The Action of the Carcinogen \(N\)-(2-Fluorenyl)acetamide on Rat Liver Catalase and Arginase in Vivo*

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SUMMARY

The oral administration of the carcinogens \(N\)-(2-fluorenyl)acetamide and \(N\)-hydroxy-2-fluorenylacetamide to male rats for 6 weeks depressed the activity of liver catalase to the extent of 20–25 per cent, but had no effect on liver arginase. The catalase activity was restored by withdrawal of the compounds from the diet. The administration of the noncarcinogenic metabolite \(N\)-(1-hydroxy-2-fluorenyl)acetamide had no effect on the activity of either liver catalase or arginase. Catalase isolated from rat liver after the intraperitoneal administration of carbon-14-labeled \(N\)-(2-fluorenyl)acetamide or \(N\)-(1-hydroxy-2-fluorenyl)acetamide contained only negligible quantities of bound \(^{14}C\). The evidence supports the view that the covalent binding of \(N\)-(2-fluorenyl)acetamide or of one of its metabolites is not a probable mechanism for the inhibition of catalase in vivo. Determinations of the total iron content of the liver after the feeding of \(N\)-(2-fluorenyl)acetamide gave no indication that a disturbance of the over-all iron metabolism was implicated in the lowering of the catalase activity. Since oral feeding of \(N\)-(2-fluorenyl)acetamide or \(N\)-hydroxy-2-fluorenylacetamide, but not of \(N\)-(1-hydroxy-2-fluorenyl)acetamide, decreased the protein content of the liver, it is suggested that the partial inactivation of catalase in vivo is due to a selective action of these carcinogens on enzyme synthesis or destruction.

The irreversible binding of a number of carcinogens to proteins has given rise to the concept that the biological activity of these compounds might be attributed to their combination with certain key enzymes which control in some way the normal growth of the cell (28). It has been suggested that the enzyme(s) to which the carcinogen is bound is inactivated and subsequently deleted (28). The permanent loss of the enzyme(s) from the cell may be the causative factor in the transformation of the normal into a malignant cell (28). There is as yet scant experimental evidence that the irreversible binding in vivo of a carcinogen (or a metabolite thereof) will lead to enzyme inactivation. However, recent model studies on the mode of action of the carcinogen \(N\)-(2-fluorenyl)acetamide have indicated that the \(o\)-quinone imine, 2-imino-1,2-fluorenoquinone, which is a transitory metabolite in vitro (32, 33) (and possibly in vivo), interacts irreversibly with the enzymes catalase and arginase and that the interaction results in the inactivation of these enzymes (20). The present study is an extension of this work. An attempt has been made to determine whether \(N\)-(2-fluorenyl)acetamide, \(N\)-hydroxy-2-fluorenylacetamide, and \(N\)-(1-hydroxy-2-fluorenyl)acetamide, all of which may be considered as precursors of 2-imino-1,2-fluorenoquinone (21, 24), would decrease the activity of rat liver catalase and arginase in vivo and, if so, whether the inactivation could be accounted for by the formation of a protein adduct. Accordingly, the activities of these enzymes were measured after the oral administration of the compounds to the rat. In addition, rat liver catalase was isolated by an immunological technic after administration of \(^{14}C\)-labeled \(N\)-(2-fluorenyl)acetamide or \(N\)-(1-hydroxy-2-fluorenyl)acetamide, and the isolated enzyme was examined for bound radioactivity.

MATERIALS AND METHODS

Animals and diets.—Male albino rats, weighing 40–80 gm. (Holtzman Rat Company, Madison, Wisconsin), were caged individually at 26°C and given water ad libitum. They were maintained on a semisynthetic 20 per cent casein diet in which the vitamins were added either individually (11) or in a commercially available (Nutritional Biochemical Company, Cleveland, Ohio)
mixture (22 gm/kg of diet). The final concentration of the vitamins was approximately the same in either case. The diet containing the commercial vitamin mixture was used for the immunochemical experiments and the experiment in which the effect of AAF on the level of total iron in the liver was determined. The diet was stored at 4°C. and was prepared fresh each 7–10 days. The test compounds were incorporated into the diet in an acetone solution. The acetone was removed by stirring the food in air.

