The Metabolism of the Carcinogen N-(2-Fluorenyl)acetamide
by Liver Cell Fractions*

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In previous communications from this laboratory we have reported that the hydroxylation of the carcinogen N-(2-fluorenyl)acetamide can be readily demonstrated in slices (1, 2) and in homogenates fortified with diphosphopyridine nucleotide and succinate (3), but not in unfortified homogenates (1, 3). It was also found that the binding of the radioactivity of N-(2-fluorenyl)acetamide-9-C*4 to cellular proteins occurred only at a very low level in unfortified homogenates, but was increased several-fold when diphosphopyridine nucleotide and succinate were added (2, 3). These data suggested a relation between hydroxylation of N-(2-fluorenyl)acetamide and protein binding. Protein binding is currently considered to play a part in the action of chemical carcinogens and may be required for the induction of neoplasms by chemical agents (4). Since hydroxylation of N-(2-fluorenyl)acetamide seemed implicated in this reaction, a further characterization of the enzymatic system(s) concerned with the hydroxylation and concurrent deacetylation of N-(2-fluorenyl)acetamide appeared desirable.

The present report deals with the optimal conditions for the hydroxylation of N-(2-fluorenyl)acetamide and the role of triphosphopyridine nucleotide or diphosphopyridine nucleotide in this reaction. The intracellular distribution of the hydroxylation and deacetylating systems has been defined, and the effects of a number of enzyme inhibitors upon the hydroxylation and deacetylating activities have been examined. The abilities of different species to hydroxylate the carcinogen have been compared. Finally, several hydroxylated metabolites of N-(2-fluorenyl)acetamide have been identified by paper chromatography after incubation of liver cell fractions with the compound.

EXPERIMENTAL

Animals—All animals were 3 to 6 months old and, with the exception of the Syrian hamsters and the bantam rooster, were albinos. The rats, weighing approximately 200 gm., were purchased from the Holtzman Rat Company, Madison, Wisconsin. All other animals were obtained locally.

Preparation of Cell Fractions—The animals were killed by a blow to the head. The livers were removed rapidly, cooled in ice, minced, and homogenized in a Potter Elvehjem type homogenizer with a solution of 1.1 per cent KCl which was 0.001 M with respect to ethylenediaminetetraacetate and 0.01 M with respect to phosphate buffer, pH 7.4. 3 to 4 ml. of homogenizing medium were used for each gram of wet tissue. The homogenates were centrifuged in an International refrigerated centrifuge (model PR-2) at 800 x g (Rm) for 10 minutes to remove debris and cell nuclei. For the preparation of mitochondria, the supernatant liquid was centrifuged at 10,000 x g (Rm) for 15 minutes in a Spinco refrigerated ultracentrifuge. The mitochondria were washed twice with homogenizing medium. 5 ml. of wash liquid were used for the mitochondria from 1 gm. of liver. For the preparation of the microsomes and the soluble fraction, the supernatant liquid, from which the mitochondria had been separated, was centrifuged at 80,000 x g (Rm) for 45 minutes. After removal of the supernatant liquid (soluble fraction) the precipitate (microsomes) was washed as described for the mitochondria, and recentrifuged at 80,000 x g for 60 minutes.

Preparation of Substrates and Cofactors—FA, m.p. 121-128° (5), AFA, m.p. 196-198° (6), 7-OH-AFA, m.p. 230-231° (7), 5-OH-AFA, m.p. 215-217° (8), and 1-OH-AFA, m.p. 210-212° (9) were prepared essentially by published methods. For the acetylation of the last two compounds, the conventional procedure (10) proved to be more satisfactory than the method employed by Weisburger and Weisburger (9). 7-OH-FA, 5-OH-FA, and 1-OH-FA were used in the form of their respective hydrochlorides. The hydrochlorides were purified by recrystallization from dilute hydrochloric acid (activated charcoal added) or according to Fieser (11). 7-F-AFA, m.p. 201-203°, was prepared according to Miller et al. (12). The intermediate, 2-nitro-7-fluorofluorene, was purified by chromatography on acid-washed, activated alumina with benzene or benzene-chloroform (1:1) as eluent instead of by repeated crystallizations. AFA-9-C*4 was prepared by the previously published synthesis (2). Glucose-6-P (Sigma Chemical Company) and isocitrate (California Foundation for Biochemical Research) were used as the di- and trisodium salts, respectively. Sodium succinate was prepared by neutralizing a solution of succinic acid to pH 7.4 (glass electrode) with dilute sodium hydroxide. DPN, DPNH, TPN, and TPNH of 90 to 95 per cent purity and a glucose-6-P dehydrogenase preparation from yeast were obtained from Sigma Chemical Company. In a number of experiments TPNH was pre-

