On the Mechanism of Protein Binding of N-2-Fluorenylacetamide. The Deacetylation of N-(1-Hydroxy-2-fluorenyl)acetamide and the Effect of Ethionine

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SUMMARY

Homogenates of six rat tissues metabolized the o-amidofluorenol, N-(1-hydroxy-2-fluorenyl)acetamide, as judged by the disappearance of the phenolic hydroxyl group of the compound from the incubation systems. By the same criterion, N-(7-hydroxy-2-fluorenyl)acetamide was resistant to metabolic attack.

The deacylation of N-(1-hydroxy-2-fluorenyl)acetamide, N-(7-hydroxy-2-fluorenyl)acetamide, N-2-fluorenylbutyramide, and N-2-fluorenylacetamide by twelve rat tissues was examined with the use of spectrophotometric technics and radioactive tracer methods. Only N-(1-hydroxy-2-fluorenyl)acetamide was deacetylated by all tissues.

The action of 0.1 M potassium fluoride upon deacetylation of N-(1-hydroxy-2-fluorenyl)acetamide-1-C\textsubscript{14} and upon protein labeling by the radioactivity of N-2-fluorenylacetamide-9-C\textsubscript{14} was examined in rat liver homogenates; 0.1 M potassium fluoride inhibited deacetylation as well as protein labeling. It was confirmed in separate experiments that fluoride under these conditions enhanced hydroxylation as previously described.

The data support a mechanism of protein binding of the carcinogen previously suggested on the basis of in \textit{vitro} evidence. This mechanism involves o-hydroxylation of N-2-fluorenylacetamide followed by deacetylation and subsequent oxidation of the resulting o-aminophenol to the o-quinoneimine which in turn may combine with protein.

The role of the inhibition of protein synthesis by Dl-ethionine upon the binding of N-2-fluorenylacetamide-9-C\textsubscript{14} was tested. No effect of this antimetabolite upon binding was observed.

Previous experiments from our laboratory indicated that the o-aminophenol, 2-amino-1-fluorenol (1-OH-FA), was oxidized \textit{in vitro} by cytochrome c-cytochrome oxidase to yield the o-quinoneimine, 1,2-fluorenoquinone-2-imine, and it was concluded on the basis of spectrophotometric data that this oxidation product combined with bovine serum albumin (10). Theoretically, 1-OH-FA may arise either from the hydroxylation of \textit{2-fluorenamine} or from the deacetylation of N-1-hydroxy-2-fluorenyl acetamide (1-OH-FAA), which is one of the products of the hydroxylation of N-2-fluorenylacetamide (FAA) in the rat (1, 15, 17). However, no published evidence was available previously that 1-OH-FAA, which was neither oxidized by cytochrome c-cytochrome oxidase (10) nor bound

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to protein (8), was deacetylated by the tissues. Without such experimental evidence, it may be questioned whether 1-OH-FA can be regarded as an intermediate in the metabolism of FAA. The present experiments show that 1-OH-FAA is readily deacetylated by a large number of rat tissues. Therefore the data support the view that 1-OH-FA is a product of the metabolism of FAA and that protein binding may indeed be the result of the oxidation of this o-aminofluorenol, as had been suggested previously (10).

Experiments with the model o-quinonemide, 1,2-fluorenoquinone-2-acetamide, showed that the ε-amino group of lysine added rapidly to the quinonoid system (8). Since this addition reaction is nonenzymatic, it appeared probable that active protein synthesis, which is necessary for protein binding of 3'-methyl-4-dimethylaminoazobenzene (9) and related azo dyes (4), is not a requirement for protein binding of FAA. To test this point, the binding of the radioactivity of FAA-9-C\textsuperscript{14} to liver proteins was determined in ethionine-treated rats at levels of the antimetabolite which interfered significantly with azo dye binding (3).

**MATERIALS AND METHODS**

**Animals and diets.—** Male and female albino rats weighing 180–220 gm. were purchased from the Holtzman Rat Company, Madison, Wisconsin. Male animals were used in all experiments, except where otherwise stated. The animals were given commercial food pellets and water ad libitum.

**Substrates.—** FAA, m.p. 196°–198°(14); N-(7-hydroxy-2-fluorenyl)acetamide, m.p. 230°–231° (17); and N-2-fluorenylbutyramide, m.p. 176°–179° (9), were synthesized by published methods. The radioactive substrates, FAA-9-C\textsuperscript{14} (8), with a specific radioactivity of 3.2 \times 10^6 counts/min/mg, and N-(1-hydroxy-2-fluorenyl)acetamide-1-C\textsuperscript{14}(8), with a specific radioactivity of 1.4 \times 10^6 counts/min/mg, were also prepared by the previously published syntheses.