With the exception of the immunochemical experiments, each animal receiving AAF was paired with a litter-mate which was fed only the basal diet. The rats were pair-fed in such a way that the animal which had consumed the greater amount of food during the preceding 24 hours was, during the next 24 hours, restricted to the amount which its litter-mate had consumed in the preceding period. The relative effects of the compounds were determined by feeding diets containing the test compounds in equimolar amounts. The diets were pair-fed to groups of rats each of which included one animal for each of the compounds and one control litter-mate. Equal food consumption in each group was assured by controlling the intake as described above. The experimental design (paired-feeding technique and use of litter-mates) minimized differences in tissue enzyme levels due to the genetic makeup of the animals or to variation in the food intake. Male rats were used, since their liver is especially susceptible to the hepatocarcinogenic action of AAF (42).

Albino male rabbits purchased from a local dealer were caged individually and maintained at a temperature of 26°C. They were fed a diet of rabbit pellets (Purina, St. Louis, Missouri). Water was given ad libitum and lettuce occasionally.

Liver arginase and catalase activities.—After the animals used in these experiments had been maintained by the paired-feeding technique for the times specified in the tables they were sacrificed by a blow to the head. The livers were removed quickly, cooled with ice, blotted, weighed, and homogenized in a Waring Blender at 4°C with 7 volumes of cold distilled water. The homogenization was carried out for three 1-minute periods interrupted by 1-minute cooling periods. The arginase and catalase activities of the homogenates were determined as described previously (20).

Immunochemical isolation of rat liver catalase.—Purification of rat liver catalase: Preliminary purification of catalase from rat liver was carried out by the method of Price and Greenfield (36) with the following modifications. Catalase (S₆ of Price and Greenfield [36]) by dialysis overnight at 4°C against 20 per cent ethanol which was 0.1 M with respect to acetate and sodium chloride (pH 5.7) (37). The precipitate was collected by centrifugation, and the supernatant liquid was discarded. The catalase was then dissolved in sodium phosphate (0.01 M, pH 7.4) and purified by chromatography on DEAE (20). The effluent catalase fractions with a ratio of absorption at 407 μM to that at 276 μM (E₄₀₇: E₂₇₆) of 0.87 or more were pooled, dialyzed against Tris buffer (0.01 M, pH 7.4), and rechromatographed on DEAE (3). The effluent fractions from the Tris-DEAE column which had a E₄₀₇: E₂₇₆ ratio of 0.87 or more were dialyzed against distilled water and then chromatographed on a mixed-bed column of Celite, calcium phosphate, and DEAE (35). The catalase which emerged from this column had absorption maxima at 276 and 407 μM and minima at 232 and 312 μM (E₂₇₆: E₄₀₇, 0.96; E₃₃₂: E₂₇₆, 0.27; E₃₂₀: E₂₇₆, 0.72; E₄₀₇: E₃₃₂, 0.28). Price et al. have found that most catalase preparations are contaminated by ferritin (36, 37). It was calculated from their data (37) that the above preparation, hereafter referred to as RLC, was contaminated with no more than 3 per cent ferritin. Electrophoresis of RLC on starch (41), agar (9), and polyacrylamide (39) disclosed only one component, which could be stained with amido black. As a further check of the purity of RLC, immunoelectrophoresis was carried out with the use of rabbit serum antibodies which had been induced against a crude catalase preparation in the following way. Two rabbits were given injections subcutaneously every 3d day of an emulsion (0.5 ml.) of a lyophilized catalase preparation (202 mg, S₆ of Price and Greenfield [36]), Freund's complete adjuvant (10 ml, Difco, Detroit, Michigan) and phenol (30 mg) until five injections had been given. A sixth injection (1 ml) was given after another 3 days. The rabbits were bled by heart puncture 13 days after the last injection, and the antisera were obtained by centrifugation after allowing the clots to form overnight (19). Merthiolate (Eli Lilly and Company, Indianapolis, Indiana) was added to a concentration of 0.01 per cent (19), and the antisera were stored at 4°C. Immuno-electrophoresis of RLC against these antisera (Fig. 1, right) showed a sharp line of precipitation at the point corresponding to the area in which catalase moved in simple electrophoresis. A trace impurity was indicated by a faint line of precipitation, which diffused less rapidly and intercepted the front portion of the catalase arc. The pattern shown in Figure 1, left, was formed by reacting the antiserum with a less pure sample of catalase, referred to as RLCI, which was obtained from one of the tailing fractions emerging from the Tris-DEAE column. RLCI contained, in addition to the contaminant seen in RLC, a slow-moving impurity. The latter impurity was eliminated during the final chromatography. RLC had a Kat. f. (43) of 36,000 and destroyed 1.8 X 10⁵ meq. of potassium permanganate in air.

