TISSUE PROTEINS AND CARCINOGENESIS

II. ELECTROPHORETIC STUDIES ON SERUM PROTEINS DURING CARCINOGENESIS DUE TO AZO DYES

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It has been known that certain azo dyes will produce cancer of the liver. During the precancerous stages the liver appears to undergo some definite changes. The activity of these azo dye carcinogens is influenced by several dietary factors. Thus the liver can be protected partially from the effects of the dye by simultaneous intake of increased amounts of riboflavin (1). This conforms with the fact that administration of these azo dye carcinogens brings about a decrease in the amount of riboflavin normally present in the liver (2, 3). Feeding of \textit{m}'-methyl-\textit{p}-dimethylaminoazobenzene increases the desoxyribonucleic acid content of the liver (4). Opie (5) and Price et al. (6) have observed that a similar dye, \textit{p}-dimethylaminoazobenzene, reduces the ribonucleic acid content of this organ. Miller and Miller (7) have found that \textit{p}-dimethylaminoazobenzene or a metabolite of this compound is bound by a protein constituent of the liver. They found that this azo compound is not bound appreciably by other tissues. However, they observed low levels of bound dye in the proteins of the blood plasma.

In connection with studies on azo dye carcinogenesis, it was considered of interest to determine any changes which might occur in the proteins of the blood serum during the development of liver tumors, hence the investigations reported in this present paper.

EXPERIMENTAL

Effects of \textit{m}'-Methyl-\textit{p}-dimethylaminoazobenzene on Rat Serum

Procedure—First, electrophoretic experiments were performed on the blood serum from rats fed the active carcinogenic azo dye, \textit{m}'-methyl-\textit{p}-dimethylaminoazobenzene. The animals used were adult male albino rats of the Holtzman Sprague-Dawley strain. They were fed a purified diet (1) which contained an added 0.06 per cent of the \textit{m}'-methyl azo dye. At the end of various periods of time, the blood serum was analyzed for its protein constituents.

\footnote{1 Obtained from Holtzman Laboratory Animals, Inc., Madison, Wisconsin.}
Samples were taken from the heart upon sacrifice of the animal, groups of three to five rats being used. The samples were allowed to clot and were then centrifuged. The serum was pooled and diluted to 3 times its volume with a sodium diethyl barbiturate buffer of ionic strength 0.1, and a pH of 8.3. The solution so obtained was poured into a Nojax cellulose sausage casing bag and dialyzed against 1.8 liters of the same buffer for 15 or more hours until a Donnan equilibrium was attained.

The electrophoretic experiments were carried out in the Tiselius apparatus with an 11 cm. cell. The cell was filled with the protein and buffer solutions in a cold room at 1-3°C and then allowed to equilibrate in a water bath at 0.6°C, the temperature at which the electrophoresis was performed. As the serum proteins are all negatively charged at the pH used, the protein boundaries separated and migrated toward the anode. In general, the experiments were continued until the fastest moving albumin component had traveled the length of the cell. The potential gradients used came within the range of 4 to 6 volts per cm.; the lengths of the experiments were 18,000 to 25,200 seconds when the lower potential gradient was used. The initial and final boundary patterns were photographed by the schlieren scanning method of Longsworth (9).

Mobilities—The electrophoretic mobilities of the proteins of the serum were calculated from the descending pattern by measuring their migration distances from the ε, buffer-dilution boundary. Conductivity measurements were made on the outer buffer solution used for dialysis. The mobilities for the components of normal rat serum were calculated. The averages of the values for six experiments, together with the average deviation, are given for each component: albumin 5.6 ± 0.1, α-globulin anomaly 3.5 ± 0.2, β-globulin 2.6 ± 0.1, γ-globulin 1.5 ± 0.1, all in units of 10^{-5} \text{ cm.} \times (\text{volts per cm.} \times \text{second})^{-1}. An α-globulin anomaly reported by others (10, 11) was generally observed in the descending boundary pattern. Its mobility was calculated in the four out of six cases in which it appeared as a means of identification. The above mobilities appear to be in line with data given by Deutsch and Goodloe (10) and Moore (11) for rat serum at pH 8.6. The values given here are slightly lower, due to the lower pH of 8.3 used.

The mobilities calculated for the components of the many samples of

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2 The sodium diethyl barbiturate buffer of Longsworth (8), ionic strength 0.1, and calculated to have a pH of 8.3: 0.050 N NaV-0.020 N HV-0.050 N NaCl, where V = diethyl barbiturate.

