction of normal rat liver, was not dialyzable and, in contrast to the microsomal p-hydroxylation of N-2-fluorenylacetamide, did not require a NADPH-generating system. N-2-Fluorenylacetamide, the substrate for the NADPH-dependent formation of the p-amidophenols, N-(7-hydroxy-2-fluorenyl)acetamide and N-(5-hydroxy-2-fluorenyl)acetamide, did not serve as a substrate for the isomerization. The isomerization was dependent on the synergistic action of two components. One of these was inducible and associated with the microsomal fraction of rat liver. The second component, localized in the soluble fraction of normal rat liver, was not dialyzable and, in contrast to the microsomal p-hydroxylation of N-2-fluorenylacetamide, did not require a NADPH-generating system.

The first evidence that the mechanism for the formation of the o-amidophenols, N-(1-hydroxy-2-fluorenyl)acetamide and N-(3-hydroxy-2-fluorenyl)acetamide from the carcinogen, N-2-fluorenylacetamide differed from a microsomal NADPH-dependent p-hydroxylation of the arylamide came from tracer experiments in vivo by Miller and Miller (1) and by Miller, Cramer, and Miller (2). These workers showed that simultaneous administration of N-hydroxy-FAA, a carcinogenic

buffer at pH 4.6. The formation of the reduced metabolites, N-2-fluorenylacetamide and 2-fluorenamine, by each fraction paralleled the formation of the o-amidofluorensols. These results and the further observation that the products of the isomerization and of the reduction were present, in all fractions, in a constant proportion suggested a common intermediate for both reactions. On the basis of these data, the isomerization has been tentatively formulated as a two-step reaction. In the first step N-hydroxy-2-fluorenylacetamide is dehydroxylated to a positively charged amidonium ion by an enzyme in the soluble fraction of rat liver. The second step in which hydroxyl ions add to the electrophilic carbon atoms 1 and 3 of the resonance forms of the amidonium ion yields the o-amidofluorensols. The induced microsomal enzyme is presumed to participate in this step of the reaction. Alternatively, hydride ions may be transferred to the positively charged nitrogen of the amidonium ion to give N-2-fluorenylacetamide and, via deacetylation, 2-fluorenamine.

The first evidence that the mechanism for the formation of the o-amidophenols, N-(1-hydroxy-2-fluorenyl)acetamide and N-(3-hydroxy-2-fluorenyl)acetamide from the carcinogen, N-2-fluorenylacetamide differed from a microsomal NADPH-dependent p-hydroxylation of the arylamide came from tracer experiments in vivo by Miller and Miller (1) and by Miller, Cramer, and Miller (2). These workers showed that the simultaneous administration of N-hydroxy-FAA, a carcinogenic

The abbreviation used is: FAA, N-2-fluorenylacetamide. The hydroxylated derivatives are abbreviated by indicating the position of the hydroxyl group in the fluorene system or on the nitrogen atom as follows: 1-hydroxy-FAA, N-(1-hydroxy-2-fluorenyl)acetamide; 3-hydroxy-FAA, N-(3-hydroxy-2-fluorenyl)acetamide; 5-hydroxy-FAA, N-(5-hydroxy-2-fluorenyl)acetamide; 7-hydroxy-FAA, N-(7-hydroxy-2-fluorenyl)acetamide; N-hydroxy-FAA, N-hydroxy-2-fluorenylacetamide or N-2-fluorenylacetohydroxamic acid.

* This investigation was supported by United States Public Health Service Research Grant CA-02571 from the National Cancer Institute.
metabolite of FAA (3), and of FAA-9-14C resulted in the urinary
excretion of 1-hydroxy-FAA with a very much lower specific
radioactivity than that of 5-hydroxy-FAA and of 7-hydroxy-FAA
which are the products of the microsomal p-hydroxylation of
FAA (4, 5). They concluded that N-hydroxy-FAA, rather than
FAA, was the immediate precursor of 1-hydroxy-FAA and that
the o-aminophenol might be formed in \textit{vivo} via a rearrangement
in a manner analogous to the transformation of arylhydroxyl-
amines to phenolic amines in acid (6). The metabolic reaction
leading to 1-hydroxy-FAA was subsequently studied in \textit{vivo}
by Booth and Boyland (7). These investigators reported an
enzyme in the soluble fraction of rat and rabbit liver which
converted N-hydroxy-FAA to 1-hydroxy-FAA, provided that
NAD, NADH, or NADPH was available. Since other arylhydro-
xamic acids, such as N-hydroxycacetanilide, N-hydroxy-2-
acetamidonaphthalene, and N-hydroxy-4-acetamidobiphenyl,
likewise yielded o-aminophenols under these conditions (7),
the authors expressed the view that the isomerization of arylhy-
oxamic acids to o-aminophenols by a soluble enzyme requiring
the above cofactors for activity was a general metabolic reaction.
Because the binding of the o-quinone imines, derived from
1-hydroxy-FAA and 3-hydroxy-FAA, to proteins and the relation-
ship of the binding of these compounds to the biological activity
of FAA and of N-hydroxy-FAA were then under study in this
laboratory, the report of Booth and Boyland (7) was of great
interest to us. However, the data presented by Booth and Boy-
lund were merely qualitative and thus permitted no conclusion
as to the extent of the reaction. Accordingly, methods for the
isolation, purification, and estimation of l-hydroxy-FAA and of
N-hydroxy-FAA were developed and applied to a detailed
examination of the reaction. Although we were unable to
confirm the observations of Booth and Boyland, evidence was
obtained that the reaction is inducible, in rat liver, by certain
polycyclic aromatic hydrocarbons and involves two enzymatic
components. The characterization of the isomerization of N-
hydroxy-FAA as an inducible enzymatic reaction forms the basis
of this report.

