On Protein Targets of Chemical Carcinogens: Dissimilar Molecular Sizes of the Principal Protein Conjugates

Sam Sorof, Emily M. Young, Regina A. McBride, and Carol B. Coffey

The Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111

SUMMARY

The various soluble liver protein-carcinogen conjugates produced in vivo by two hepatocarcinogens have been resolved and analyzed according to molecular size. In addition, the molecular sizes of the principal soluble conjugates of three types of carcinogens have been compared in order to deduce whether they originate from a common or similar target protein.

Adult male rats were fed the liver carcinogen 3'-methyl-4-dimethylaminoazobenzene for 18 to 21 days. Gel filtration of the soluble proteins of perfused livers revealed the presence of five species of azoproteins: The principal conjugate had 62% of the soluble protein-bound dyes, belonged to the 55 class of macromolecules, and was of 60,000 to 80,000 molecular weight. This azoprotein is identical to the previously characterized principal charge species, slow h₂. A minor azoprotein (19%) was a 45 macromolecule (30,000 to 40,000) and is apparently the basic h₂ conjugate previously separated on the basis of charge. Small amounts of azo dyes (~5%) were bound to the 25 (10,000 to 15,000) and ~7.5S (~150,000) macromolecules.

In addition, adult male rats were fed the liver carcinogen N-2-fluorenylacetamide for 5 weeks, given single intragastric doses of N-2-fluorenylacetamide-¹⁴C, and killed 48 hr later. The 7.5S macromolecules (~150,000) had 40% of the soluble protein-bound fluorenyl-¹⁴C, and apparently contained the previously described principal charge species, fast h₂ and/or slow h₁ fluorenyl-¹⁴C-proteins. Each of the other classes of soluble macromolecules had considerably less conjugate.

That these two hepatocarcinogens and the skin carcinogen, polycyclic aromatic hydrocarbons, may interact with a common target protein during carcinogenesis was previously suggested by the fact that the resultant principal soluble protein conjugates of the three carcinogens are of closely similar charge (h₂ or h₂-like). It now appears that these principal conjugates are of considerably different molecular weights: aminoazo dye, 60,000 to 80,000; N-2-fluorenylacetamide ~150,000; and, as reported by Tasseron et al., the polycyclic aromatic hydrocarbons, 40,000. This diversity indicates that the target proteins of the 3 carcinogens either are different at least in molecular size or, if identical, are altered in different ways as a result of interaction with the 3 carcinogens.

INTRODUCTION

Certain cellular proteins are specific targets of 3 families of carcinogens [reviewed (2, 12, 15—17, 19, 26)]. During carcinogenesis in rat liver by the aminoazo dyes and FAA and in mouse skin by the polycyclic aromatic hydrocarbons, various carcinogen-protein conjugates are formed. Most of these are soluble [references compiled (26)]. The soluble conjugates belong mainly to a small group of relatively basic proteins, electrophoretically defined and termed h₂ or h₂-like (1, 20, 25, 26). In contrast, the consequent primary tumors (1, 25, 26) and transplanted highly differentiated hepatomas (23) do not contain these conjugates.

The evidence thus suggested that the 3 carcinogens may interact mainly with a common or similar receptor protein to form analogous principal conjugates. It was therefore deemed important to examine the similarity of these conjugates. If the likeness were great, then the existence of a common principal receptor protein would be supported. The present study accordingly undertook to examine the molecular size species of protein conjugates of the 2 hepatocarcinogens in liver extract in regard to multiplicity, relative abundance, and molecular weights. In addition, the molecular size of a principal hydrocarbon-protein conjugate of mouse skin was recently reported by Tasseron et al. (28). Comparison of the molecular sizes of principal protein conjugates of the 3 carcinogens thus was possible and is presented. A portion of this study was reported in a preliminary communication (19).