3 Obtained from the Visking Corporation, 6733 West 65th Street, Chicago 38, Illinois.

4 A modified Klett electrophoresis apparatus.

5 Average of four values.
Composition—The relative protein composition of each sample was determined. Since the area under each peak is proportional to the total concentration of that component, the percentage of each serum protein present was determined by resolving the pattern into a series of symmetrical curves. The total protein concentration was calculated from Kjeldahl nitrogen analyses. The average value obtained from twenty-nine of the undiluted serum samples was 5.41 gm. of protein per 100 ml. The average deviation was ±0.52 gm. per 100 ml; the range, 4.15 to 6.61 gm. per 100 ml. Two typical electrophoretic patterns are shown. Fig. 1 shows a pattern obtained for serum from normal rats and a characteristic diagram for serum from rats in receipt of the \( m' \)-methyl azo dye. It can be seen that the relative amount of albumin decreased, whereas the percentage of \( \gamma \)-globulin definitely increased in this second case.

The data obtained for the percentage composition of normal serum and serum from rats receiving the \( m' \)-methyl-\( p \)-dimethylaminoazobenzene are given in Table I. Since the \( \alpha \)-globulin peaks were ill defined, their areas were estimated together as the total amount of \( \alpha \)-globulin. The average values\(^6\) (and the ranges) for the components of normal rat serum, viz. 66 per cent albumin, 14 per cent \( \alpha \)-globulin, 13 per cent \( \beta \)-globulin, and 6 per cent \( \gamma \)-globulin, agree essentially with the values found by Deutsch and Goodloe (10) and Moore (11). In general, three serum samples from

\(^6\) Unless otherwise indicated the serum protein percentages reported in this paper are proportional values and not absolute concentrations.
TISSUE PROTEINS AND CARCINOGENESIS. II

the rats on the \( m'\)-methyl azo dye were analyzed after each 2 week interval of feeding. At the end of the first 2 weeks the relative amount of \( \gamma \)-globulin in the serum had definitely increased; the average value was 19 per cent, the range 13 to 27 per cent. The percentage of this component remained high as long as the dye was fed. The values found for the \( \alpha \)- and \( \beta \)-globulin components during the entire 8 week feeding period were normal. The percentage of serum albumin, however, had definitely decreased at the end of 2 weeks to an average value of 53 per cent (44 to 61 per cent). The

<table>
<thead>
<tr>
<th>Table I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of ( m')-Methyl-( p )-Dimethylaminoazobenzene on Percentage Composition of Rat Sera by Electrophoretic Analysis*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Diet</th>
<th>Albumin</th>
<th>( \alpha )-Globulin</th>
<th>( \beta )-Globulin</th>
<th>( \gamma )-Globulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>Range</td>
<td>Average</td>
<td>Range</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>6</td>
<td>Normal</td>
<td>66</td>
<td>60-74</td>
<td>14</td>
<td>11-26</td>
</tr>
<tr>
<td>1</td>
<td>1 wk., ( m')Me-DABS†</td>
<td>71</td>
<td>11</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>2 wks., &quot;</td>
<td>53</td>
<td>44-61</td>
<td>15</td>
<td>12-16</td>
</tr>
<tr>
<td>3</td>
<td>4 &quot;</td>
<td>55</td>
<td>54-56</td>
<td>14</td>
<td>13-16</td>
</tr>
<tr>
<td>3</td>
<td>6 &quot;</td>
<td>58</td>
<td>55-60</td>
<td>14</td>
<td>13-16</td>
</tr>
<tr>
<td>3</td>
<td>8 &quot;</td>
<td>55</td>
<td>54-58</td>
<td>16</td>
<td>15-17</td>
</tr>
<tr>
<td>1</td>
<td>8 &quot; + 2 wks., normal (hepatomas)</td>
<td>56</td>
<td>18</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>10 wks., ( m')Me-DAB + 4 wks., normal (hepatomas)</td>
<td>53</td>
<td>25</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>2 wks., ( m')Me-DAB + 1 wk. (normal)</td>
<td>66</td>
<td>17</td>
<td>14</td>
<td>3</td>
</tr>
</tbody>
</table>

* Buffer, sodium diethyl barbiturate, ionic strength 0.1, pH 8.3 (8.21-8.40), potential gradient, 4 to 6 volts per cm.
† Percentages are the fractional areas of the electrophoretic diagrams due to each component, and represent the percentages of the total amount of protein in the serum present as these components.
‡ \( m'\)-Methyl-\( p \)-dimethylaminoazobenzene, 0.06 per cent in the diet.

relative amount of albumin remained low as long as the dye was continued. Administration of the carcinogenic \( m'\)-methyl-\( p \)-dimethylaminoazobenzene over a period of time results in an increased percentage of serum \( \gamma \)-globulin and a concurrent decrease in serum albumin.

Serum from animals to which the dye had been fed for only 1 week was analyzed. The values obtained (Table I) show that the percentage distribution of serum proteins was normal at the end of this period of time.