Antisera to catalase.—The antigen used for the immunochemical experiments and the experiment in which the effect of AAF on the level of total iron in the liver was determined was the following way. Two rabbits were given injections subcutaneously every 3d day of an emulsion (0.5 ml.) of a lyophilized catalase preparation (202 mg, S₆ of Price and Greenfield [36]), Freund's complete adjuvant (10 ml, Difco, Detroit, Michigan) and phenol (30 mg) until five injections had been given. A sixth injection (1 ml) was given after another 3 days. The rabbits were bled by heart puncture 13 days after the last injection, and the antisera were obtained by centrifugation after allowing the clots to form overnight (19). Merthiolate (Eli Lilly and Company, Indianapolis, Indiana) was added to a concentration of 0.01 per cent (19), and the antisera were stored at 4°C. Immuno-electrophoresis of RLC against these antisera (Fig. 1, right) showed a sharp line of precipitation at the point corresponding to the area in which catalase moved in simple electrophoresis. A trace impurity was indicated by a faint line of precipitation, which diffused less rapidly and intercepted the front portion of the catalase arc. The pattern shown in Figure 1, left, was formed by reacting the antiserum with a less pure sample of catalase, referred to as RLCI, which was obtained from one of the tailing fractions emerging from the Tris-DEAE column. RLCI contained, in addition to the contaminant seen in RLC, a slow-moving impurity. The latter impurity was eliminated during the final chromatography. RLC had a Kat. f. (43) of 36,000 and destroyed 1.8 X 10⁵ meq. of potassium permanganate in air.

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1 The following abbreviations are used throughout this paper: AAF, N-(2-fluorenyl)acetamide; 1-hydroxy-AAF, N-(1-hydroxy-2-fluorenyl)acetamide; N-hydroxy-AAF, N-hydroxy-2-fluorenylacetamide; 2-IFQ, 2-imino-1,2-fluorenoquinone.
Preparation of anti-catalase.—RLC (2 ml, 12.5 mg. catalase) was emulsified with Freund's complete adjuvant (10 ml). This suspension was injected subcutaneously (1 ml) into each of three rabbits every 3d day until a total of five doses had been administered. Each rabbit was bled by heart puncture every 2 weeks thereafter until sufficient anti-catalase had been obtained. The antisera were prepared, treated with merthiolate, and stored as described above. The presence of anti-catalase in the serum of each animal was demonstrated by immunoelectrophoresis against RLC. The major catalase component and the trace contaminant, which were observed previously (Fig. 1, right) were also seen in the pattern obtained with the antisera against RLC. Assays revealed that 6 ml of the antisera precipitated the catalase from 1 gm. of rat liver quantitatively. The specificity of the antibodies induced by RLC was shown by subjecting RLCI to immunoelectrophoresis against the antisera. The third component seen previously (Fig. 1, left) in RLCI was absent from the patterns obtained with the antisera against RLC. Similarly, only two lines of precipitation were seen when a soluble rat liver preparation prepared by homogenizing the liver with water was tested by immunoelectrophoresis against the anti-catalase.