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1 The abbreviations used are: FA, 2-fluorenamine (the parent compound); AFA, N-(2-fluorenyl)acetamide (the N-acetyl derivative). The hydroxylated or fluorinated derivatives of AFA or of FA are abbreviated by indicating the position of the hydroxyl group or of the fluorine atom in the fluorene nucleus: 1-OH-AFA, N-(1-hydroxy-2-fluorenyl)acetamide; 3-OH-AFA, N-(3-hydroxy-2-fluorenyl)acetamide; 5-OH-AFA, N-(5-hydroxy-2-fluorenyl)acetamide; 7-OH-AFA, N-(7-hydroxy-2-fluorenyl)acetamide; 1-OH-FA, 2-amino-1-fluorenol; 5-OH-FA, 2-amino-5-fluorenol; 7-OH-FA, 2-amino-7-fluorenol; 7-F-AFA, N-(7-fluoro-2-fluorenyl)-acetamide.
Incubation mixtures heated for
3 minutes; cooled; centrifuged.

Fraction 1: protein precipitate; extracted
2 times with 5 ml. of 50% ethanol.

Fraction 3: protein precipitate; discarded.

Fraction 4: ethanol washings.

Fraction 2: supernatant

Fraction 5: extracted 2 times with 2 volumes of ether.

Fraction 6: ether extract; extracted with 10 ml. of 0.25 M NaOH.

Fraction 7: aqueous phase; discarded.

Fraction 8: 0.25 M NaOH containing fluorenols; quantitative Folin test on aliquot.

Fraction 9: ether extract to dryness; residue containing fluorenamines and starting compound taken up in 50 ml. of 50% ethanol; R salt test on aliquot.

FIG. 1. Extraction and partition of metabolites of AFA after incubation with tissue preparations.

pared enzymatically by a slight modification of the method of Evans and Nason (13) with the use of glucose-6-P and glucose-6-P dehydrogenase in place of isocitrate and isocitric dehydrogenase.

The standard incubation medium consisted of 15 μmoles of glucose-6-P, 0.3 μmole of TPN, 360 μmoles of nicotinamide, 150 μmoles of potassium chloride, 150 μmoles of phosphate buffer, pH 7.4, and 2.2 μmoles of the respective fluorene derivative (added in 0.1 ml. of methyl Cellosolve). To this medium was added the appropriate cell fraction or fractions equivalent to 500 mg. of wet liver to give a total volume of 6 ml. The mixtures were placed into open flasks and incubated for 30 minutes at 37° with constant shaking. After incubation, the mixtures were transferred to 15-ml. centrifuge tubes and heated for 3 minutes in a boiling water bath, cooled, centrifuged, and processed according to the scheme shown in Fig. 1.