**Preparation of tissues and incubation media.—** The animals were anesthetized with ether and the tissues removed rapidly, cooled on ice, rinsed with homogenizing medium, minced, and homogenized in a Potter-Elvehjem type homogenizer with a solution of 1.1 per cent potassium chloride which was 0.01 M with respect to phosphate buffer, pH 7.4. Four ml. of homogenizing medium were used for each gram of wet tissue. The homogenates were centrifuged in a Spinco-refrigerated ultracentrifuge at 10,000 \times g (R\textsubscript{av}) for 15 minutes, and the supernatant liquid, which consisted of microsomes and the soluble fraction, was used for the experiments summarized in Table 3.

The standard incubation medium for hydroxylation, which contained glucose-6-phosphate, triphosphopyridine nucleotide, nicotinamide, potassium chloride, phosphate buffer, pH 7.4, and the substrate (in 0.1 ml. of methyl cellosolve), as well as the conditions of incubation, have been described (15). Incubations for measurement of deacetylation (Table 2) were carried out in 0.03 M phosphate buffer, pH 8.0, at 37° in air, with equal quantities of tissue for each of the substrates examined. Since none of the cofactors necessary for the formation of reduced triphosphopyridine nucleotide was added, decylation was considered the predominant reaction under these conditions.

For measurement of protein binding in vivo after administration of pL-ethionine, 4.7 mg. of FAA-9-C\textsuperscript{14} having a specific radioactivity of 1.88 \times 10^6 counts/min/mg was dissolved in 0.5 ml. of corn oil, and this solution was injected intraperitoneally.

**Spectrophotometric and radioactivity measurements.—** The methods for extraction and assay of the free bases, liberated from FAA, N-2-fluorenylbutyramide, and N-(7-hydroxy-2-fluorenyl)acetamide, by the "R" salt test have been described previously (15). The measurements of the recovery of the compounds containing the phenolic hydroxyl group, which are presented in Table 1, were performed with the Folin-Ciocalteu phenol reagent (2), as described previously (15).

The deacetylation of 1-OH-FAA was estimated by determining the quantity of labeled acetic acid which was liberated from N-(1-hydroxy-2-fluorenyl)acetamide-1-C\textsuperscript{14}. This is the most reliable method for measuring the extent of this reaction because of the disappearance of 1-OH-FA from the incubation system (15). The radioactive substrate was added to the incubation mixtures in 0.1 ml. of methyl cellosolve. After incubation for 1 hour, 165 mg. of sodium acetate trihydrate was added to each flask, and the reaction was terminated by heating 3 minutes in a boiling water bath. Control flasks with contents identical to those of the respective experimental flasks were heated prior to the addition of substrate and then incubated. The contents of the flasks were centrifuged, and the aqueous layers were extracted twice with 3 volumes of ether to remove unreacted substrate. The aqueous phases were then frozen until they were further processed as follows. The extracted aqueous layers were thawed, acidified with 3 ml. of concentrated sulfuric acid, and then subjected to steam distillation. The distillate was titrated with...
a 0.25 N sodium hydroxide solution and then evaporated to dryness under reduced pressure. The residue was taken up in hot 95 per cent ethanol and filtered. The filtrate was diluted with 3 volumes of ether and cooled in ice. The precipitated sodium acetate was collected, dried to a constant weight at 125 °C, and the radioactivity of an appropriate aliquot was determined. Radioactive proteins and sodium acetate-1-C\textsuperscript{14} were subjected to wet combustion, and the resulting barium carbonate precipitates were collected and counted according to the micro method (12).

**RESULTS**

Previous experiments indicated that rat liver microsomes plus the soluble fraction metabolized 39 per cent of added 1-OH-FAA, as judged by the disappearance of the phenolic hydroxyl group (8, 15). On the other hand, N-(7-hydroxy-2-fluorenyl)acetamide was recovered quantitatively from the incubation systems by this method of assay (15). In the present study, the disappearance of the two substrates from the homogenates of liver, kidney, spleen, brain, and lung was measured (Table 1). The instability of 1-OH-FAA, previously noted with liver cell fractions, was also observed with liver homogenates as well as with homogenates of other tissues. In contrast, N-(7-hydroxy-2-fluorenyl)acetamide proved to be stable under these conditions.