Isolation of catalase from the livers of rats given injections of the test compounds by precipitation with anti-catalase.—The test compounds were dissolved in corn oil:acetone (3:1) and injected intraperitoneally. Sixteen hours later, at which time the radioactivity of the soluble fraction had attained a peak value (40), the animal was sacrificed by distilled water (6 ml.). Each aliquot contained the aliquot (10 ml.) which served as a control was treated with two aliquots of the soluble fraction (10 ml.). A third aliquot (13 ml.) was treated with normal rabbit serum (6 ml.). In a further control, water (6 ml.) was added to that portion of the soluble fraction which had been diluted with water instead of normal rabbit serum in the first step of the isolation. The three mixtures stood overnight at 4° C. The supernatant liquids were separated from the precipitates by centrifugation. The rat liver catalase was precipitated by anti-catalase (6 ml.) from one of the aliquots (13 ml.) of the supernatant liquid whose nonspecific antigens had been removed. As a control, the second aliquot (13 ml.) was treated with normal rabbit serum (6 ml.). In a further control, water (6 ml.) was added to that portion of the soluble fraction which had been diluted with water instead of normal rabbit serum in the first step of the isolation. The three mixtures stood overnight at 4° C. to permit the catalase to precipitate, and they were then centrifuged. There was no catalase activity in the supernatant fraction to which anti-catalase had been added. On the other hand, the supernatant fractions of the controls contained, after correction for dilution, all the catalase activity which had been present in the rat liver homogenate. Prior to radioassay the precipitates were washed 3 times with cold 0.9 per cent sodium chloride (9 ml.) and once with acetone (3 ml.). Enzyme assays of the sodium chloride solution indicated that less than 1 per cent of the catalase was removed from the precipitate in this procedure. The bound radioactivity was determined by dissolving the precipitates in warm sodium hydroxide (2.5 ml, 0.2 n) and counting 1-ml samples of the solution by a liquid scintillation technic with the use of thixotropic gel (16). The higher of the counts given by the two control samples was subtracted from the radioactivity of the catalase-anticatalase precipitate. This small correction (10-100 counts/min/gm liver) allowed for the radioactivity of proteins which, although they were not a part of the catalase-anticatalase complex, were precipitated as the result of side reactions.

Preparation of compounds and analytical methods.—AAF, m.p., 196°-198° (38); N-hydroxy-AAF, m.p., 150°-152° (6, 25); and 1-hydroxy-AAF, m.p., 214°-216° (30)4 were prepared by the standard methods used routinely in this laboratory. 3,4-Benzpyrene, m.p., 179°-180°, Lot #B100B-82, was obtained from the Sigma Chemical Company, St. Louis, Missouri. AAF-9-C4 (3.0 mc/mmole) was purchased from tracerlab, Incorporated, Waltham, Massachusetts. The material migrated as a single component on chromatograms developed either with cyclohexane: t-butyl alcohol:acetic acid:water (16:4:2:1) (45) (Rf = 0.72), n-butyl alcohol:ethanol:water (5:1:4) (Rf = 0.90) or benzene: 95 per cent ethanol:water (1:4:1) (10) (Rf = 0.88), and scanned with an Atomic Accessories, Incorporated, model RSC-5A, chromatogram scanner. 1-Hydroxy-AAF-1-C4 (33.6 mc/mmole) was prepared according to a recently published procedure (17).5 The labeled o-amidofluorenol gave a single radioactive peak when it was chromatographed in the cyclohexane system described above (Rf = 0.64) or in sec-butyl alcohol:3 per cent ammonium hydroxide (3:1) (45) (Rf = 0.84), and subsequently scanned. All radioactive samples were counted in duplicate with a standard error of 5 per cent or less (16). Corrections for quenching were made with the use of internal standards. A micro-Kjeldahl method was used for nitrogen determinations (29). Iron was determined spectrophotometrically as the thiocyanate complex (34).

RESULTS

The effect of the oral administration of AAF, N-hydroxy-AAF, and 1-hydroxy AAF on rat liver catalase and arginase.—The action of AAF on catalase and arginase was tested at a dietary level of 0.03 per cent, which is a tumor-producing concentration in the male rat (46). Five pairs of litter-mates were sacrificed at the end of 3, 6, and 9 weeks. Assays of the liver catalase showed a 20-25 per cent drop of the catalase activity, which had become established 6 weeks after feeding the carcinogen, irrespec-

4 The starting material for this compound, 4-(3'-indenyl)-butyronitrile, was prepared by an unpublished procedure of Dr. H. T. Nagasawa for which we are indebted.