Spectrophotometric and Radioactivity Measurements—Free amine and remaining starting compound were determined in an aliquot of the ethanol solution by the modified R salt test which permitted measurement of 0.01 μmole of FA (14). Total fluor-enols were measured spectrophotometrically by use of the Folin-Ciocalteu reagent for phenols (15). Turbidities in the final extract (Fraction 8) due to trace amounts of ethanol or ether were prevented by heating the final extract for 3 minutes in a boiling water bath or by washing the ether extract (Fraction 6) with distilled water before extraction with 0.25 N sodium hydroxide. Heating of the final extract could then be omitted. The Folin-Ciocalteu reagent permitted the detection of 0.01 μmole of fluorenol. The optical densities were expressed in terms of 7-OH-AFA by reference to a calibration curve. These measurements are considered to give a satisfactory approximation of the total hydroxylation of AFA by rat liver for the following reasons. The color given by equimolar amounts of 7-OH-AFA, 5-OH-AFA, and 7-OH-FA with the Folin reagent was virtually identical throughout a 10-fold range of concentrations, whereas the color yield of 5-OH-FA was approximately 20 per cent and that of 1-OH-AFA was 85 per cent greater. However, under our experimental conditions, the only metabolite detectable chromatographically in extracts of rat liver homogenates or cell fractions incubated with AFA was 7-OH-AFA. The estimation of fluor-enols in experiments with preparations from hamster liver was undoubtedly less accurate, since 7-OH-AFA and 5 OH-AFA as well as 1-OH-AFA and 3-OH-AFA were found by paper chro-matography. However, it was apparent from inspection of the chromatograms that 7-OH-AFA and 5-OH-AFA were the pre-

We are indebted to Dr. J. H. Weisburger, National Cancer Institute, for a gift of this compound.
ponderant metabolites. Consequently, the method of expressing the data in terms of 7-OH-AFA is likely to give a reasonable estimate of total hydroxylation by hamster liver. The color given with the phenol reagent in extracts of liver cell fractions incubated with 7-F-AFA was also expressed in terms of 7-OH-AFA. Since no hydroxylated derivatives of this substrate have been prepared, no reference calibration curve was available. Accordingly, no quantitative significance can be attached to these data, and no comparison of the extent of hydroxylation of 7-F-AFA and of AFA is intended. The data, as presented, indicate in a qualitative manner how the hydroxylation of 7-F-AFA was affected by variations of the experimental conditions. Recoveries of 7-OH-AFA, 5-OH-AFA, 3-OH-AFA, 1-OH-AFA, and the corresponding aminofluorenols as well as recoveries of FA and AFA added to incubation mixtures were essentially quantitative at zero time. The radioactive cell fractions were subjected to wet combustion and the resulting barium carbonate precipitates counted according to the semi-micro method (16).

The individual metabolites formed by liver cell fractions were identified by paper chromatography as follows. Microsomes and soluble fraction obtained from 2.5 gm. of liver were incubated in an appropriately sealed incubation system in the presence of 0.1 M potassium fluoride and the fluorenols isolated according to the scheme of Fig. 1. The ether extract (Fraction 6) was washed with distilled water before extraction with dilute alkali. The alkaline extract was carefully neutralized with 1 M sulfuric acid and then extracted with diethyl ether. After solvent evaporation the residue was taken up in a small volume of ethanol. This solution was applied to a 1:3 dilution of the Folin reagent followed by a spray with 20 per cent sodium carbonate (17). In each experiment, the synthetic fluorenols were run simultaneously, each on a separate strip, as controls. The 

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R_f \text{values of 7-OH-AFA, 5-OH-AFA, 3-OH-AFA, and 1-OH-AFA were 0.27, 0.35, 0.45, and 0.51, respectively. With the same solvent system, } R_f \text{ values of 0.07, 0.18, and 0.13 were obtained for 7-OH-FA, 5-OH-FA, and 1-OH-FA, respectively. In the experiments with hamster liver preparations, the identity of 7-OH-AFA and 5-OH-AFA was confirmed by ultraviolet spectrophotometry as follows. Sections of untreated chromatograms corresponding to the location of these metabolites on sprayed chromatograms were cut out and extracted with dilute sodium hydroxide. The ultraviolet spectra of these solutions were compared with the ultraviolet spectra of the synthetic products.}