\textbf{EXPERIMENTAL PROCEDURE}

\textbf{Preparation and Properties of Labeled and Unlabeled
Compounds—}N-Hydroxy-FAA-9-14C with a specific radioactivity of
1.42 \times 10^8 \text{ dpm per mmole} was obtained from Tracerlab,
Inc., Waltham, Massachusetts. The radioactivity of the arylhy-
xamic acid was shown by descending chromatography on
Whatman No. 1 paper with the upper phase of a mixture of
cyclohexane-t-butyl alcohol-glacial acetic acid-water (16: 1: 1: 1)
as the solvent. A scan of the developed chromatogram1 showed
1730 Isomerization of N-Hydroxy-2-fluorenylacetamide to o-Amidophenols in Vitro Vol. 244, No. 7

\begin{align*}
\text{C}_7\text{H}_5\text{NO}_2 \\
\text{Calculated: C 72.98, H 5.37, N 4.08} \\
\text{Found: C 72.93, H 5.31, N 4.78}
\end{align*}

{$\nu_{\text{max}}$} 1790 (O–C=O), 1690 (N–C=O) cm$^{-1}$; {$\lambda_{\text{max}}^{\text{max}}$} 275 (e, 23,900), 289 (e, 16,500), 302 (e, 16,500) \mu m.

\textbf{Inducers of Hepatic Drug-metabolizing Enzymes and Co-}
\textbf{factors—}Benzo[a]pyrene, m. p. 175.5–177$,\text{ dibenz[a,h]anthracene,}$
m. p. 269–271$, testosterone, m. p. 153–156$, and 19-nortesto-
sterone, m. p. 118–120$, were obtained from Sigma. 2-Chloro-
10-(3-dimethylaminopropyl)phenothiazine (SKF-2601), m. p. 54–
55$, and 4-chloro-19-nortestosterone (SKF-6611), m. p. 176–
178.5$, were gifts of the Smith, Kline and French Laboratories.
5 Ethyl 5 phenylbarbituric acid, m. p. 177.5–178.5$, and the
sodium salt of 5-ethyl-(1-methylbutyl)barbituric acid were
purchased from Merek and Abbott Laboratories, respectively.
The above compounds were judged to be pure on the basis of
their melting points and they were used without further purifi-
cation. 3-Methylcholanthrene m. p. 181–182$, and phenothiazine
were purchased from Eastman Kodak, and the phenothiazine
was recrystallized from butan-1-ol before use, m. p. 186–187$.5
The monosodium salt of NADP and the dipotassium or the
sodium salt of 5-ethyl-5-(1-methylbutyl)barbituric acid were
prepared by published methods.

\textbf{Preparation of Cell Fractions—}The animals were exsanguinated
by decapitation and the livers were removed without prior
commercial food pellets (Purina chow) and water except where noted
otherwise in Table II.

\textbf{Preparation of Cell Fractions—}The animals were exsanguinated
by decapitation and the livers were removed without prior

\text{Lotlikar et al. (16), who first prepared N-acetoxy-2-fluorenyl-
acetamide, reported a melting point of 109–111$^\circ$ for this com-
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method of O-acetylation of N-hydroxy-FAA and no elemental
analysis was given.}

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analysis was given.}
perfusion \(^3\) and chilled on ice. All subsequent operations were carried out at \(4^\circ\). The livers were washed with homogenizing buffer, blotted with filter paper, and minced with scissors. To equalize differences in enzyme content, the cell fractions for each experiment were obtained from the pooled livers of four to five rats. The mince was homogenized in a Potter-Elvehjem glass homogenizer equipped with a Teflon pestle (0.004-inch clearance). The homogenizing medium (4.0 ml per g of wet liver) was that described previously (4) except that the EDTA was omitted. In the experiments depicted in Fig. 6, a 1.1\% solution of potassium chloride was used as the homogenizing medium. The resulting 20\% homogenate was centrifuged at 600 \(\times g\) for 15 min (Fig. 1) and the supernatant liquid equivalent to 4.0 g of wet liver (20 ml) was utilized in the incubations as the source of the isomerizing enzyme. In the experiments dealing with the intracellular localization of the enzyme 20 ml of the 600 \(\times g\) supernatant liquid were fractionated, by centrifugation at 105,000 \(\times g\) for 1 hour, into the microsomal-mitochondrial sediment and into the soluble fraction (Fig. 1). Prior to incubation the particulate fraction was dispersed in homogenizing medium, recentrifuged, and resuspended in 20 ml of homogenizing medium. In the initial experiments the incubation medium consisted of 15 \(\mu\)moles of glucose-6-P, 300 \(\mu\)moles of nicotinamide, 0.3 \(\mu\)mole of NADP, 150 \(\mu\)moles of potassium chloride, and 150 \(\mu\)moles of sodium phosphate buffer, pH 7.4 (Medium A). The microsomal hydroxylation of the fluorene nucleus proceeded optimally in this medium (4). In later experiments the compounds and cofactors necessary for hydroxylation of the aromatic ring were omitted from Medium A and the incubation medium consisted only of 40 \(\mu\)moles of sodium phosphate buffer, pH 7.4 (Medium B). The appropriate cell fraction (2.0 ml, equivalent to 0.4 g of wet liver) and the substrate dissolved in 0.1 ml of methyl Cellosolve were added to Medium A or B to give a total volume of 6.1 ml per flask. A total of 10 flasks (containing the equivalent of 4.0 g of wet liver) was used routinely in each experiment. The flasks which were open to air were shaken in a water bath maintained at 37\(^\circ\). After 1 hour the contents of the flasks were combined and the proteins were precipitated by the addition of 0 volumes of ethanol. After the mixture stood at 4\(^\circ\) for 0.5 hour, the precipitate was separated by centrifugation and the metabolites in the supernatant liquid were partitioned by solvent extraction as shown in Fig. 2. In the radioactive tracer experiments, the carrier compounds dissolved in methanol were added, with vigorous shaking, prior to the precipitation of the proteins.