5 We thank Mr. S. F. Chang of this laboratory for preparing the labeled compound.
TABLE 1

THE EFFECT OF DIETARY AAF (0.03 PER CENT) ON THE ACTIVITIES OF LIVER CATALASE AND ARGINASE

<table>
<thead>
<tr>
<th>DURATION OF FEEDING (weeks)</th>
<th>mMOLES OF AAF INGESTED</th>
<th>ENZYME ACTIVITIES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Catalase units††</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>3</td>
<td>0.31 ± 0.01</td>
<td>0.74 ± 0.12</td>
</tr>
<tr>
<td>6</td>
<td>0.64 ± 0.04</td>
<td>0.72 ± 0.17</td>
</tr>
<tr>
<td>9</td>
<td>0.90 ± 0.03</td>
<td>0.61 ± 0.06</td>
</tr>
</tbody>
</table>

* A catalase unit is defined as the milliequivalents of NaBO₄ destroyed during the standard assay period of 5 minutes (8).
† Each value represents the average and the average deviation of measurements on five animals.
‡ P indicates the probability that the differences between the control and experimental values might have been due to chance (15).
§ An arginase unit is defined as the jinuleles of urea formed during the standard assay period of 20 minutes (20).

TABLE 2

THE ACTION OF AAF (0.050 PER CENT), N-HYDROXY-AAF (0.054 PER CENT), AND 1-HYDROXY-AAF (0.054 PER CENT) ON LIVER CATALASE AND ARGINASE, LIVER NITROGEN, AND TOTAL BODY WEIGHT

<table>
<thead>
<tr>
<th>DIET FED*</th>
<th>ENZYME ACTIVITIES†</th>
<th>LIVER NITROGEN‡</th>
<th>INCREASE IN BODY WEIGHT§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Catalase units†</td>
<td>Arginase units‡</td>
<td>(mg/gm liver)</td>
</tr>
<tr>
<td></td>
<td>Per mg. liver</td>
<td>Per mg. N</td>
<td>Per mg. liver</td>
</tr>
<tr>
<td>Basal</td>
<td>0.89 ± 0.07</td>
<td>23 ± 2</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Basal + AAF</td>
<td>0.55 ± 0.04</td>
<td>17 ± 1</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Basal + N-hydroxy-AAF</td>
<td>0.58 ± 0.04</td>
<td>18 ± 1</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Basal + 1-hydroxy-AAF</td>
<td>0.90 ± 0.14</td>
<td>22 ± 3</td>
<td>0.12 ± 0.01</td>
</tr>
</tbody>
</table>

* The compounds were pair-fed for 6 weeks as described under "Methods." Each experimental animal consumed 0.54 ± 0.04 mmole of test compound.
† Each value represents the mean and the average deviation of measurements on five animals. P indicates the probability that the differences between control and experimental values were due to chance (15).
‡ A catalase unit is defined as the milliequivalents of sodium perborate destroyed during the standard assay period of 5 minutes (8).
§ An arginase unit is defined as the jinuleles of urea formed during the standard assay period of 20 minutes (20).

Approximately the same depression of the enzyme activity was recorded when the level of carcinogen in the diet was increased to 0.05% (Table 2). This observation is probably due to the closely similar intake of carcinogen (140 and 120 mg., respectively) on either diet. In contrast to catalase, the activity of arginase was not affected significantly.

Since it was one of the objectives of these experiments to obtain a clue whether the transitory o-quinone imine, 2-IFQ, was implicated in any enzyme inhibition in vivo, two metabolites of AAF and probable precursors of 2-IFQ, N-hydroxy-AAF and 1-hydroxy-AAF, were also tested by oral feeding for their action on liver catalase and arginase. N-hydroxy-AAF has been shown to give rise to 1-hydroxy-AAF, presumably through a rearrangement (24, 26). 1-Hydroxy-AAF may be converted to 2-IFQ by deacetylation and subsequent enzymatic oxidation of the ensuing o-aminofluorenol (13, 32). Pair-feeding of equimolar quantities of N-hydroxy-AAF (0.054 per cent) or of AAF (0.050 per cent) for 6 weeks resulted in a 25-35 per cent depression of the catalase activity with either compound, whereas the intake of 1-hydroxy-AAF (0.054 per cent) had no effect. As in the previous experiments with AAF at the 0.03 per cent level, the activity of arginase remained unimpaired following the administration of AAF at a concentration of 0.050 per cent. N-Hydroxy-AAF or 1-hydroxy-AAF at equimolar concentrations likewise failed to affect liver arginase activity (Table 2).
TABLE 3