Two electrophoretic experiments were performed on sera from animals which had developed liver tumors. In the first case, the rats developed
tumors after being fed the diet containing the \textit{m}'-methyl azo dye for 8 weeks followed by a normal diet for 2 weeks. In the second case, the tumors had developed after 10 weeks on the diet containing the \textit{m}'-methyl dye followed by 4 weeks on a normal diet. The percentage of \(\gamma\)-globulin in both of these samples was in the upper part of the normal range, while the percentage of albumin was still somewhat low. The relative amount of \(\alpha\)-globulin appeared to be high in the second sample, although one normal sample had been obtained with an equally high concentration. It is interesting to compare these results with those obtained by Seibert \textit{et al.} (12) from electrophoretic analyses of sera from clinical cases of carcinoma. They found no increase, and often a decrease, in \(\gamma\)-globulin. In cases with metastases to the liver which resulted in jaundice, however, they found an abnormally high \(\gamma\)-globulin. The \(\alpha_2\)-globulin was found to be increased in practically all cases, while the concentration of albumin has been found to be low in carcinoma sera (12, 13).

In an effort to determine whether the drop in the \(\gamma\)-globulin of the sera from the rats with liver tumors could be due to the return to a normal diet, another electrophoretic analysis was made of serum from animals fed the azo dye diet for 2 weeks, followed by a normal diet for the succeeding 3rd week (Table I). In this case, the relative amounts of proteins were normal. Evidently the composition of the blood serum returns rapidly to normal when feeding of the carcinogenic azo dye is discontinued.

\textit{Effects of Structurally Related Azo Compounds and One Non-Azo Compound}

Electrophoretic studies were made of sera obtained from rats fed two other azo compounds related structurally to the \textit{m}'-methyl azo dye and one non-azo, but carcinogenic, compound, 2-acetylaminofluorene.

\textit{Azo Compounds}—The serum from rats which had been fed \(p\)-dimethylaminoazobenzene was studied. This compound is intermediate in carcinogenic activity between the active \textit{m}'-methyl-\(p\)-dimethylaminoazobenzene and the relatively non-active azobenzene. The tabulated results (Table II) show that the percentage of \(\gamma\)-globulin found in the serum samples after 4 weeks and 8 weeks of feeding was slightly above the normal values. At the end of 3 months of feeding, the serum composition had apparently returned to normal. Administration of an increased amount of \(p\)-dimethylaminoazobenzene in the diet (0.09 per cent as compared to the usual 0.06 per cent) resulted in approximately 13 per cent of \(\gamma\)-globulin at the end of 2 week and 4 week periods. These amounts were the same as those found with the lower concentration of carcinogen.

The relatively non-carcinogenic compound, azobenzene, produced sera that were essentially normal at the end of 2 and 4 week intervals. The values of 11 and 12 per cent obtained for the \(\gamma\)-globulin were slightly above
normal. It appears from the electrophoresis of sera from rats fed these three structurally related azo compounds that the magnitude of the increase in the percentage of $\gamma$-globulin is in line with the relative order of activity of the carcinogenic agents. The most active derivative, $m'$-methyl, definitely produced the most marked effect.

**Non-Azo Compound, 2-Acetylaminofluorene**—Two electrophoretic analyses were made of serum from animals which had been fed the non-azo com-

| Table II |

| Effects of Three Azo Compounds and One Non-Azo Carcinogen on Percentage Composition of Rat Sera |

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Diet</th>
<th>Time wks.</th>
<th>Albumin Average Range per cent</th>
<th>$\alpha$-Globulin Average Range per cent</th>
<th>$\beta$-Globulin Average Range per cent</th>
<th>$\gamma$-Globulin Average Range per cent</th>
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</thead>
<tbody>
<tr>
<td>6</td>
<td>Normal</td>
<td>66</td>
<td>60-74</td>
<td>14</td>
<td>11-26</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>m'Me-DAB†</td>
<td>4</td>
<td>55</td>
<td>54-56</td>
<td>14</td>
<td>13-16</td>
</tr>
<tr>
<td>1</td>
<td>DAB‡ (0.06%)</td>
<td>4</td>
<td>61</td>
<td>14</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>1</td>
<td>“ (0.06%)</td>
<td>8</td>
<td>59</td>
<td>18</td>
<td>12</td>
<td>12</td>
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<tr>
<td>2</td>
<td>“ (0.09%)</td>
<td>12</td>
<td>66</td>
<td>63-70</td>
<td>16</td>
<td>15-17</td>
</tr>
<tr>
<td>1</td>
<td>“ (0.09%)</td>
<td>2</td>
<td>61</td>
<td>14</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>1</td>
<td>“ (0.09%)</td>
<td>4</td>
<td>57</td>
<td>17</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>Azo benzene</td>
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<td>61</td>
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<td>16</td>
<td>16-17</td>
</tr>
<tr>
<td>1</td>
<td>AAF§</td>
<td>2</td>
<td>63</td>
<td>16</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>1</td>
<td>“</td>
<td>6</td>
<td>62</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

* Percentages are the fractional areas of the electrophoretic diagrams due to each component, and represent the percentages of the total amount of protein in the serum present as these components.
† $m'$-Methyl-$p$-dimethylaminoazobenzene.
‡ $p$-Dimethylaminoazobenzene.
§ 2-Acetylaminofluorene.