MATERIALS AND METHODS

Rats, Diets, and Carcinogens. In 5 experiments, 2 to 5 adult male rats (Carworth CFN, New City, N. Y.) were fed, ad libitum for 18 to 21 days, a synthetic version of Diet 3 of Miller et al. (13) containing 18% casein, 1.0 mg riboflavin/kg, and 0.058% of the liver azocarcinogen, 3'-Me-DAB (Eastman Kodak Co., Rochester, N. Y.). In 3 control experiments, azo dye was absent from the diet. Rats were then fasted for 17 to 22 hr and killed.

In 2 experiments, rats (128 to 148 g) like those above were fed, ad libitum for 5 weeks, a grain diet (14) containing 0.036% of the liver carcinogen, FAA (Eastman Kodak). After 13 hr on the control diet (without carcinogen) to lessen metabolic stores of fluorenyl metabolites, the rats were given 1

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2 The abbreviations used are: 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene [N,N-dimethyl-p-(m-tolylazo)aniline]; FAA, N-2-fluorenylacetamide (2-acetaminofluorene).
single intragastric doses of FAA-9\textsuperscript{14}C (6.83 mCi/m mole; Tracerlab, Waltham, Mass.) in 1,2-propanediol (0.5 ml/100 g).

In 1 experiment, 3 rats (168, 177, and 203 g) received a total of 98.7 $\mu$Ci; in another, 2 rats (216 and 228 g) were given a total of 67.8 $\mu$Ci. The rats were then fed the control diet for 48 hr and killed. Two other similar experiments are also reported in part (mainly in Table 1).

**Preparation of Liver Extracts.** Liver extracts were prepared at 1–4°C as previously described (22, 26). Livers were perfused portally with 0.08 M sodium phosphate buffer, pH 7.8, containing 0.01 M sodium phosphate buffer, pH 7.4. Within 3.5 hr after rat sacrifice, 6-mI samples of liver extract were applied to the columns and resolved at 2.2° according to molecular size.

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Peaks in the profiles were extrapolated into symmetrical values approximating the sedimentation rates of macro molecules at the modes. The recoveries of constituents absorbing at 280 m\(\mu\) in the column eluates were 97 ± 3% in 5 dye profiles, 104 ± 5% in 3 control profiles, and 103 ± 6% in 4 FAA profiles. The value 96 ± 4% was previously obtained with normal rat liver extract (22, 24).

It was previously determined in a zonal electrophoretic study that, if liver fluorenyl-\textsuperscript{14}C-proteins were counted directly, rather than after extensive solvent extraction, then considerably more bound carcinogen was detected, without changing the qualitative nature of the profile (25). Accordingly, for assay of fluorenyl-\textsuperscript{14}C-proteins, samples of liver extracts and fractions throughout molecular size profiles were transferred to 20-ml vials, freeze-dried, and counted in Thixin gel by \(\beta\)-scintillation spectrometry at an efficiency of 62% with a 1 to 2% standard deviation (25). The recovery of fluorenyl-\textsuperscript{14}C in the 2 profiles was 77 and 82%.

## RESULTS

### 3\textsuperscript{\prime}-Me-DAB and Control Livers

The soluble liver proteins consisted of at least 8 size classes (Chart 1, Table 1). The macromolecules spanned from the components termed 15–20S to the 2S. In addition, there were later eluting small molecules. The molecular size profiles from rats fed the azo-carcinogen or control diet resembled each other and those formerly encountered with normal rat liver (22, 24).

The principal azoprotein was at the mode of the 5S size class of macromolecules. It contained about three-fifths of all the azo dyes bound to macromolecules in the liver extracts. This is shown in Chart 1 and in Table 2, which lists the relative contents of bound dyes in the various molecular size classes. The 4S azoprotein contained one-fifth of the soluble bound azo dyes. At still lower levels (~5%) were the 2S, 6S, and 7.5S conjugates. Because of incomplete resolution from the principal 5S species, the 6S species probably contained less dye than was apparent, and belonged to the remaining group at near baseline levels. Azo metabolites among the small molecules (600 to 720 ml in Chart 1, upper) were barely, if at all, detected. This conclusion is based on their low relative dye content (Table 2), and on the absence of an absorption maximum in 88% formic acid in the region 510 to 525 m\(\mu\), which is characteristic of azo dye. The 5S, 4S, and 2S azoproteins absorbed maximally at 521 m\(\mu\), and those of the 7.5S absorbed at 518 m\(\mu\).