**Purification and Determination of Metabolites**—The residue which remained after evaporation of the solvent of Fraction F-1 (Fig. 2) was dissolved in methanol, and the solution was applied as a band (1 \(\times\) 15 cm) to Whatman No. 3MM paper. Authentic 1-hydroxy-FAA, 3-hydroxy-FAA, 5-hydroxy-FAA, 7-hydroxy-FAA, and N-hydroxy-FAA were spotted as markers at the origin. The chromatogram was developed with the upper phase of the solvent system described above after equilibration with both phases for 18 to 20 hours. The separated compounds (Fig. 3) were located by exposure of the chromatogram to ultraviolet light (2537 A), and the compounds were eluted by descending chromatography with methanol. 1-Hydroxy-FAA and 3-hydroxy-FAA were further purified by thin layer chromatography on Silica Gel GF254 (20 \(\times\) 20 cm plates, 0.25 mm thickness of adsorbent). Since 5-hydroxy-FAA and 7-hydroxy-FAA migrated on Whatman No. 3MM paper with identical rates, these compounds were separated subsequently by thin layer chromatography on plates (20 \(\times\) 20 cm) coated to a thickness of 0.25 mm with silicic acid (Mallinkrodt, 200 mesh) mixed with calcium sulfate (13\%) and zinc silicate (1\%). The thin layer chromatograms were developed without previous equilibration.

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\(^3\) The livers were not perfused prior to removal since serum, when added to the microsomal-mitochondrial sediment from the livers of 3-methylcholanthrene-treated rats to give a serum protein concentration of 0.61 mg per ml of incubation system, converted 1.9\% of the substrate, N-hydroxy-FAA-9-\(^{14}\)C, to 1-hydroxy-FAA-9-\(^{14}\)C. The soluble proteins from the same rat (5.7 mg per ml of incubation system) were located by exposure of the chromatogram to ultraviolet light (2537 A), and the compounds were eluted by descending chromatography with methanol. 1-Hydroxy-FAA and 3-hydroxy-FAA were further purified by thin layer chromatography on Silica Gel GF254 (20 \(\times\) 20 cm plates, 0.25 mm thickness of adsorbent). Since 5-hydroxy-FAA and 7-hydroxy-FAA migrated on Whatman No. 3MM paper with identical rates, these compounds were separated subsequently by thin layer chromatography on plates (20 \(\times\) 20 cm) coated to a thickness of 0.25 mm with silicic acid (Mallinkrodt, 200 mesh) mixed with calcium sulfate (13\%) and zinc silicate (1\%). The thin layer chromatograms were developed without previous equilibration.
Incubation system; deproteinized with 6 volumes of ethanol; centrifuged at 0° for 10 min

Precipitate (discarded)  
Supernatant liquid, ethanol distilled at reduced pressure; volume adjusted to 60 ml; pH adjusted to 4; extracted three times with 60 ml of ether

Either extracted two times with 25 ml of 0.25 N NaOH; washed with water  
Aqueous phase (discarded)

Ether (F-2) contains FAA and 2-fluorenamine; compounds separated by thin layer chromatography  
Aqueous phase washed with ether; acidified (pH 2) with 8 N HCl; extracted two times with 25 ml of ether

Ether (F-1) contains acidic-phenolic metabolites; washed with distilled water; solvent removed at reduced pressure; residual compounds separated by paper chromatography and by thin layer chromatography  
Aqueous phase (discarded)

Fig. 2. The partition of the metabolites of N-hydroxy-FAA or of FAA by solvent extraction after incubation of these substrates with cell fractions from rat liver.

Fig. 3. The separation of the acidic and phenolic compounds in Fraction F-1 (Fig. 2) by descending paper chromatography on Whatman No. 3MM paper with the upper phase of a mixture of cyclohexane-t-butyl alcohol-glacial acetic acid-water (16:1:1:1) as a solvent. The bands were visualized by the quenching of the fluorescence on exposure of the developed chromatogram to ultraviolet light (2537 Å) or by spraying with dilute (1:3) Folin reagent.

in glass tanks lined with Whatman No. 3MM paper. The mobilities of the compounds in various systems are listed in Table I. The compounds were eluted from the gel by repeated extractions with methanol (2 × 2.0 ml) and the ultraviolet absorption spectra of the eluted compounds were checked against those of authentic samples. Purification by thin layer chromatography was repeated until the spectra of the isolated metabolites were superimposable on those of authentic samples. The quantities of 1-hydroxy-FAA and of 3-hydroxy-FAA in the extract of the final chromatogram were then determined by means of the appropriate extinction coefficients listed above. These values were corrected for loss of compound during the isolation and purification by means of experimentally determined values for the recoveries of the compounds. The recoveries were estimated by adding known amounts of the compounds to the complete incubation system and by precipitating the proteins immediately. The separation, purification, and estimation of the compounds were then carried out as described above. The average recoveries of 1-hydroxy-FAA and of 3-hydroxy-FAA in three different experiments were 27.1 ± 1.6% and 32.6 ± 2.8%, respectively. The lower limit for the spectrophotometric estimation of the isomerization of N-hydroxy-FAA to the o-amidofluorenoles was 0.30%. In the radioactive tracer experiments the carrier compounds, which had been isolated by solvent extraction and separated by paper chromatography, were purified to constant (±10%) specific radioactivity by repeated thin layer chromatography. The quantities of metabolite formed were calculated in the usual manner from the final specific radioactivity and from the amounts of carrier added. Under the experimental conditions the isomerization of as little as 0.01% of N-hydroxy-FAA-9,10C was measurable by inverse isotopic dilution.
Experiments were designed to characterize the soluble component in rat liver which participated in the isomerization of N-hydroxy-FAA as shown below. The soluble fraction obtained from 11.0 g of wet liver was subjected to gel filtration on Bio-Gel P-6. The gel was equilibrated overnight with 0.01 M phosphate buffer, pH 7.4, and was then poured into a column (3.3 x 44 cm). The soluble fraction was applied to the gel and elution was carried out with the above phosphate buffer at a flow rate of 0.01 M per hour. Fractions of 0 ml were collected and the absorbances of each fraction at 260 and 280 mµ were determined with a Hitachi Perkin-Elmer model 319 spectrophotometer. The elution profile exhibited two peaks. The first of these, which supported the formation of 7-hydroxy-FAA, was used as a carrier in several of the experiments described in the text.