\section*{RESULTS}

\textbf{Requirements for Hydroxylation Activity from Rat Liver—Table I shows the relative effectiveness of several systems, which generate DPNH or TPNH, in promoting the hydroxylation of AFA. The combination of glucose-6-P (50 \text{ pmole}) and the soluble fraction, together with the microsomes, gave the most rapid hydroxylation, the rates being approximately linear with time for periods up to 30 minutes (Fig. 2). Further increase in the concentration of TPN to a level of 1.5 \text{ pmole} had no effect on the rate of hydroxylation. Replacement of glucose-6-P and TPN by succinate and DPN which had been used in earlier experiments (3) resulted in appreciably lower rates of hydroxylation. Reduced coenzyme was present throughout the entire incubation period in all experiments, since the 340 nm band due to reduced coenzyme could be demonstrated in aliquots removed at intervals from the incubation systems. Furthermore, independent assays of each of the DPNH- or TPNH-generating systems used in this work indicated that all of the oxidized coenzyme was converted in less than 1 minute to the reduced form and kept in the reduced state for periods up to 60 minutes. The consistent differences in the}
rates of hydroxylation of AFA were therefore not due to differences in the quantities of reduced coenzyme which were generated, and further study is required for an adequate explanation. Omission of nicotinamide from the incubation system reduced the rate of hydroxylation of AFA to 0.02 amole per hour per gm. of wet liver. In contrast, Conney et al. (18) reported little effect on the hydroxylation of 3,4-benzopyrene after the deletion of nicotinamide. Replacement of air by nitrogen abolished hydroxylation of AFA.

Enzymatically prepared TPNH and microsomes alone supported hydroxylation of AFA at approximately two-thirds of the rate obtained with the complete system, but commercial TPNH, which is prepared by reduction of TPN with sodium hydroxysulfite, and microsomes gave hydroxylation only at one-fourth of the rate observed under optimal conditions. Serial additions of 1 μmole of commercial TPNH at zero time as well as 10 and 20 minutes after the start of the incubation gave no increase in the rate of hydroxylation over a single addition of 2 μmoles at zero time. It would appear that enzymatically generated TPNH was the most effective means for hydroxylation of AFA. The lower rates of hydroxylation noted when TPNH preparations replaced the complete system could conceivably have been due to removal of the compound by other reactions occurring in the microsomes. However, the disappearance of TPNH from the microsomal system (obtained from 300 mg. of rat liver) measured in the absence of AFA was only 0.5 micromole of TPNH in 30 minutes leaving sufficient TPNH for hydroxylation of AFA. DPNH and microsomes were ineffective for hydroxylation of AFA, and DPNH did not increase the hydroxylation of AFA by TPNH and microsomes. Failure of DPNH to support hydroxylation has also been observed with 3,4-benzopyrene as substrate (18). On the other hand, Booth and Boyland (19) reported that hydroxylation of 2-acetamidonaphthalene by rat liver microsomes was accomplished with DPNH at one-half of the rate obtained with TPNH.

A glucose-6-P dehydrogenase preparation, although active in generating TPNH in the presence of glucose-6-P, supported hydroxylation only partially. The low rate of hydroxylation was most probably due to the presence of an inhibitor in the crude dehydrogenase, since the rate of hydroxylation was further depressed when the amount of dehydrogenase in the hydroxylation system was increased. The pH optimum for the hydroxylation of AFA was at 7.4 to 7.6 (Fig. 3). The decrease in hydroxylation at lower and higher pH values is presumably referable to lower activity of the microsomal system, since glucose-6-P dehydrogenase is active from 6.5 to 9.5 (20) and enzymatically produced TPNH is stable under our experimental conditions (21). The data of Fig. 3 were obtained in incubation systems buffered with 0.025 M phosphate. At pH 5 and 8.5, with the use of 0.025 M acetate, Veronal, borate, or tris(hydroxymethyl)aminomethane buffers, phosphate was added to give a concentration of 0.020 M. Substitution of phosphate buffers by borate, tris(hydroxymethyl)aminomethane, Veronal, or glycylglycine buffers reduced hydroxylation of AFA to approximately one-half of the rate obtained in the presence of phosphate. However, the original activity was restored by the addition of phosphate to a final concentration of 0.02 M. Inorganic phosphate has been noted to be required for optimal activity of the microsomal system from rabbit liver which hydroxylates acetanilide (22).

Under the conditions which were optimal for the hydroxylation of AFA deacetylation was not maximal, but proceeded lin-
0.04 pmole of FA formed per hour per gm. of wet tissue by careful tissue.