### Table 1

Sizes of soluble molecules of preneoplastic and control livers

<table>
<thead>
<tr>
<th>Liver extract</th>
<th>No. of profiles</th>
<th>Macromolecule (% ± a.d.)</th>
<th>Small (% ± a.d.)</th>
<th>% of total molecules</th>
<th>% of macromolecules (± a.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>74 ± 7</td>
<td>26 ± 7</td>
<td>2 ± &lt;1</td>
<td>24 ± &lt;1</td>
</tr>
<tr>
<td>3\textsuperscript{\prime}-Me-DAB</td>
<td>5</td>
<td>81 ± 2</td>
<td>19 ± 1</td>
<td>3 ± &lt;1</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>FAA + FAA-9\textsuperscript{14}C</td>
<td>4</td>
<td>66 ± 5</td>
<td>34 ± 5</td>
<td>3 ± 1</td>
<td>18 ± 4</td>
</tr>
</tbody>
</table>

\(\text{a.d., average deviation of the mean.}\)
Protein-Carcinogen Conjugates

Chart 1. Molecular size profiles of the soluble liver proteins and azoproteins. Rats were fed the azocarcinogen, 3'-Me-DAB, for 18 to 20 days. Soluble proteins of perfused livers were resolved by sieving through Sephadex G-200 gel in sodium chloride-sodium phosphate buffer, pH 7.4. Proteins $A_{280\text{nm}}$ eluted at between 200 and 580 ml (upper) or 200 and 700 ml (lower); the later appearing constituents were small molecules (22, 24). $A_{280\text{nm}}$ measured azo dyes in formic acid in the 3'-Me-DAB experiment (upper) and nonspecific constituents in the control experiment (lower); $A_{400\text{nm}}$ denotes absorbance at 400 nm by interfering chromogens. Reprinted in part from a preliminary account (19) by permission.

Table 2

<table>
<thead>
<tr>
<th>Component</th>
<th>3'-Me-DAB liver$^a$</th>
<th>FAA + FAA-9$^b$ C liver$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(amount in component)</td>
<td>(amount in component)</td>
</tr>
<tr>
<td>15—20S</td>
<td>&lt;1</td>
<td>5</td>
</tr>
<tr>
<td>10—15S</td>
<td>&lt;1</td>
<td>4</td>
</tr>
<tr>
<td>7.5S</td>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td>6S</td>
<td>5</td>
<td>4</td>
</tr>
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<td>3S</td>
<td>62</td>
<td>13</td>
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<td>4S</td>
<td>19</td>
<td>14</td>
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<td>3S</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>2S</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Small molecules</td>
<td>&lt;1</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$ Values are based on three 3'-Me-DAB liver profiles and corrected for nonspecific absorption by use of two control liver profiles ("Materials and Methods").

$^b$ Values are based on two profiles.

The molecular weight of the principal azoprotein is estimated to be 60,000 to 80,000. This azoprotein was at the mode of the 5S class among macromolecules that sediment at 4.6 to 5.3 S (22, 24). [With simple artificial mixtures as standards, the molecular weight at the 5S mode was previously estimated to be about 60,000 (22, 24). Additional evidence suggested that the range 60,000 to 80,000 is more accurate. This reevaluation is based on the locations of known proteins (half-molecule of hemoglobin, lactic dehydrogenase, main arginase molecule) within the size profile of the soluble macro- molecules of rat liver (21).]

