The studies of Miller, Miller, and co-workers (1, 3, 5, 7–10) on the occurrence of protein-bound dye in the livers of rats fed the hepatic carcinogen 4-dimethylaminoazobenzene (DAB) or certain of its derivatives have directed attention to the possible causal role of these proteins in aminoazo dye carcinogenesis. The protein-bound dye appears in the liver a few days after the initiation of dye-feeding and is found in the non-neoplastic portions throughout the stages of tumor formation and growth, as long as the animals are maintained on the dye. However, the protein-bound dye cannot be detected in the aminoazo dye-induced tumors, and this lack can be interpreted as indicating a qualitative difference between the proteins of liver and those of the tumors.

Over one-half of the bound dye in these livers is combined with the soluble proteins (7–10). The present investigation was undertaken to ascertain the electrophoretic group(s) of the soluble rat liver proteins which were combined with the dye following the ingestion of aminoazo dyes that have different carcinogenic activities. In previous studies (12, 13), it was found that the soluble proteins of normal rat liver, of livers from rats fed 4-dimethylaminoazobenzene for varying lengths of time short of neoplasia, and of liver tissue adjacent to DAB-induced hepatomas had similar electrophoretic patterns. On the other hand, the soluble proteins of DAB-induced hepatomas, their metastases, and a group of other tumors showed electrophoretic patterns which were very similar to each other but different from these livers. In contrast to the non-neoplastic liver tissues, all the tumors studied exhibited a striking decrease in the slow moving h components and contained more of the A and, to a lesser extent, the N components. It will be shown in the present report that the major portion of the protein-bound azo dye migrates electrophoretically with the same h components (probably h1) which were previously found to be markedly reduced in the DAB-induced tumor (13).

METHODS

Male rats of the Sprague-Dawley strain, weighing 175–220 gm., were fed ad libitum a semi-synthetic diet (2, diet 3). The aminoazo dyes were incorporated into the diets at a level of 0.058 per cent for 3’-methyl-4-dimethylaminoazobenzene (3’-Me-DAB) and 2-methyl-4-dimethylaminoazobenzene (2-Me-DAB), and 0.064 per cent for 4’-methyl-4-dimethylaminoazobenzene (4’-Me-DAB). The diet with 3’-Me-DAB contained 1.0 mg riboflavin/kg, while the other two diets contained 2.0 mg/kg. Two rats from each group were killed for each analysis. Two analyses were made on the livers from four rats fed 3’-Me-DAB for 2.5 weeks, two on the livers from four rats fed 4’-Me-DAB for 8 weeks, and one each on the livers of two rats fed 2-Me-DAB for 11, 14, or 16 weeks. At these times the levels of bound dye were approximately at maximum (3). For each analysis the soluble proteins were prepared with a Potter-Elvehjem-type lucite homogenizer and electrophoretically analyzed, by the technics previously described (11–13). After the patterns were photographed, the bottom cell section was closed off to isolate the two vertical limbs. By means of a 5-ml syringe, equipped with a 15-inch, 20-gauge stainless steel needle and attached to an adjustable screw rack, fractions throughout the entire cell were removed in the order of the Roman numerals shown in Chart 1. The volumes were noted, and the entire samples were transferred quantitatively to tared 15-ml centrifuge tubes. The protein was precipitated with tri...
chloroacetic acid at a final concentration of 9 per cent. The sedimented protein was washed once with 1 M, pH 5 acetate buffer and extracted 4 times for 5 minutes each with 6 ml. of ethanol at 75° C. After the protein samples were dried in vacuo over CaCl₂ and finally over Na₂SO₄, they were weighed and analyzed for total bound dye (1, 3). Bound dye analyses on similar weights of protein extracted in the above manner agreed closely with analyses on larger samples of the same liver protein, which had been subjected to exhaustive extraction with hot ethanol as previously described (1, 3).

RESULTS

As previously found with DAB (13), the soluble liver proteins from rats fed 3'-Me-DAB, 4'-Me-DAB, or 2-Me-DAB for periods of time short of hepatoma development yielded electrophoretic patterns (Table 1) similar to those for normal liver (12). In most cases light yellow boundaries were observed with unfiltered light to move with the h₁ component. In two anomalous runs this light yellow boundary migrated with the h₂ component (see footnote of Table 1). These yellow fronts have not been seen in preparations from rats not fed dye.

In agreement with previously reported results (7—10), all the soluble protein preparations contained high concentrations of bound dye. Chart 1 depicts the mapping of the pipetted electrophoretic fractions, while Table 2 shows the concentrations of total bound dye per milliliter for two representative experiments with 3'-Me-DAB. The dye contents are expressed in E values, i.e., the optical densities of acid solutions of the dyes obtained following extraction of alkaline hydrolyzates of the

![Chart 1](chart.png)

**CHAR T 1.—** The typical mapping of an electrophoretic fractionation of the soluble liver proteins from rats fed aminoazo dyes.