Hydroxylation of AFA is associated with the soluble proteins and microsomes and soluble fraction from rat liver as described under "Experimental." Nearly maximal hydroxylation of AFA is associated with the soluble proteins and microsomes and soluble fraction from testis, kidney, bladder, and intestine. These data substantiate the view that the enzymatic system which generates the TPNH required for hydroxylation of AFA was obtained when liver microsomes were incubated with the soluble fraction from testis, kidney, bladder, and intestine were combined with the soluble fraction from the livers of the various species. The deacetylation of AFA was inhibited by ethanol, ethylene diaminetetraacetate, a,a'-dipyridyl, and by potassium fluoride. Complete inhibition was observed at a concentration of 0.1 M F⁻ (Fig. 4). The concomitant rise of hydroxylation was not due to a direct stimulation of the microsomal hydroxylation system by fluoride ion, since incubation of FA in the presence of 0.1 M F⁻ did not increase the hydroxylation of this compound (Fig. 2). The stimulatory effect of F⁻ is most reasonably explained by the increase in the amounts of AFA which were available for hydroxylation when deacetylation was blocked. These results indicate that the presence of F⁻ in the incubation system is required for an estimate of the total capacity of the liver microsomal system to hydroxylate AFA and probably other acylamines.

**Species Comparison of Hydroxylation System**—A comparison of the ability of microsomal systems from several species to hydroxylate AFA as well as FA is presented in Table IV. All species examined hydroxylated AFA at 2- to 4-fold faster rates than FA. The most active hydroxylation was contained in hamster liver microsomes. Hydroxylation of 7-F-AFA was observed only with microsomal preparations from the livers of the various species examined (liver, kidney, bladder, testis, and intestine), only liver homogenates or microsomes deacetylated AFA at measurable rates. Since it has recently been shown by radioactive tracer methods (23) that intestinal strips in a hanging-phosphate buffer containing glucose readily deacetylated AFA, it would appear that the activity of the intestinal deacetylase is markedly influenced by the experimental conditions.

* The compound was added to the standard system containing microsomes and soluble fraction from rat liver as described under "Experimental."† Expressed as µmoles of 7-OH-AFA formed per hour per gm. of wet tissue.‡ Expressed as µmoles of FA formed per hour per gm. of wet tissue.

![Fig. 4. The action of fluoride ion on the hydroxylation and deacetylation of AFA by rat liver microsomes and soluble fraction. The incubation conditions are described under Experimental.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Species comparison of hydroxylation activities</th>
<th>Fluorenl formed by</th>
<th>Rat (30)</th>
<th>Guinea pig (4)</th>
<th>Rabbit (3)</th>
<th>Rooter (1)</th>
<th>Hamster (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFA + 0.1 m F⁻</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-F-AFA</td>
<td>0.36</td>
<td>0.28</td>
<td>0.20</td>
<td>0.18</td>
<td>0.18</td>
<td>0.36</td>
</tr>
<tr>
<td>7-F-AFA + 0.1 m F⁻</td>
<td>0.05</td>
<td>0.06</td>
<td>0</td>
<td>0.18</td>
<td>0.18</td>
<td>0.05</td>
</tr>
<tr>
<td>FA</td>
<td>0.08</td>
<td>0.08</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Liver microsomes and soluble fraction from the various species were added to the standard incubation system as described under "Experimental."† AFA, 7-F-AFA, and FA were used in equimolar concentrations. The results are expressed as µmoles of 7-OH-AFA formed per gm. of wet tissue per hour. The numbers in parentheses refer to the number of animals used.

† Hydroxylation ranged from 0.10 to 0.50 µmoles of fluorenel formed per hour per gm. of wet tissue.