Radioactivity Measurements—The radioactivity of samples soluble in organic solvents was determined in a scintillation solution (10 ml) consisting of 2, 5-diphenyloxazole (4 g) and 1, 4-bis[2-(phenyloxazoyl)]benzene (0.1 g) in toluene (1 liter). The radioactivities of aqueous solutions were determined as described previously (8). All samples were counted in duplicates with a liquid scintillation spectrometer at a level of confidence of at least 95%. Corrections for quenching were made by the channel ratio procedure (18).

RESULTS

Incubation of N-hydroxy-FAA with the soluble fraction or with the 600 x g supernatant liquid of rat liver in Medium A which supported the formation of 7-hydroxy-FAA by microsomes of rat liver (4) gave only trace amounts of 1-hydroxy-FAA and of 3-hydroxy-FAA (Table 11). The amounts of the a-amino-fluorenols formed under these conditions were of the same order of magnitude as the quantities formed when N-hydroxy-FAA was incubated in 0.01 M phosphate buffer, pH 7.4, in the absence of any cell fraction. The detection of small amounts of 1-hydroxy-FAA and of 3-hydroxy-FAA was therefore referable to a nonenzymatic isomerization. The formation of sizeable amounts of 7-hydroxy-FAA observed on incubation of the 600 x g supernatant liquid with N-hydroxy-FAA in Medium A was due to the microsomal hydroxylation of FAA which was present in the incubation systems and isolated from Fraction F 1 (Fig. 2). The enzymatic reduction of N-hydroxy-FAA to FAA by rat liver homogenates (19) and the microsomal, NADPH-
Isomerization of N-Hydroxy-2-fluorenylacetamide to o-Amidophenols in Vitro

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A420 o -..____... 0

2 2.0 - 5

0

1.2 6.0

3 c 0 5

1.0

4b ' sb Ii0 . ' 260 ' 240

Tuba Numbsr

1.2 6.0

3 c 0 5

1.0

4b ' sb Ii0 . ' 260 ' 240

FIG. 4. DEAE-cellulose chromatography of the soluble fraction of rat liver (upper) and the relative magnitudes of the isomerization and of the reduction of N-hydroxy-FAA by Subfractions A, B, C, D, and E combined with the microsomal-mitochondrial sediment from the livers of 3-methylcholanthrene-treated rats (lower). The soluble fraction from 64 g of wet liver was obtained as described in Fig. 1 and dialyzed against 0.005 M NaHCO3 buffer, pH 9.5, for 20 hours prior to ion-exchange chromatography. Protein elution was carried out in a stepwise manner with 650 ml of each of five buffers. The numbers on the top of the figure correspond to the following buffers: I, 0.005 M NaHCO3, pH 9.5; II, 0.02 M Na2HPO4, pH 9.2; III, 0.02 M Na2HPO4, pH 8.5; IV, 0.07 M NaH2PO4, pH 4.6; V, 0.05 M NaH2PO4 + 1.0 M NaCl, pH 4.6. Fractions of 16 ml per tube were collected at a rate of 200 ml of effluent per hour and the absorbances of each fraction at 260 and 280 nm were determined. The contents of Tubes 16 to 40 (Subfraction A, A280:A260 = 1.68), Tubes 66 to 85 (Subfraction B, A280:A260 = 1.45), Tubes 86 to 120 (Subfraction C, A280:A260 = 1.51), Tubes 121 to 184 (Subfraction D, A280:A260 = 1.73), and Tubes 185 to 214 (Subfraction E, A280:A260 = 0.93) were dialyzed for 20 hours against 0.01 M sodium phosphate buffer, pH 7.4. Aliquots (55 ml) of each of the dialyzed subfractions were incubated with the microsomal-mitochondrial sediment from 4.0 g of wet liver of 3-methylcholanthrene-treated rats and with N-hydroxy-FAA-9-14C (0.14 pmole per ml of incubation mixture). The amounts of 1-hydroxy-FAA, 3-hydroxy-FAA, FAA, and 2-fluorenamine (FA) were determined by inverse isotopic dilution. In the figure 1-hydroxy-FAA and 3-hydroxy-FAA are referred to as the o-hydroxylated metabolites and FAA and 2-fluorenamine as the reduced metabolites.

dependent hydroxylation of FAA to 7-hydroxy-FAA have already been described (4, 5). Attempts to stimulate the isomerization of N-hydroxy-FAA by the oral administration of FAA for 8 weeks at a level which induced the N-hydroxylation of FAA were unsuccessful. However, the yields of the o-amidofluorens in Medium A (Experiment 6, Table II) were increased about 6-fold by a single, intraperitoneal injection of 3-methylcholanthrene to the rat 24 hours prior to the preparation of the 600 x g supernatant liquid. A 15- to 20-fold increase in the formation of 1-hydroxy-FAA was observed when 2-diethylaminomethyl-2,2-diphenylvalerate hydrochloride (SKF 525-A) was added to the incubation systems which contained the 600 x g supernatant liquid from the livers of 3-methylcholanthrene-treated rats.