The disappearance of 1-OH-FAA could be interpreted to result from the deacetylation of the compound and the subsequent oxidation of 1-OH-FAA. Accordingly, the capacity of twelve tissues to deacetylate N-(1-hydroxy-2-fluorenyl) acetamide-1-C\textsuperscript{14}, N-(7-hydroxy-2-fluorenyl)acetamide, FAA, and N-(2-fluorenyl)acetamide-1-C\textsuperscript{14}, N-(7-hydroxy-2-fluorenyl)acetamide, FAA, and N-1-fluorenylbutyramide was tested with the results shown in Table 2. All tissues deacylated the \(p\)-amido-...
dofluorenol proved to be resistant to deacetylation by many of the tissues under study. FAA and N-2-fluorenylbutyramide likewise were not deacetylated by all the tissues. Thus, N-(7-hydroxy-2-fluorenyl)acetamide was deacylated by only three of ten tissues, N-2-fluorenylbutyramide by only six of twelve tissues, and FAA by only seven of eleven tissues. The inertness of N-(7-hydroxy-2-fluorenyl)acetamide in homogenates, which was noted above, may thus be attributed to the fact that the compound was in most instances not deacetylated. Moreover, it is questionable whether 2-amino-7-fluorenol, even when formed, is further oxidized. The labeling of liver proteins by administered N-(7-hydroxy-2-fluorenyl-9-C14)acetamide (20), which seemingly contradicts its apparent inertness in homogenates, will be discussed below. It will be observed that intestinal homogenates deacylated FAA as well as N-2-fluorenylbutyramide. Therefore, the present results qualitatively confirmed the previous data on the hydrolytic cleavage of these two substrates by intestinal strips (9).

The comparatively facile deacetylation of 1-OH-FAA raised the question whether o-hydroxylation increased the susceptibility of aromatic N-acylamides toward enzymatic deacetylation. This point is of some interest in connection with the observation that the radioactivity of N-(2-fluorenyl-9-C14)benzamide was bound to liver slice proteins to the same extent as that of FAA-9-C14, although deacylation of the former compound to 2-fluorenamine was very much smaller (6). However, the possibility has not been ruled out that N-2-fluorenylbenzamide was o-hydroxylated and subsequently deacylated to 1-OH-FA. This reaction requires further study.

If the deacetylation of 1-OH-FAA to 1-OH-FA and the subsequent oxidation of the o-amino-phenol is, in fact, the mechanism which underlies protein binding of FAA, inhibition of either of the two reactions should have an inhibitory effect on protein labeling by FAA-9-C14. It has recently been found in our laboratory that the fluoride ion at 0.1 m concentration inhibited the deacetylation of FAA but stimulated the hydroxylation of the compound by rat liver microsomes and the soluble fraction. Consequently, 0.1 m fluoride was used in these experiments to demonstrate the dependence of protein labeling on the deacetylation of 1-OH-FAA. The data of Table 3 show that binding of the radioactivity of FAA-9-C14 to microsomal and 5 Exposures of liver slices incubated with FAA-9-C14 to 0.005 m sodium cyanide inhibited protein binding of carbon-14 approximately 75 per cent (6). Poisoning of liver slices, incubated with N-(1-hydroxy-2-fluorenyl-1-C14) acetamide, by 0.005 m sodium cyanide likewise resulted in a marked inhibition of protein labeling of the individual cell fractions (H. T. Nagasawa and H. R. Gutmann, unpublished observations). These data indicated that the inhibition of cellular oxidations by cyanide depressed protein binding. 0.005 m sodium cyanide had no inhibitory effect on rat liver deacylase (15).
soluble proteins of rat liver was greatly diminished by potassium fluoride at a concentration which reduced deacetylation of 1-OH-FAA-1-C14. Recently, Cramer, Miller, and Miller have reported that 0.1 M fluoride depressed, rather than stimulated, the hydroxylation of FAA in vitro (1). Since the interpretation of the data of Table 3 depended upon the fact that fluoride inhibited deacetylation, but not hydroxylation, it became necessary to re-examine the action of fluoride upon hydroxylation of FAA in the light of the data of Cramer, Miller, and Miller (1). It should be noted that the experimental conditions employed by these investigators differed from ours with respect to homogenate preparation and composition of the incubation medium. Furthermore, they estimated hydroxylation indirectly from the disappearance of the 228-mu band of FAA rather than from direct measurements of the formation of hydroxylated products (15). To clarify the discrepancy referred to above, comparative measurements were made in which the action of fluoride upon hydroxylation of FAA was tested in the incubation system of Cramer, Miller, and Miller (1), as well as under the conditions previously described (15). The data of Table 4 show that under optimal conditions approximately the same amounts of hydroxylated products were formed in both systems. Addition of 0.1 M fluoride to the system of Cramer, Miller, and Miller resulted indeed in a slight inhibition of hydroxylation, while the stimulation of hydroxylation previously described and observed repeatedly (15) was again noted under our conditions. The absence of the stimulation of hydroxylation by fluoride in the system of Cramer, Miller, and Miller may have conceivably been due to the fact that under their conditions hydroxylation proceeded at a maximal rate without fluoride, no further increase in rate being attainable. Since under the less favorable conditions of our incubation system hydroxylation proceeded only at a suboptimal rate in the absence of fluoride, any stimulating effects would manifest themselves in an increase of the rate of hydroxylation. The inhibition of protein labeling observed under conditions which enhanced hydroxylation of FAA, but decreased deacetylation of 1-OH-FAA, supports the view that deacetylation of the latter compound to 1-OH-FAA is an essential step in the mechanism of protein binding of the carcinogen.