**TABLE 1**

<table>
<thead>
<tr>
<th>Dye fed</th>
<th>3'-Me-DAB</th>
<th>4'-Me-DAB</th>
<th>2-Me-DAB</th>
<th>2-Me-DAB</th>
<th>2-Me-DAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks fed</td>
<td>2.5</td>
<td>8</td>
<td>11</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>No. of rats</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No. of analyses</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**ANALYSIS OF THE SOLUBLE LIVER PROTEINS FROM RATS FED AMINOAZO DYES**

<table>
<thead>
<tr>
<th>Components</th>
<th>N</th>
<th>A</th>
<th>a₁</th>
<th>a₂</th>
<th>b</th>
<th>g₁</th>
<th>g₂</th>
<th>h₁</th>
<th>h₂</th>
<th>i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobility</td>
<td>6.9 (3.4—9.5)</td>
<td>9.9 (8.4—11.3)</td>
<td>29.1 (24.0—31.7)</td>
<td>17.4 (16.5—18.2)</td>
<td>9.4 (8.5—10.2)</td>
<td>7.3 (6.0—8.3)</td>
<td>3.7 (2.5—5.0)</td>
<td>7.2 (5.8—10.0)</td>
<td>5.2 (3.9—6.1)</td>
<td>1.7 (1.4—2.1)</td>
</tr>
<tr>
<td>Area (per cent)</td>
<td>6.9 (3.4—9.5)</td>
<td>9.9 (8.4—11.3)</td>
<td>29.1 (24.0—31.7)</td>
<td>17.4 (16.5—18.2)</td>
<td>9.4 (8.5—10.2)</td>
<td>7.3 (6.0—8.3)</td>
<td>3.7 (2.5—5.0)</td>
<td>7.2 (5.8—10.0)</td>
<td>5.2 (3.9—6.1)</td>
<td>1.7 (1.4—2.1)</td>
</tr>
</tbody>
</table>

*Descending electrophoretic data of preneoplastic liver supernatants of rats fed 3'-Me-DAB, 4'-Me-DAB, and 5-Me-DAB.*

†These data have been directly affected by a spiking of the descending h₂ component (cf. [11]). Where more than one determination is available, such figures are omitted.

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**Cancer Research**

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proteins (1). The values \( E (h_1\text{-corrected})/ml \) for the border fractions (II and X) were calculated by correcting the concentrations, \( E/ml \), of the bound dye to take into account the dilutions caused by the relative electrophoretic position of the \( h_1 \) component within these fractions. This corrected value would be the approximate concentration, boundary (fractions VI, XI, and XII) as 100 per cent, the \( E (h_1\text{-corrected})/ml \) value of the border fraction (II) can be used to calculate the percentage of the bound dye migrating with the \( h_1 \) component. Another method of calculation, by difference, involves the determination of the percentage of the bound dye (\( E/ml \) of VI, XI, and

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>THE CONCENTRATIONS OF PROTEIN-BOUND DYE IN THE ELECTROPHORETIC FRACTIONS OF THE SOLUBLE LIVER PROTEINS FROM RATS FED 3'-METHYL-4-DIMETHYLAMINOAZOBENZENE</strong></td>
</tr>
<tr>
<td><strong>No.</strong></td>
</tr>
<tr>
<td>E/ml</td>
</tr>
<tr>
<td>E(h$_1$-corr)/ml</td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>100 mg. protein</td>
</tr>
<tr>
<td>E/ml</td>
</tr>
<tr>
<td>E(h$_1$-corr)/ml</td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>100 mg. protein</td>
</tr>
</tbody>
</table>

*Concentrations of protein-bound azo dye in the pipetted electrophoretic fractions of the soluble liver proteins of rats fed 3'-Me-DAB.*

E/ml in the border fractions, if they could be pipetted in such a way as to exclude portions of the cell not containing proteins of the \( h(h_1) \) components. It is seen that the protein-bound azo dyes were present at high concentrations in fractions II—VI and X—XII.

Considering the range of \( E/ml \) values for the fractions of the cell containing protein without any a) Moving with \( h \) components (calculated as \( h_1 \)):

\[
\frac{E (h_1\text{-corr}) \text{ ml of fraction II}}{E/ml \text{ of fraction XII}} = 0.15 \times 100 = 75 \text{ per cent}.
\]

\[
\frac{E (h_1\text{-corr}) \text{ ml of fraction II}}{E/ml \text{ of fraction XI}} = 0.15 \times 100 = 68 \text{ per cent}.
\]

b) Moving faster than \( h \) components on ascending side:

\[
\frac{E/ml \text{ of fraction IX}}{E/ml \text{ of fraction XII}} = 0.01 \times 100 = 5 \text{ per cent}.
\]

\[
\frac{E/ml \text{ of fraction IX}}{E/ml \text{ of fraction XI}} = 0.01 \times 100 = 5 \text{ per cent}.
\]

On the latter basis, 95 per cent of the total bound dye in the supernatant fluid moved with a mobility slower than that of the electrophoretic components in fraction IX. This slower mobility corresponds to that of the \( h \) components, again ignoring the salt and minor boundaries. Therefore, as the overall range, 68—95 per cent of the protein-bound dye derived from 3'-Me-DAB and contained in the supernatant fluid moved with the \( h \) components (probably \( h_1 \)). Table 3 contains data obtained in a similar manner from the experiments performed with the soluble protein-bound dyes from the livers of rats fed 3'-Me-DAB, 4'-Me-DAB, and 2-Me-DAB.