Since SKF 525-A inhibits the NADPH-dependent, microsomal hydroxylation of aromatic compounds to some extent (20), the isomerization of N-hydroxy-FAA to 1-hydroxy-FAA appeared to be essentially different from the microsomal hydroxylation of FAA which takes place on carbon atom 7 of the fluorene nucleus and requires NADPH or a NADPH-generating system (4, 5). This view was strongly supported by the finding that the induced formation of the o-amidofluorens proceeded optimally in media, such as phosphate buffer, Tris buffer, or deionized water, which lacked the compounds necessary for aromatic hydroxylations (Experiments 9, 10, and 11, Table II). It should also be noted that 7-hydroxy-FAA was detectable in the incubation systems under these conditions (Experiment 9, Table II). The possibility that FAA, arising via the enzymatic reduction of N-
hydroxy-FAA, served as the substrate in the isomerization of N-hydroxy-FAA was ruled out by experiments in which N-hydroxy-FAA-9-14C and FAA-9-14C were incubated separately and concurrently, in Medium B, with equal portions of the 600 × g supernatant liquid from the livers of 3-methylcholanthrene-treated rats. Scans of the paper chromatograms of Fraction F-1 prepared from the incubation systems with N-hydroxy-FAA-9-14C as the substrate showed a radioactive peak, RF = 0.29, coincident with authentic 1-hydroxy-FAA (Fig. 5). In contrast, no radioactive peak was detected in this region on chromatograms obtained from incubation systems which contained FAA-9-14C as the substrate. The possibility that N-acetoxy-2-fluorenylacetamide, a hypothetical intermediate in the metabolism of N-hydroxy-FAA and the precursor of a highly reactive amidonium ion (10), was the substrate of the isomerization was also examined. In these experiments the formation of 1-hydroxy-FAA and of 3-hydroxy-FAA by an aliquot of the 600 × g supernatant liquid, from the livers of 3-methylcholanthrene-treated rats was compared with the formation of the o-amidofluorenoles by another aliquot of the same cell fraction (Experiments 2 and 3, Table III). The data indicated that 2.7 and 0.5% of N-acetoxy-2-fluorenylacetamide were metabolized to 1-hydroxy-FAA and 3-hydroxy-FAA, respectively. These values take account of the spontaneous conversion of N-acetoxy-2-fluorenylacetamide to 1-hydroxy-FAA and 3-hydroxy-FAA which amounted to 4.6 and 4.4%, respectively (Experiment 1, Table III). Since N-hydroxy-FAA, under the same conditions and with the same cell fraction, yielded 7.8 and 5.1% of 1-hydroxy-FAA and of 3-hydroxy-FAA, respectively, the arylhydroxamic acid was a better substrate for the induced formation of the o-amidofluorenoles than was the acetate ester.

The isomerization of N-hydroxy-FAA was dependent on pH and the tissue fractions were obtained from 4.0 g of wet liver. For the spectrophotometric estimation of 7-hydroxy-FAA in Experiment 8 the formation of the metabolites was determined by inverse isotopic dilution.

**Table II**

<table>
<thead>
<tr>
<th>Experiment and cell fraction</th>
<th>Preliminary treatment of rats</th>
<th>Incubation medium</th>
<th>Conversion of N-hydroxy-FAA</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td></td>
<td>Medium B</td>
<td>To 1-hydroxy-FAA</td>
<td>0.33</td>
</tr>
<tr>
<td>2. 105,000 × g supernatant liquid</td>
<td>None</td>
<td>Medium A</td>
<td>To 3-hydroxy-FAA</td>
<td>0.12</td>
</tr>
<tr>
<td>3. 600 × g supernatant liquid</td>
<td>None</td>
<td>Medium A</td>
<td>To 7-hydroxy-FAA</td>
<td>0.16</td>
</tr>
<tr>
<td>4. 600 × g supernatant liquid</td>
<td>None</td>
<td>Medium A</td>
<td></td>
<td>0.27</td>
</tr>
<tr>
<td>5. 600 × g supernatant liquid</td>
<td>2 FAA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Medium A</td>
<td></td>
<td>0.28</td>
</tr>
<tr>
<td>6. 600 × g supernatant liquid</td>
<td>3-MC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Medium A</td>
<td></td>
<td>1.81 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>7. 600 × g supernatant liquid</td>
<td>3-MC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Medium A + 5 × 10&lt;sup&gt;-3&lt;/sup&gt; m SKF 525-A</td>
<td>5.57 ± 0.27&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>8. 600 × g supernatant liquid</td>
<td>3-MC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Medium A + 10&lt;sup&gt;-3&lt;/sup&gt; m SKF 525-A</td>
<td>4.88 ± 0.17&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>9. 600 × g supernatant liquid</td>
<td>3-MC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Medium B</td>
<td></td>
<td>6.31 ± 2.57&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>10. 600 × g supernatant liquid</td>
<td>3-MC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Deionized water</td>
<td></td>
<td>7.57</td>
</tr>
<tr>
<td>11. 600 × g supernatant liquid</td>
<td>3-MC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.01 M Tris buffer, pH 7.4</td>
<td>7.66</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Not determined.

<sup>b</sup> FAA was administered in the diet at a level of 0.08% (3) for 8 weeks prior to the preparation of the cell fraction.

<sup>c</sup> 3-Methylcholanthrene (3-MC) (2.0 mg/100 g of body weight) was injected intraperitoneally in 0.35 ml of corn oil 24 hours prior to the preparation of the cell fraction.