‡ Hydroxylation ranged from 0.60 to 0.70 µmoles of fluorenel formed per hour per gm. of wet tissue.
rabbit, the rat, and the hamster, the latter species yielding again the most active preparations. In contrast, guinea pig and rooster livers were unable to hydroxylate 7-F-AFA. Since the 7 position is blocked in 7-F-AFA, use of this substrate tests the ability of the liver microsomal system to effect hydroxylation of the fluorene nucleus in positions other than this "extended" para position. Since chromatography of the urine of guinea pigs failed to reveal the presence of ortho amido- or amino-fluorens, it has been concluded that this species is incapable of hydroxylation AFA in a position ortho to the amido group (24). Incubation of 7-F-AFA with the guinea pig liver microsomal system likewise gave no hydroxylated products as indicated by the negative Folin test. These data suggest that comparative measurements of the hydroxylation of AFA and 7-F-AFA permit differentiation between ortho- and para-hydroxylation enzymes. The separate existence of these enzymes has recently been postulated (24, 25).

Identification of Hydroxylated Metabolites—The hydroxylated products obtained from the incubation of liver microsomal systems with AFA were identified by paper chromatography as outlined in the "Experimental" section. To insure maximal hydroxylation, the incubation mixtures contained 0.1 mM F-.

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Four hydroxylated metabolites of AFA were detected in extracts of hamster liver microsomal preparations after incubation with AFA and were identified as the 7-, 5-, 3-, and 1-hydroxy derivatives of AFA.

Chromatography of mixtures incubated with 7-F-AFA yielded three spots, which gave a positive Folin test (RF = 0.24, 0.34, and 0.45). Because of a lack of standards, these hydroxylated metabolites could not be further characterized. In contrast, only one hydroxylated derivative of AFA (7-OH-AFA, RF = 0.27) and one hydroxylated metabolite of 7-F-AFA (RF = 0.23) could be detected on chromatograms of extracts of rat liver microsomal preparations. The greater number of metabolites observed on chromatograms from hamster liver preparations thus coincided with the greater extent of hydroxylation by this species revealed by the colorimetric test.

The absence of 1-OH-AFA and of 3-OH-AFA on chromatograms of extracts of hydroxylation systems from rat liver may be partly attributed to the low level of ortho hydroxylation which was manifest in the experiments with 7-F-AFA as substrate (Table IV). Moreover, these compounds disappeared rapidly from actively hydroxylating and deacytlyating microsomal systems (Table V) and were presumably further metabolized. Preliminary experiments with 1-OH-AFA, labeled with carbon-14 at the carbonyl carbon, have shown that this ortho-amidofluorenol is rapidly deacytlyated by microsomal preparations from rat liver. This is presumptive evidence that the disappearance of 1-OH-AFA is conditional upon its deacytlyation to 1-OH-FA. In contrast, 7-OH-AFA and 5-OH-AFA proved to be stable, since they were not deacytlyated and were recovered quantitatively. These compounds are therefore regarded as metabolic end products under the experimental conditions. Addition of mitochondria to the hydroxylating system nearly doubled the initial rate of disappearance of 1 OH FA. It is of interest that addition of mitochondria to an actively hydroxylation system also increased the quantities of carbon-14 derived from AFA-9-C14, which were irreversibly bound to protein as shown in Table VI. However, measurable binding occurred even in the absence of mitochondria.

### Table V

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Disappearance of compound† at 30 minutes</th>
<th>Disappearance of compound† at 60 minutes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>N-(7-hydroxy-2-fluorenyl)acetamide</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>N-(5-hydroxy-2-fluorenyl)acetamide</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>2-Amino-7-fluorenone</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2-Amino-5-fluorenone</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>N'(3-hydroxy-2-fluorenyl)acetamide</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>N'(1-hydroxy-2-fluorenyl)acetamide</td>
<td>26</td>
<td>39</td>
</tr>
<tr>
<td>2-Amino-1-fluorenone</td>
<td>34</td>
<td>68</td>
</tr>
<tr>
<td>2-Amino-1-fluorenone†</td>
<td>60</td>
<td>73</td>
</tr>
</tbody>
</table>

* 0.5 pmole of each compound was added in 0.1 ml. of methyl Cellosolve to the standard incubation system containing microsomes and soluble fraction as described under "Experimental."† Based on comparison with a sample at zero time.