The molecular weight of the 4S azoprotein is 30,000 to 40,000. Macromolecules at the same profile location, the 4S mode, sediment at 3.8 to 4.1 S (22, 24). These were estimated to have a molecular weight of 34,000 (22, 24), and later of 30,000 to 40,000 (21). Similarly, the 2S azoprotein was at the mode of its class. Such macromolecules sediment at 1.8 to 2.2 S, and have a molecular weight of 10,000 to 15,000. They were previously estimated to have a molecular weight equal to 10,000 (22, 24). The 7.5S azoprotein has a molecular weight of at least 150,000. Macromolecules at the mode of this class sedimented at 7.3 to 8.0 S, and were formerly found to have a molecular weight of 125,000 (22, 24).

Macromolecular size profiles of 3'-Me-DAB and control liver extracts exhibited 4 peaks of absorption at 400 nm in 88% formic acid: at the 15—20S, at the 10—15S, at the largest molecules within the 7.5S component (where azo dyes were in dye experiments), and at the region between the 3S and 4S classes (Chart 1). The 1st position coincided with a concentration of nucleic acids there, as judged by ratio of absorbances at 260 and 280 nm (cf. Refs. 22 and 24). The 2nd may in part be due to catalase (5.4, 24). The 3rd may belong to an unresolved 9S class in the 7.5S component (22, 24). The 4th may be predominantly due to half-molecules of hemoglobin present in small amount despite liver perfusion (22, 24). Only the 3S and 15—20S components had significant concentrations of nucleic acids ($A_{260\text{nm}}/A_{280\text{nm}}$) among the macromolecules. These components together possessed 3% of
the macromolecular azo dyes (Table 2). The other macromolecules, which contained still less nucleic acid, if any, had the remainder of the azoproteins.

**FAA Livers.** The soluble liver proteins of rats given FAA exhibited molecular size distributions similar to those of normal (22, 24), azo dye, and control livers. Comparisons of the protein profile in Chart 2 and the relative composition data in Table 1 with the corresponding results of these other types of livers bear this out. The only difference noted was that the FAA liver extracts had relatively more ultraviolet-absorbing small molecules than did the other extracts.

The principal fluorenyl-14C-protein was a 7.5S macromolecule. It contained 40% of the fluorenyl-14C derivatives in the liver extracts. This is shown in Chart 2 and Table 2. The latter lists the relative contents of fluorenyl-14C in the various molecular size classes. At one-third to one-fifth of the amount of fluorenyl-14C in the 7.5S were the 2S, 3S, 4S, and 5S fluorenyl-14C-proteins. They each contained 8 to 16% of all the soluble fluorenyl-14C derivatives. Of these 4, only the 2S conjugates were discrete. The remaining fluorenyl-14C-proteins, while present in significant amounts, were at near baseline levels. The presence of small molecular fluorenyl-14C was not detected.

The molecular weight of the 7.5S fluorenyl-14C-protein is approximately 150,000. Macromolecules at the same profile position were previously found to sediment at 7.3 to 8.0 S and were estimated to have a molecular weight of 125,000 (22, 24).

**DISCUSSION**

The carcinogens, aminoazo dyes, FAA, and polycyclic aromatic hydrocarbons generate principal soluble protein-conjugates of like charge in preneoplastic organs ("Introduction"). This similarity previously suggested that these carcinogens interact mainly with common or similar target proteins. However, the molecular sizes of these conjugates are now known to be different. The one resulting from aminoazo dye is 60,000 to 80,000, FAA ~150,000 (Ref. 19; this report), and that of polycyclic aromatic hydrocarbons is 40,000 (28). This diversity of molecular sizes weighs heavily against the possibility that these conjugates derive from a common or similar target protein.

In rat liver undergoing carcinogenesis by aminoazo dye, the principal soluble azoprotein is a 5S macromolecule. Macromolecules of this class sediment at 4.6 to 5.3 S and have a molecular weight of 60,000 to 80,000 (22, 24). This conjugate is identical with the principal charge species of azoprotein, the slow h2 azoprotein (26). Their identity is supported by their similar content of bound carcinogen relative to that in liver extract: the 5S has 62% (Table 2); the slow h2 azoprotein has 47% (26). All other azoproteins have much less. Furthermore, the slow h2 azoprotein has been purified and was found to be a 5S azoprotein (unpublished).