<sup>d</sup> This value is the mean and the average deviation from the mean of 11 experiments.

<sup>e</sup> This value is the mean and the average deviation from the mean of two experiments.

<sup>f</sup> This value is the mean and the average deviation from the mean of nine experiments.

<sup>g</sup> Not detectable by the spectrophotometric assay.

**Fig. 5.** Evidence for the specificity of N-hydroxy-FAA as a substrate in the formation of 1-hydroxy FAA by the 600 × g supernatant liquid. Equal amounts of the 600 × g supernatant liquid, prepared from the livers of 3-methylcholanthrene-treated rats as described in the text, were incubated in Medium B for 1 hour (a) with N-hydroxy-FAA-9-14C (4.2 pmoles, 4.0 × 10<sup>6</sup> dpm) or (b) with FAA-9-14C (4.2 pmoles, 4.0 × 10<sup>6</sup> dpm). The incubation mixtures were processed by solvent extraction as shown in Fig. 2. Fraction F-1 was chromatographed on Whatman No. 3MM paper by the descending technique with the upper phase of cyclohexane-butyl alcohol-glacial acetic acid-water (10:1:1:1), and the chromatograms were scanned as described in the text. The upper tracing shows the distribution of the radioactivity when N-hydroxy-FAA-9-14C was used as the substrate. The lower tracing is a scan of the chromatogram when FAA-9-14C was used as the substrate.
Relative yields of 1-hydroxy-FAA and of 3-hydroxy-FAA formed from N-acetoxy-2-fluorenylacetamide (N-acetoxy-FAA) and from N-hydroxy-FAA by 600 \times g supernatant liquid of 3-methylcholanthrene-treated rats

<table>
<thead>
<tr>
<th>Experiment and substrate(a)</th>
<th>Incubation medium</th>
<th>Preliminary treatment of rats</th>
<th>Conversion of substrate</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. N-Acetoxy-FAA (\ldots) B</td>
<td>None</td>
<td>4.63</td>
<td>4.40</td>
<td></td>
</tr>
<tr>
<td>2. N-Acetoxy-FAA (\ldots) B</td>
<td>3-MC</td>
<td>7.32</td>
<td>7.41</td>
<td></td>
</tr>
<tr>
<td>3. N-Hydroxy-FAA (\ldots) B</td>
<td>3-MC*</td>
<td>7.80</td>
<td>5.09</td>
<td></td>
</tr>
</tbody>
</table>

\(\ast\) The concentrations of N-acetoxy-FAA and of N-hydroxy-FAA in the incubation systems were 0.12 \(\mu\)mole per ml and 0.14 \(\mu\)mole per ml, respectively.

\(\ast\) The quantities of 1-hydroxy-FAA and of 3-hydroxy-FAA were estimated spectrophotometrically as described in the text.

\(\ast\) 3-Methylcholanthrene (3-MC) (2.0 mg/100 g of body weight) was injected intraperitoneally in 0.35 ml of corn oil 24 hours prior to the preparation of the cell fractions.

<table>
<thead>
<tr>
<th>Experiment and substrate</th>
<th>Conversion of N-hydroxy-FAA</th>
<th>Conversion of 3-hydroxy-FAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of inducer (\times 10^3)</td>
<td>1-hydroxy-FAA</td>
<td>3-hydroxy-FAA</td>
</tr>
<tr>
<td>1. Benz[a]pyrene</td>
<td>2.5</td>
<td>14.65</td>
</tr>
<tr>
<td>2. Dibenzo[a,l]anthracene</td>
<td>2.8</td>
<td>7.44</td>
</tr>
<tr>
<td>3. Phenothiazine</td>
<td>2.0</td>
<td>2.30</td>
</tr>
<tr>
<td>4. 2-Chloro-10-(3-dimethylamino-propyl)phenothiazine (SKF-2601)</td>
<td>3.5</td>
<td>(&lt;0.30)</td>
</tr>
<tr>
<td>5. 3-Chloro-19-nortestosterone</td>
<td>2.4</td>
<td>(&lt;0.30)</td>
</tr>
<tr>
<td>6. Sodium 5-ethyl-5(1-methyl-buty)barbiturate</td>
<td>2.3</td>
<td>(&lt;0.30)</td>
</tr>
<tr>
<td>7. Testosterone</td>
<td>2.9</td>
<td>(&lt;0.30)</td>
</tr>
<tr>
<td>8. 19-Nortestosterone</td>
<td>2.7</td>
<td>(&lt;0.30)</td>
</tr>
<tr>
<td>9. 1-Chloro 10 nortestosterone (SKF-0611)</td>
<td>3.7</td>
<td>(&lt;0.30)</td>
</tr>
</tbody>
</table>

\(\ast\) Each compound was administered as a suspension in 0.35 ml of isotonic 0.9% NaCl containing 1.75% acacia. The single injections were given 24 hours prior to the preparation of the homogenates.
treated rat after recombination with the soluble fraction from the livers of untreated or 3-methylcholanthrene-treated rats (Table V). Neither the soluble fraction nor the microsomal-mitochondrial sediment from the livers of 3-methylcholanthrene-treated rats alone was able to isomerize N-hydroxy-FAA. However, the microsomal-mitochondrial sediment from the livers of 3-methylcholanthrene-treated rats, in conjunction with the soluble fraction from the livers of untreated or 3-methylcholanthrene-treated rats, performed the reaction at a rate comparable to that of the 600 X g supernatant liquid of 3-methylcholanthrene-treated rats. It was evident that the isomerization of N-hydroxy-FAA required the synergistic action of two components. One of these was inducible by certain polycyclic aromatic hydrocarbons and resided in the particulate fraction of the liver cell, while the second component was noninducible and present in the soluble fraction of rat liver. Assays of mitochondria and of microsomes which had been separated by differential centrifugation at 10,000 X g (0.25 hour) and at 105,000 X g (1 hour) indicated that 33% of the isomerizing activity was associated with the mitochondria and 67% with the microsomes. This distribution of the activity suggested that the inducible component was a microsomal enzyme. Since the separation of the mitochondria from the microsomes by differential centrifugation is incomplete (26), the activity in the mitochondria was likely due to contamination with microsomes.