This compound is an active carcinogen; it produces cancer of the liver as well as of other tissues. The analyses showed that at the end of a 2 week and a 6 week interval a normal proportion of 8 per cent of $\gamma$-globulin was present. The values for the other serum components were also normal. The absence of any effect of this carcinogen on the blood serum, compared to the increase in $\gamma$-globulin caused by the azo dye carcinogens, is noteworthy. However, it may be that longer administration of 2-acetylaminofluorene would produce changes in the composition of the serum.
DISCUSSION

Administration of the active carcinogenic azo dye, \( m'-methyl-p \)-dimethylaminoazobenzene, in the diet of rats over a period of time resulted in a definite increase in the serum \( \gamma \)-globulin accompanied by a decrease in serum albumin. These results may be considered in the light of other studies related to this work. It has been found, in general, that many pathological conditions result in a reduction of serum albumin and an increase in globulin, usually \( \gamma \)-globulin (13–17). It is also well known that formed antibodies travel with the \( \gamma \)-fraction (18–25). Thus an increased \( \gamma \) component is often associated with antibody formation. Gray and Barron (26) and others (27–29) have shown that liver disease in humans results in a decrease of albumin with a compensatory increase in globulin, usually \( \gamma \). The magnitude of these changes depends on the degree of liver damage. Therefore, the decreased amount of albumin and the increased \( \gamma \)-globulin content found in the sera of rats fed carcinogenic azo dyes are non-specific changes. However, it appears very interesting that these changes can be correlated with the order of carcinogenic activity of these compounds.

It is also of interest to note the time sequence of liver protein response. The electrophoretic experiments on the blood sera of rats fed \( m'-methyl-p \)-dimethylaminoazobenzene showed marked changes in protein composition at the end of 2 weeks. The first visible signs of liver cirrhosis were observed at 4 weeks. Miller and Miller (7) have found that the binding of the carcinogenic dye, \( p \)-dimethylaminoazobenzene, first occurs in 4 days. The maximum amount of this dye is found to be bound in the liver at 4 weeks; then the amount of bound dye slowly diminishes to a very small amount at 20 weeks, although the dye is continually being ingested. Since the \( m'-methyl \) azo dye is a more active carcinogen, it also might be expected to be bound in the liver within 4 days at least.

The protein composition of sera from rats on the carcinogenic non-azo-2-acetylaminofluorene was normal after 2 and 6 weeks. Although the livers of these animals were not completely normal, very little damage could be observed visually.

SUMMARY

Electrophoretic studies of the relative protein composition of blood serum from rats fed carcinogenic azo dyes led to the following results.

1. Three azo compounds, \( m'-methyl-p \)-dimethylaminoazobenzene, \( p \)-dimethylaminoazobenzene, and azobenzene, and the non-azo carcinogen, 2-acetylaminofluorene, had no noticeable effect on the mobilities of the serum proteins.
2. Administration of the active carcinogen, \( m'\)-methyl-\( p\)-dimethylaminoazobenzene, in the diet resulted in an increased percentage of \( \gamma \)-globulin and a decreased percentage of albumin in the blood serum at the end of 2 weeks. These changes were observed as long as the feeding of the dye was continued. The composition of the serum returned to normal 1 week after the dye was discontinued.

3. Feeding of a non-azo carcinogen, 2-acetylaminofluorene, produced no apparent change in the serum composition after 6 weeks.

4. Comparative studies were made of the effects of three structurally related azo compounds on the composition of the serum. \( m'\)-Methyl-\( p\)-dimethylaminoazobenzene is strongly active as a carcinogen, \( p\)-dimethylaminoazobenzene is intermediate, and azobenzene is relatively non-active. The \( m'\)-methyl derivative definitely produced the most marked effects on the relative amounts of serum albumin and \( \gamma \)-globulin. The \( p\)-dimethylaminoazobenzene produced a much smaller effect, while the azobenzene produced little, if any. The magnitude of the changes produced by these azo dyes appears to be in the order of their carcinogenic activity.

It is with pleasure that the authors acknowledge Miss Eleanore Frey's contribution to this research by conducting the series of Kjeldahl nitrogen determinations. We wish to express our appreciation to the American Cancer Society, the Rockefeller Foundation, and the United States Public Health Service for grants which helped to make this work possible.

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