The 4S azoprotein is a minor conjugate. It has about one-third as much bound dyes as has the 5S azoprotein. The 4S macromolecules sediment at 3.8 to 4.1 S and have molecular weights of 30,000 to 40,000 (21, 22, 24). The 4S azoprotein is apparently the h3 charge species of azoprotein, the most basic soluble azoprotein. This azoprotein was previously isolated by boundary electrophoresis in free solution (27), but it usually was not recovered after column electrophoresis (26). The identity of the 4S and h3 azoproteins is supported by their similar contents of bound azo dyes relative to that in liver extract: the 4S azoprotein has 19% (Table 2); the h3 azoprotein has 15% (27). Furthermore, elimination of h3 azoprotein from mixtures containing slow h2 and h3 azoproteins removes the 4S azoprotein (unpublished).

Ketterer et al. (9) have reported the purification of an azoprotein from rat livers isolated 16 hr after i.p. administration of the azocarcinogen, 4-dimethylaminoazobenzene. The protein was characterized as homogeneous, isoelectric at pH 8.4, sedimenting at 3.5 S, and having a molecular weight of 45,000; it was suggested to be the principal azoprotein (slow
Protein-Carcinogen Conjugates

$h_2$ of liver cell sap. On the other hand, in our experience, the $SS$ (slow $h_2$), azoprotein is the principal soluble liver azoprotein present 48 hr after intragastric administration of single doses of 3'-Me-DAB to rats (unpublished), the same as after feeding for 2.5 to 3 weeks (present study). The sedimentation rate, molecular weight, and basicity of the azoprotein of Ketterer et al. do not correspond to the characteristics of the principal azoprotein (slow $h_2$), but they instead appear to be of the minor $4S$ ($h_3$) azoprotein (Ref. 19; this report). This indication is also compatible with their observation that two-thirds of the protein-bound azo dyes in liver extract was lost in the first step (pH 4.5, Ca", Cu", NaCl) of their isolation procedure (9). In our experience, the slow $h_2$ azoprotein irreversibly coprecipitates with other proteins when liver extract is acidified to pH 5 or below. Further, Morey and Litwack (10, 18) have isolated the azoprotein of Ketterer et al. and have proposed that it is identical to the liver protein which non-covalently binds metabolites of cortisol 45 min after i.p. administration of that hormone. In our experiments (unpublished), cortisol or its metabolites are bound to the $h_3$ proteins, among others, but not to slow $h_2$ proteins. It thus appears that both the azoprotein of Ketterer et al. and, if identical, the cortisol metabolite-binding protein of Morey and Litwack pertain to a minor azoprotein ($h_3$), rather than to the principal azoprotein (slow $h_2$). Further identification of the purified azoprotein is warranted, especially since chemical (8), immunological (3), and biochemical (10) investigations based on it have been reported. In addition, Ketterer et al. (9) partly fractionated a near-neutral azoprotein having a molecular weight of 13,800, which has also been suggested to be another principal azoprotein (8). On the other hand, in the present study, azoproteins of this molecular weight (28; M.W. 10,000 to 15,000) contain only 5% of all the soluble protein-bound azo dyes in liver extract (Table 2).

In rat liver undergoing carcinogenesis by FAA, the principal soluble fluorenylprotein is a 7.5$S$ macromolecule. Such macromolecules sediment at the rate 7.3 to 8.0 S and have a molecular weight of ~150,000 (22, 24). The 7.5$S$ fluorenylprotein is apparently the same as the principal charge species of fluorenylprotein(s), previously described as the fast $h_2$ or slow $h_1$ (25). Their identity is supported by the fact that both species contain similar relative quantities of bound carcinogen among the soluble liver proteins: the 7.5$S$ fluorenylprotein(s) has 40% (Table 2); the fast $h_2$ and/or slow $h_1$ fluorenylprotein(s) have 32 to 43% (25). No other species contains more than one-third as much fluorenyl residue. Barry et al. (4, 5) have described a chromatographic Fraction B, composed of weakly basic soluble liver proteins and suggested to be $h_2$ proteins, which retained a gradually increasing concentration of bound carcinogen following the administration of FAA over a period of 18 days. As previously noted (25), the 4.1 $S$ sedimentation coefficient and estimated molecular weight of 60,000 reported by them for the protein(s) of Fraction B differ from those of the principal species (7.5$S$ M.W. ~150,000) in the present investigation.