EFFECT OF THE WITHDRAWAL OF DIETARY AAF (0.050 PER CENT) AND N-HYDROXY-AAF (0.054 PER CENT) ON RAT LIVER CATALASE*

<table>
<thead>
<tr>
<th>Diet fed</th>
<th>Units/mg liver</th>
<th>Enzyme activity†</th>
<th>Units/mg N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At 6 weeks</td>
<td>At 8 weeks</td>
<td>At 10 weeks</td>
</tr>
<tr>
<td>Basal</td>
<td>0.77 ± 0.17</td>
<td>0.77 ± 0.11</td>
<td>0.89 ± 0.06</td>
</tr>
<tr>
<td>Basal + AAF</td>
<td>0.65 ± 0.06 (P &lt; 0.10)</td>
<td>0.83 ± 0.05</td>
<td>0.89 ± 0.07</td>
</tr>
<tr>
<td>Basal + N-hydroxy-AAF</td>
<td>0.58 ± 0.08 (P &lt; 0.05)</td>
<td>0.82 ± 0.06</td>
<td>0.91 ± 0.12</td>
</tr>
</tbody>
</table>

* The rats were pair-fed as described in the text, and each experimental animal consumed 0.52 mmole of test compound during 6 weeks. After 6 weeks the experimental animals were transferred to the basal diet.

† A catalase unit is defined as the milliequivalents of sodium perborate destroyed during the standard assay period of 5 minutes (8).

‡ Each value represents the mean and the average deviation of measurements obtained on five animals. P indicates the probability that the difference between the experimental and control values were due to chance (15).

TABLE 4

IMMUNOCHEMICAL EXPERIMENTS ON THE BINDING IN VIVO OF AAF AND 1-HYDROXY-AAF TO RAT LIVER CATALASE

<table>
<thead>
<tr>
<th>Compound injected</th>
<th>Dose injected (µmoles/100 gm body weight)*</th>
<th>Pretreatment</th>
<th>Total counts/min/gm liver</th>
<th>Catalase-bound counts/min/gm liver†</th>
<th>Catalase-bound C4 Catalase†</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAF-9-C14‡</td>
<td>5.8</td>
<td>None</td>
<td>1.1 × 10^4</td>
<td>270</td>
<td>0.02</td>
</tr>
<tr>
<td>AAF-9-C14‡</td>
<td>13.0</td>
<td>None</td>
<td>2.0 × 10^4</td>
<td>320</td>
<td>0.03</td>
</tr>
<tr>
<td>AAF-9-C14</td>
<td>9.9</td>
<td>AAF-fed §</td>
<td>2.3 × 10^4</td>
<td>50</td>
<td>0.006</td>
</tr>
<tr>
<td>AAF-9-C14</td>
<td>12.0</td>
<td>3,4-Benzpyrene-injected¶</td>
<td>1.4 × 10^4</td>
<td>300</td>
<td>0.03</td>
</tr>
<tr>
<td>1-hydroxy-AAF-1-C14¶</td>
<td>5.4</td>
<td>None</td>
<td>8.4 × 10^4</td>
<td>20</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* The animals weighed from 140 to 280 gm.

† The amount of catalase in each liver was calculated from an assay of the enzymatic activity of the liver homogenate (20) and the specific activity of RLC.

‡ The specific activity of AAF-9-C14 was 2.2 × 10^4 counts/min/µmole.

§ The specific activity of 1-hydroxy-AAF-1-C14 was 2.5 × 10^4 counts/min/µmole.

¶ This animal received AAF (0.05 per cent) in the diet for 6 weeks. It was transferred to the basal diet 1 week prior to injection of AAF-9-C14.

To decide whether the depression of the catalase activity induced by the administration of AAF or N-hydroxy-AAF was a temporary or a permanent alteration, each of these compounds was pair-fed to three groups of rats for 6 weeks. At the end of 6 weeks one group was sacrificed, and the liver catalase activity was measured. The remaining groups were transferred to the basal diet and the measurements of the catalase activity were repeated 2 and 4 weeks after withdrawal of the compounds. The catalase assays (Table 3) showed a prompt reversal of the catalase inhibition. The return of the activities to the level of the controls upon withdrawal of AAF or N-hydroxy-AAF indicated clearly that the partial inactivation of catalase was only temporary and cannot be regarded as a true deletion.