The noninducible soluble component of the isomerizing activity was investigated by dialysis experiments, by gel filtration, and by ion exchange chromatography of the soluble proteins. In these experiments the assay systems consisted of the dialyzed or fractionated macromolecules to which standard amounts of the microsomal-mitochondrial sediment from the livers of 3-methylcholanthrene treated rats were added. Dialysis experiments indicated that virtually none of the isomerizing activity was dialyzable (Table VI). The soluble component was further characterized as a compound with a molecular weight >4600 by gel filtration of the soluble fraction on polyacrylamide gel. In these experiments 90% of the activity recovered in the effluent was found in the fraction excluded from the gel which contained the compounds with a molecular weight in excess of 4600.

Evidence that the soluble component of the isomerizing activity was associated with a protein or proteins was provided by the fractionation of the soluble proteins of rat liver on DEAE-cellulose which yielded the five subfractions (A, B, C, D, and E) previously described (9). Measurements of the Am85:Am90 ratios confirmed that Subfractions A, B, and D which were eluted from the ion exchanger with buffers of increasing ionic strength and decreasing pH consisted of proteins (8). Subfraction E which was eluted with 1.0 M sodium chloride at pH 4.6 contained nuclear acids in addition to proteins. By far the largest amounts of 1-hydroxy-FAA and 3-hydroxy-FAA were produced by the assays systems which contained the acidic proteins eluted at pH 4.6 (Subfraction D). It may also be seen that the reduction of N-hydroxy-FAA to FAA and 2-fluorenamine proceeded optimally in this assay system and that the yields of these metabolites paralleled those of the o-amidofluorenols in each fraction (Fig. 4). Reduction of N-hydroxy-FAA to FAA by liver homogenates and cell fractions has been reported previously (19, 27). The 2-fluorenamine arose either by demethylation of FAA (1) or by reduction of 2-fluorenylhydroxylamine (15). The sum of FAA and 2-fluorenamine represented therefore the total reduced metabolites derived from N-hydroxy-FAA. Subfraction D also

![Graph](http://www.jbc.org/)

**Table V**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Assay of cell fractions or combination of cell fractions</th>
<th>Conversion of N-hydroxy-FAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obtained from the 3-MC-treated rat</td>
<td>Obtained from the untreated rat</td>
</tr>
<tr>
<td>1</td>
<td>600 X g supernatant liquid</td>
<td>6.31</td>
</tr>
<tr>
<td>2</td>
<td>Microsomal-mitochondrial sediment</td>
<td>&lt;0.30</td>
</tr>
<tr>
<td>3</td>
<td>Soluble fraction</td>
<td>0.58</td>
</tr>
<tr>
<td>4</td>
<td>Microsomal-mitochondrial sediment + soluble fraction</td>
<td>5.20</td>
</tr>
<tr>
<td>5a</td>
<td>Soluble fraction</td>
<td>&lt;0.30</td>
</tr>
<tr>
<td>5b</td>
<td>Microsomal-mitochondrial sediment</td>
<td>6.73</td>
</tr>
</tbody>
</table>

Each of the fractions was obtained from 4.0 g of wet liver. The fractions, or combination of fractions, were incubated with N-hydroxy-FAA in Medium B, as described in the text. The substrate concentration in all experiments was 0.070 μmole per ml. 1-Hydroxy-FAA and 3-hydroxy-FAA were determined by the spectrophotometric method. 3-Methylcholanthrene (2.0 mg/100 g of body weight) was injected intraperitoneally 24 hours prior to preparation of the cell fractions.
was collected and concentrated to 22.5 ml by lyophilization. The quantities of 1-hydroxy-FAA and of 3-hydroxy-threne-treated rats were incubated in 0.01 M phosphate buffer, pH 7.4, with N-hydroxy-FAA (0.14 μmole per ml of incubation mixture). The quantities of 1-hydroxy-FAA and of 3-hydroxy-FAA formed were determined spectrophotometrically as described in the text.

### Table VI

<table>
<thead>
<tr>
<th>Cell fraction assayed</th>
<th>Conversion of N-hydroxy-FAA</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>600 × g supernatant liquid (undialyzed)</td>
<td>4.35</td>
<td>1.29</td>
</tr>
<tr>
<td>Dialyzed soluble fraction + microsomal-mitochondrial sediment*</td>
<td>6.74</td>
<td>1.95</td>
</tr>
<tr>
<td>Dialyze + microsomal-mitochondrial sediment</td>
<td>0.66</td>
<td>&lt;0.30</td>
</tr>
</tbody>
</table>

* The soluble fraction (22.5 ml) was placed into Visking NoJax cellulose casing size 18 and dialysed in a rocking dialyzer for 16 hours against 1.6 liters of deionized water. The dialyzing liquid was collected and concentrated to 22.5 ml by lyophilization. The concentrate is referred to in the table as the dialyzate.