Similar conclusions are also reached by calculating the data in terms of bound dye per unit weight of protein. As shown in Table 2 for two experiments with 3'-Me-DAB, the proteins in fractions II and III (composed largely of the \( h \) components) contained the highest concentrations of bound dye. The values then decreased progressively through the fractions clockwise around the cell, as shown in Chart 1.

Therefore, if the protein-bound dye is consid-
ered to be migrating with the h₁ component, the observed electrophoretic mobility (in veronal buffer, 0.1 M, at pH 8.6) of the majority of the soluble proteins bearing the dye was \(-0.98 \times 10^{-4}\) cm²/volt sec. (average of all analyses in Table 2). The mobility value for the corresponding com-
ponent in normal rat liver (13) was previously found to be \(-0.99 \pm 0.09 \times 10^{-4}\) cm²/volt sec. The range of values for normal rats (18) is \(-0.35\) to \(-0.99 \times 10^{-4}\) cm²/volt sec.

### TABLE 3

<table>
<thead>
<tr>
<th>Dye fed</th>
<th>Per cent of protein-bound dye migrating with the h components†</th>
<th>Carcinogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-Me-DAB</td>
<td>10–12</td>
<td>69–75</td>
</tr>
<tr>
<td>4'-Me-DAB</td>
<td>&lt;1</td>
<td>61–75</td>
</tr>
<tr>
<td>2-Me-DAB</td>
<td>0</td>
<td>43–100</td>
</tr>
</tbody>
</table>

† Each range represents the values obtained by the two methods of computation for each experiment. The lower figure was obtained by calculation (a) and the higher figure by calculation (b) as described in the text.

The proteins combined with derivatives of 3'-Me-DAB, a very potent carcinogen, have the same electrophoretic mobility as those combined with derivatives of the very weak carcinogenic dyes 4'-Me-DAB and 2-Me-DAB indicates that (a) the three aminoazo dyes give rise to the same azo dye-containing protein(s) of the h components or (b) electrophoresis under these conditions cannot detect the existent differences between the protein(s) bound to the derivatives of these three azo dyes. It is possible that other conditions of electrophoresis or other methods could distinguish between these proteins. In any case, considerable differences in the rate of accumulation of these protein-bound dyes exist in vivo and can be correlated with the carcinogenic activities of the parent dyes (3).

The finding that the soluble proteins bound to the azo dyes migrate with the protein(s) of the h components does not necessarily mean that protein(s) of the h components from normal rat liver are bound to the azo dyes. Conceivably, soluble protein(s) of other electrophoretic components, or even insoluble proteins, may act as precursor(s) of these observed aminoazo dye-protein(s) that have the mobility range of the h components. In any case, the finding that the greater portion of the protein-bound dye travels with a small fraction (Table 2; normal rat liver [13]: h₁ = 8.0 per cent, and h₁ + h₂ = 14.7 per cent) of the total soluble liver proteins suggests that **metabolic derivatives of these aminoazo dyes bind specific protein(s)**.

### DISCUSSION

Miller and Miller and associates (1, 3, 6) have suggested that the aminoazo dyes might induce liver tumors by reacting with certain essential liver proteins (for example, those required for the control of growth) in such a manner that cells might arise which lacked these proteins. The lack of protein-bound dye in the tumors produced by the aminoazo dyes was one of the major facts which led to these general considerations. The findings in the present communication are in harmony with this protein depletion mechanism of azo dye carcinogenesis in that the proteins of the electrophoretic group, which include the aminoazo dye-protein(s), are those greatly reduced in amount in liver tumor (12). Of great general interest is the observation that the same components are also low in a variety of other tumors (18).

1 Standard deviation = \(\sqrt{\frac{\sum (d_i^2)}{n - 1}}\).

### REFERENCES


3. MILLER, E. C.; MILLER, J. A.; SAPP, R. W.; and WEBER, 

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*The per cent of the total soluble protein-bound dye migrating with the h components for rats fed 3'-Me-DAB, 4'-Me-DAB, and 2-Me-DAB in the indicated diets.*


Electrophoretic Studies on the Soluble Proteins from Livers of Rats Fed Aminoazo Dyes

Sam Sorof, Philip P. Cohen, E. C. Miller, et al.


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