Experiments on the binding in vivo of AAF-9-C14 and 1-hydroxy-AAF-1-C14 to rat liver catalase.—The lowering of the activity of liver catalase after the feeding of AAF or N-hydroxy-AAF and the dependence of the decrease in activity on the continual presence of the compounds in the diet raised the possibility that a protein-carcinogen complex, formed in vivo, might account for the lowered catalase activity. This idea was tested by isolating the liver catalase by an immunochemical method after the injection of AAF-9-C14 into rats. In an attempt to increase the chances for adduct formation in vivo, three different sets of conditions were selected for the administration of AAF-9-C14.

In the first case, two different quantities (5.8 and 13 µmoles/100 gm of body weight, respectively) of AAF-9-C14 were injected without pretreatment of the animals. As might be expected, the amounts of C4 in the liver increased as a function of the administered dose (Table 4, column 4). In the second case, a rat was fed AAF at the 0.05 per cent level for 6 weeks and then transferred to the basal diet for 1 week prior to the administration of AAF-9-C14.

In the third case, the amount of C4 in the liver increased as a function of the administered dose (Table 4, column 4). In the second case, a rat was fed AAF at the 0.05 per cent level for 6 weeks and then transferred to the basal diet for 1 week prior to the administration of AAF-9-C14. This regimen induces the enzyme which N-hydroxylates AAF (6) and thus favors the formation of N-hydroxy-AAF-C14 from AAF-C14. In the third
instance a rat was pretreated with 3,4-benzpyrene prior to the injection of AAF-9-C\textsubscript{14} to promote ring hydroxylation of the fluorene moiety (4, 5) and thus to increase the formation of 1-hydroxy-AAF-\textsubscript{C\textsubscript{14}}. Although 1-hydroxy-AAF which had no effect is not (31). The number of fluorene derivatives which have thus far been examined for their action on rat or mouse liver catalase was carcinogenic. It is of interest that two of the three compounds which inhibited rat liver catalase after a single application of AAF (0.015 mmole) to the skin. The greater inactivation of catalase in the present experiments is possibly due to the greater amounts of compound ingested over a longer period of time as well as to the route of administration. Adams and Roe noted that nineteen of the 21 compounds which inhibited mouse liver catalase were carcinogens. It is of interest that two of the three compounds which inhibited rat liver catalase in the present experiments, AAF and \(N\)-hydroxy-AAF, are carcinogens, whereas 1-hydroxy-AAF which had no effect is not (31). The number of fluorene derivatives which have thus far been examined for their action on rat or mouse liver catalase is not sufficient to permit a correlation between carcinogenicity and catalase inhibition. Nevertheless, there is a possibility that the depression of catalase might be exploited for a test of the carcinogenic potential of a compound. It would seem that skin painting is preferable to oral feeding, because the latter procedure requires an elaborate feeding technique for meaningful results.

There are several mechanisms which may account for the depression of catalase \textit{in vivo} after the administration of AAF. One of these, the irreversible binding of 2-IFQ to catalase, appears highly improbable because of the negligible amounts of radioactivity in the isolated catalase. It seems unlikely that an adduct had been formed but was disrupted by the mild conditions of the isolation procedure, which involved only a one-step precipitation of the catalase in the soluble fraction by the anti-catalase. It should be noted that the catalase-2-IFQ adduct had survived the enzymatic activity in the liver homogenates after treatment with anti-catalase, if a catalase adduct which was unreactive to anti-catalase had been present in major amounts. The fact that no activity was detected argues against the existence of a modified catalase which was not precipitable by anti-catalase.
far more drastic conditions used previously for its purification (20). In view of the fact that the inhibition of catalase by AAF in vivo appears to be caused by a different mechanism than the inactivation in vitro (20), it would be of interest to learn what mechanism underlies the profound inhibition in vivo of catalase by 3-amino-1,2,4-triazole (14). Such experiments would be especially relevant, because 3-amino-1,2,4-triazole forms an irreversible complex with the enzyme in model systems (22). A second possibility, which would account for the lowering of catalase activity in vivo, is the formation of a dissociable catalase inhibitor complex between AAF and catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase. This mode of action does not seem plausible from a rough estimate of the probable concentration of catalase.


The Action of the Carcinogen N-(2-Fluorenyl)acetamide on Rat Liver Catalase and Arginase *in Vivo*

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