The binding of the radioactivity of N-(7-hydroxy-2-fluorenyl-9-C14) acetamide to proteins of rat liver, which has recently been described by Weisburger, Weisburger, and Morris (20) does not invalidate the mechanism of protein binding delineated above. As has been pointed out by these authors, the structure of the bound metabolite of N-(7-hydroxy-2-fluorenyl)acetamide is unknown, but they have suggested that the compound may be further hydroxylated in the 1 or 3 position (or both) of the fluorene nucleus (20). If this is the case, the resulting \sigma-hydroxylated amido-fluorenediol might be expected to be deacetylated as easily as 1-OH-FAA, and the deacetylated product would then be in a readily oxidizable state. Since unfortified homogenates, which were used in the

### Table 4

<table>
<thead>
<tr>
<th>Incubation system</th>
<th>Concentration of potassium fluoride (M)</th>
<th>Hydroxylated product formed (ug.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>41</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>45</td>
</tr>
</tbody>
</table>

*The incubations were done at 37° C. for 30 minutes with air as the gas phase. The homogenates were prepared from the pooled livers of two 300-gm. male rats. The homogenate for incubation system A was prepared from 5 gm. of liver in 50 ml. of 0.25 M sucrose (1). The homogenate for incubation system B was prepared from 5 gm. of liver in 55 ml. of 1.1 per cent potassium chloride-0.01 M phosphate buffer, pH 7.4. The homogenates were centrifuged at 10,000 X g (Hr.) for 30 minutes. The supernatant liquid from 0.5 gm. of liver was used as the enzyme source for each of the incubation systems. The complete incubation system A had the following composition: 5 ml. sucrose homogenate, 2 mg. each of TPN and DPN, 12 mg. ATP, 146 mg. nicotinamide, 2 ml. M potassium chloride, 0.1 M phosphate buffer, pH 7.8, distilled water to volume of 30 ml., and sufficient potassium fluoride to give the final concentrations indicated in the table. The substrate, 500 mg. FAA, was added in 0.1 ml. of methanol. Incubation system B had the following composition: 2.5 ml. KCl homogenate, 1.5 mg. TPN, 280 mg. nicotinamide, 0.75 ml. M potassium chloride, 7.5 ml. 0.1 M phosphate buffer, pH 7.4, distilled water to a total volume of 30 ml., and the concentrations of potassium fluoride listed in the table. The substrate, 500 ug. FAA, was added in 0.1 ml. of methyl cellosolve. The reaction was stopped by heating for 3 minutes on a boiling water bath. The extraction and assay of the hydroxylated products were carried out as described (15).
experiments of Table 2, exhibited only a very low level of hydroxylating activity (15), further o-hydroxylation, which we believe to facilitate subsequent deacetylation, was very probably excluded.

To test whether the formation of protein or of peptide bonds is a requirement for protein binding of the carcinogen, the experiments summarized in Table 5 were carried out. The intraperitoneal administration of FAA-9-C14 was preceded by the injection of DL-ethionine. The amounts of DL-ethionine which were employed (50–250 mg.) have been found to decrease azo dye binding very significantly, the inhibitions ranging from 50 to 85 per cent (3). However, it will be seen from Table 5 that DL-ethionine had no measurable effect on the labeling of liver proteins which had been carefully washed and extracted with organic solvents.

It has been reported by Cramer, Miller, and Miller that the administration of ethionine had no effect upon the enzymes of rat liver which hydroxylate FAA (1). We have been able to confirm this result. Thus, a portion of the liver from the rat which had been given injections of 100 mg. of ethionine (Table 4) was assayed for hydroxylation of FAA, a value of 0.52 μmoles/hr/gm of wet tissue being obtained. This value was within the range observed previously for hydroxylation of FAA (15). Thus, there would appear to be no short-term effect of ethionine upon the activity of FAA hydroxylase. Since the hydroxylase activity remained unaffected after ethionine administration, the mechanism of protein binding proposed above appears valid also in the ethionine-treated animal.