### Table VI

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Dry weight mg.</th>
<th>Specific activity c.p.m./mg.</th>
<th>Total counts c.p.m.</th>
<th>Compound bound/mg. protein μmoles</th>
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</thead>
<tbody>
<tr>
<td>Microsomes and soluble fraction</td>
<td>100</td>
<td>13</td>
<td>12</td>
<td>1.200</td>
</tr>
<tr>
<td>Microsomes, soluble fraction, and mitochondria</td>
<td>180</td>
<td>21</td>
<td>20</td>
<td>3.600</td>
</tr>
</tbody>
</table>

### DISCUSSION

The question has been raised whether 7-OH-FA, which has been identified in rat liver preparations incubated with AFA (3, 19), arises from the deacytlyation of 7-OH-AFA or from the direct hydroxylation of FA which is always found in rat tissue preparations exposed to AFA (1). The present evidence indicates that 7-OH-AFA is relatively resistant to deacytlyation, but that FA is hydroxylated directly by rat liver, although at a slower rate than AFA.

There is as yet no definite link between the metabolism of carcinogenic aromatic amines and their carcinogenic activity. However, recent evidence suggests that ortho hydroxylation of the amine or amide is implicated in the carcinogenic process (26, 27). On the basis of the data presented here, one might
conjecture that the hamster, whose liver contains an active ortho-hydroxylating system, would also be susceptible to liver tumor induction by AFA, and this appears to be the case. On the other hand, the guinea pig, which apparently lacks an ortho-hydroxylating enzyme, is resistant to the carcinogenic action of AFA (28).

It has recently been proposed from this laboratory that the binding of AFA to cellular proteins involves the intermediate formation of 2-imino-1,2-fluorenoquinone (29). This ortho-quinone imine arises from the oxidation of 1-OH-FA by mitochondria or the cytochrome c-cytochrome oxidase system and is rapidly and irreversibly bound to protein.4 The data relative to the increased rate of disappearance of 1-OH-FA from actively hydroxylating systems and the increased protein binding of the radioactivity of AFA-9-C14 upon addition of mitochondria are in harmony with this view. Since disappearance of 1-OH-FA and measurable protein binding was observed in the absence of mitochondria, it is suggested that microsomes or the soluble proteins of the cytoplasm or both are also capable of oxidizing 1-OH-FA through an as yet undetermined mechanism.

SUMMARY

1. A method for the measurement of the enzymatic hydroxylation of the carcinogen N-(2-fluorenyl)acetamide has been described.

2. Optimal conditions for the hydroxylation have been determined and some of the properties of the microsomal enzymes of liver which hydroxylate and deacetylate the compound have been investigated. Reduced triphosphopyridine nucleotide and oxygen were required for hydroxylation.

3. Data are presented on the hydroxylation of N-(2-fluorenyl)acetamide, 2-fluorenamine, and N-(1-fluoro-2-fluorenyl)acetamide by the hydroxylation systems of several species. The liver of the hamster yielded the most active preparations. The data suggest the existence of separate enzymes for ortho and para hydroxylation.

4. Evidence has been obtained that the ortho-hydroxylated compounds, N-(1-hydroxy-2-fluorenyl) acetamide, N-(3-hydroxy-2-fluorenyl) acetamide, and 2-amino-1-fluorenol, are further metabolized as indicated by their disappearance from hydroxylating systems of rat liver. The rate of disappearance was markedly enhanced by the addition of mitochondria.

5. It has been suggested that the disappearance of the ortho-hydroxylated products and the binding of N-(2-fluorenyl)acetamide to cellular proteins which was demonstrated in actively hydroxylating preparations is due to oxidation of these compounds to orthoquinone imines.

Acknowledgment—The authors wish to thank Mr. Leonard Sutton for technical assistance.

REFERENCES

4. Data concerning the susceptibility of the hamster to the carcinogenic action of AFA were kindly made available to us by Dr. P. Shubik, Department of Oncology, Chicago Medical School, Chicago, Illinois.
The Metabolism of the Carcinogen N-(2-Fluorenyl) acetamide by Liver Cell Fractions
Ulysses S. Seal and Helmut R. Gutmann


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