### Table VII

<table>
<thead>
<tr>
<th>Cell fraction testeda, b</th>
<th>Conversion of N-hydroxy-FAA</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subfraction A + microsomal-mitochondrial sediment</td>
<td>0.30</td>
<td>4.81</td>
</tr>
<tr>
<td>Subfraction B + microsomal-mitochondrial sediment</td>
<td>0.93</td>
<td>16.19</td>
</tr>
<tr>
<td>Subfraction C + microsomal-mitochondrial sediment</td>
<td>0.92</td>
<td>12.15</td>
</tr>
<tr>
<td>Subfraction D + microsomal-mitochondrial sediment</td>
<td>1.66</td>
<td>24.48</td>
</tr>
<tr>
<td>Subfraction E + microsomal-mitochondrial sediment</td>
<td>0.80</td>
<td>8.48</td>
</tr>
</tbody>
</table>

The soluble fraction (22.5 ml) was placed into Visking NoJax cellulose casing size 18 and dialysed in a rocking dialyzer for 16 hours against 1.6 liters of deionized water. The dialyzing liquid was collected and concentrated to 22.5 ml by lyophilization. The concentrate is referred to in the table as the dialyzate.

### DISCUSSION

The induced isomerization of N-hydroxy-FAA by rat liver has been tentatively formulated by the two-step reaction sequence shown in Fig. 8. In the first step, N-hydroxy-FAA is dehydroxylated by an enzyme in the soluble fraction to a positively charged amidonium ion which may subsequently undergo reduction or isomerization. The dehydroxylation was assigned to the soluble fraction rather than to the particulate fraction, because reduction of N-hydroxy-FAA to FAA, which involves a dehydroxylation (27) and the addition of a hydride ion, was performed by the soluble fraction at a 25-fold faster rate than by the microsomal-mitochondrial sediment from the livers of 3-methylcholanthrene-treated rats (Table VIII). The dehydroxylation enzyme was most active in a protein fraction eluted from DEAE-cellulose at pH 4.6 (Fraction D). The presence of considerably smaller amounts of dehydroxylation activity in other protein fractions may be due to the stepwise elution of the proteins from DEAE-cellulose. Accordingly, additional procedures will be used for the purification of the dehydroxylation activity. The fact that chromatography did not separate the soluble proteins into fractions which catalyzed either reduction or isomerization alone supports the view that a single compound (i.e. the amidonium ion) was the intermediate common to both reactions. The observation that the products of both reactions were, in all fractions, formed in a constant proportion (Table VII) likewise argues for this interpretation of the data. The role of the induced microsomal enzyme remains at present conjectural. It may complete the isomerization by catalyzing the nucleophilic attack of hydroxyl ions from the medium on the positive carbon atoms 1 and 3 of the fluorene nucleus as shown in Fig. 8. Alternatively, the induced enzyme may stabilize the amidonium ion and its resonance forms. In this event, the addition of hydroxyl ions to the electrophilic centers at carbon atoms 1 and 3 may proceed nonenzymatically.

The formation in vitro of an amidonium ion derived from N-hydroxy-FAA has been inferred previously (16, 29). In these experiments, the amidonium ion arose from the nonenzymatic decomposition of the acetate ester of N-hydroxy-FAA (16). Sulfate and phosphate esters of N-hydroxy-FAA have also been considered as the immediate precursors of the amidonium ion (29). The present data suggest the direct dehydroxylation of N-hydroxy-FAA, not requiring previous esterification, as an alternate mechanism for the formation of the amidonium ion. Two lines of evidence indicate that the amidonium ion was not derived from an ester of N-hydroxy-FAA in the present experiments. The first is that the isomerization of N-hydroxy-FAA to 1-hydroxy-FAA and 3-hydroxy-FAA proceeded maximally in phosphate buffer, Tris buffer, or deionized water. In contrast, the formation of a phosphate or sulfate ester of N-hydroxy-FAA by preparations of rat liver in vitro has been reported to require high concentrations of ATP and of divalent magnesium and sulfate ions (30). The second line of evidence came from the experiments cited above (Table III) which indicated that...
Formation of F-4 from N-hydroxy-FAA-9-14C by cell fractions or combinations of cell fractions from livers of 3-methylcholanthrene-treated or untreated rats

The cell fractions from 4.0 g of wet liver were incubated in 0.01 M phosphate buffer, pH 7.4, with N-hydroxy-FAA-9-14C (0.070 μmol per ml of incubation mixture). The quantities of FAA obtained here indicates that the isomerization of N-hydroxy-FAA plays a role in the carcinogenic activity of the arylhydroxamic acid. It would appear, at first glance, that this compound has as yet been found which would serve this function. Even if such an inducer were found, the products of the isomerization, 1-hydroxy-FAA and 3-hydroxy-FAA, when tested for carcinogenic activity, were inactive (15, 31). However, the experiments presented here provide indirect evidence for the formation of an amidonium ion, by the enzymatic dehydroxylation of N-hydroxy-FAA, without involving intermediate acyl esters of N-hydroxy-FAA. The same amidonium ion arising from the spontaneous decomposition of acyl esters of N-hydroxy-FAA has been shown to arylate various tissue nucleophiles (28, 32), and the arylation of nucleic acids or proteins, or both, by this unstable intermediate is currently presumed to be one of the initial molecular events in the induction of neoplasia by N-hydroxy-FAA (28). The idea that the amidonium ion derived from N-hydroxy-FAA by enzymatic dehydroxylation is the common intermediate for the reduction and isomerization of the arylhydroxamic acid as well as for the arylation of tissue constituents will be further pursued.

Acknowledgments—The authors thank Mrs. B. Zakis and Mrs. J. Thaker for technical assistance and Dr. E. J. Barry of this laboratory for help with the ion exchange chromatographies and for discussion.

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The Conversion of the Carcinogen N-Hydroxy-2-fluorenylacetamide to o-Amidophenols by Rat Liver in Vitro: AN INDUCIBLE ENZYMATIC REACTION

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J. Biol. Chem. 1969, 244:1729-1740.

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