## DISCUSSION

Previous studies in vitro have focused attention on 1-OH-FA as a substrate for the further oxidation to the α-quinonimine, 1,2-fluorenoquinone-2-imine, by cytochrome c and mitochondria or by cytochrome c-cytochrome oxidase. This oxidation product may in turn combine with protein (10, 11). These studies also disclosed that the α-amido-fluorenol, 1-OH-FAA, was not oxidized by cytochrome c-cytochrome oxidase (10). The present experiments establish the wide distribution of a deacetylase in rat tissues which is capable of deacetylati

### Table 5

**Effect of DL-Ethionine on In Vitro Protein Binding of FAA-9-C14 by Rat Liver**

<table>
<thead>
<tr>
<th>Time of injection</th>
<th>Time of sacrifice</th>
<th>Total amount of FAA-9-C14 injected</th>
<th>Protein binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Of ethionine (hr.)</td>
<td>Of FAA-9-C14 (hr.)</td>
<td>Control (mg.)</td>
<td>Experimental (mg.)</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>0, 2.5†</td>
<td>4</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

* DL-Ethionine was dissolved in 0.9 per cent sodium chloride in the quantities indicated and injected intraperitoneally. Each animal received 4.7 mg. of FAA-9-C14 dissolved in corn oil. The livers were removed, rinsed, and portions were homogenized in 2–3 volumes of water. Five volumes of cold acetone were added. The precipitated proteins were washed 4 times with acetone and then extracted with 5 per cent trichloro-acetic acid, ethanol, ethanol-ether (3:1), and ether (18). Samples of the dried protein were then subjected to combustion and assayed for radioactivity as described under “Materials and Methods.”† In this experiment, 250 mg. of DL-ethionine was injected in two equal, divided doses.

The oxidation of 1-OH-FAA has been carried out in model enzyme studies by cytochrome c-cytochrome oxidase or by cytochrome c and mitochondria, and it has been possible to demonstrate binding of the oxidation product to bovine serum albumin in this enzymatic system.
in the case of FAA, 1-OH-FA appears implicated in the binding reaction (10, 11) and in the absence of mitochondria, would strongly support such a view (7). The present data (Table 8) as well as previous results (15) also indicate that binding of the radioactivity of FAA-9-C¹⁴ to liver proteins may occur in hydroxylating systems without mitochondria, although binding may be enhanced by the addition of mitochondria (15). It remains to be established whether protein labeling in hydroxylating systems and in the absence of mitochondria proceeds through an oxidative reaction.

If the mechanism of protein binding of the carcinogen outlined above is to hold in liver as well as elsewhere, it would appear necessary that either 1-OH-FA or 1-OH-FAA be transported from the liver to the other tissues, since under normal circumstances hydroxylation is restricted to the liver (1, 15). 1,2-Fluorenoquinone-2-imine may be excluded on account of its very great instability, which thus far has made chemical isolation of the compound impossible (11). Since 1-OH-FA also displays a high degree of instability as shown above, it likewise would not appear suited for transport, although unpublished evidence cited by the Weisburgers suggests that unspecified amino-α-fluorens and their glucuronides are found in trace quantities in rat urine (16). A decision whether 1-OH-FA is, in fact, carried to the tissues in appreciable quantities must await actual isolation of the compound from blood. It is probable, however, that 1-OH-FAA is carried to the tissues, since the free amidofluorene as well as the respective glucuronide has been isolated from urine (19). The action of tissue deacylase on 1-OH-FAA may then release the substrate, 1-OH-FA, which in turn may be further metabolized as suggested.

The failure of ethionine to inhibit binding of FAA-9-C¹⁴ to liver proteins in vivo contrasts markedly with the inhibitory effect of this antinutrient on protein binding of azo dyes. This suggests that the mechanisms by which carcinogen aromatic amines and azo dyes interact with cellular proteins are not identical. This view is also supported by the data of Gelboin, Miller, and Miller, who have shown that the orthohydroxy-α-aminoazodyes, 3-hydroxy-4-α-aminoazobenzenes and 3-hydroxy-4-monomethylaminoazobenzenes, are not bound by rat liver preparations (4). However, in the case of FAA, 1-OH-FA appears implicated in the binding reaction (10, 11).

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