The particular species of carcinogen-protein conjugates which have been isolated by others thus far have certain similar properties. This agreement may be in part a reflection of the relative uniqueness of these particular conjugates in cell sap, resulting from the combination of their relatively small size and basicity. The liver azoprotein of Ketterer et al. (8, 9), present 16 hr after a single dose of azo dye, sediments at 3.5 S and has a molecular weight of 45,000. The liver protein(s) of Peak B of Barry et al. (4, 5), which accumulate bound fluorenyl metabolites up to 18 days of administration of FAA, sediment at 4.1 ± 0.6 S and have a molecular size equal to 60,000 ± 9,000. The skin hydrocarbon-protein conjugate of Tasseron et al. (28), present 48 hr after a single application of hydrocarbon, has a molecular weight of 40,000. As indicated, these conjugates were isolated soon after the start of administration of carcinogen. Accordingly, it seems worthwhile to determine whether they are mainly relevant to early effects of the carcinogens and whether the conjugates of the present investigation, which were examined considerably later, are connected with subsequent events.

The diversity of the molecular sizes of the principal conjugates of the 3 types of carcinogens indicates that their target proteins either are different at least in size or, if identical or similar, are altered in different ways by the 3 carcinogens. In the former alternative, which is the simpler and perhaps more likely, different target proteins would probably have distinguishable biological activities. The latter alternative (different alterations of an identical protein) requires that the reactive intermediates formed from the carcinogens arylate and rupture peptide bonds (11, 16) of the same protein at different sites and/or to different extents. Alterations which are sufficiently different to produce the observed diversity of molecular sizes of conjugates, starting with a common protein, would likely give rise to conjugates of either different or no activity. Thus with either alternative, the principal soluble protein conjugates of the 3 carcinogens would likely have distinguishable or no biological activities. The azo dyes (Refs. 19, 26; this report), FAA (Refs. 4, 19, 25; this report), and polycyclic aromatic hydrocarbons (1, 28) generate numerous carcinogen-protein conjugates in target organs. Therefore, in considering conjugates of different carcinogens in search of possible common target proteins, it is essential that only similar conjugates be compared. Unfortunately, failure to do this has resulted in some confusion in recent years. This caution is particularly appropriate in view of the present indication that the 3 types of carcinogens may have different principal target proteins.

The indication that different carcinogens may have different principal target proteins may be separate from the question of whether protein-carcinogen interactions may be involved in oncogenesis. The apparent diversity of the target proteins may have its counterpart in the lack of detectable homology of the DNA's of the small oncogenic papovaviruses, namely polyoma, SV40, Shope rabbit papilloma, and human papilloma viruses. The DNA or transcribed RNA of one virus does not hybridize with the DNA of another virus [reviewed (6)]. The inference is that these viral DNAs give rise to small numbers of different gene products (RNA's or proteins), one or very few of which act on a cellular system(s) to effect transformation and tumorigenesis. Likewise, even if the 3 chemical carcinogens interact with different principal target proteins, there may be common end results, particularly if the targets are functionally related to each other. That they may be so is suggested by the similar basic charge of the 3 specific carcinogen-protein con-
jugates. Various enzymes of like functions have similar charge (22). Accordingly, even if the target proteins are different molecular species, but are functionally related, their alterations by carcinogens might nevertheless be able to bring about similar effects in cellular system(s).

REFERENCES

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