CHANGES IN THE ACTIVITIES OF GLUCOSE-6-PHOSPHATASE, ALDOLASE, AND ALKALINE PHOSPHATASE DURING AZO-DYE CARCINOGENESIS*1

(Plate IV)

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Synopsis

The activities of glucose-6-phosphatase, aldolase, and alkaline phosphatase were measured in rat liver during the early stage in the feeding of 3’-methyl-4-(dimethylamino)azobenzene. Enzyme activities changed quantitatively and qualitatively according to histological alteration in the cell population.

The fluctuation of enzyme activities occurred in three stages. The first stage was observed within 3 weeks after dye feeding. Activities of three enzymes decreased markedly, except for a slight increase in Mg2+-insensitive alkaline phosphatase at the second week. The second stage was seen between 4 and 8 weeks with a peak at the 6th week. Enzyme activities were elevated, and the muscle-type aldolase and Mg2+-insensitive alkaline phosphatase increased. In the third stage, after 8 weeks on, the altered patterns of these enzymes were maintained in hepatocyte fractions throughout the precancerous stage. It was suggested that the hepatocytes regenerated during 4〜8 weeks acquire different characters from those of the original hepatocytes.

INTRODUCTION

In the preceding report,10) we described the histological and electron microscopic alteration of rat liver in the course of hepatocarcinogenesis induced by 3’-methyl-4-(dimethylamino)azobenzene (3’-Me-DAB). The significant observation on the early stage of hepatocarcinogenesis by 3’-Me-DAB was the regeneration of the hepatic tissue through transformation of proliferated cholangiolar cells.

Under our experimental conditions, the proliferation of cholangiolar cells became a maximum in the 4th week followed by increase in the number of small hepatocytes which commenced to appear among and adjacent to cholangiolar cells. Following the increase in number and size of small hepatocytes, the ability of dye binding and the glucose-6-phosphatase activity elevated, whose peaks were seen in the 6th week. After 8 weeks of dye feeding, most of the original hepatocytes appeared to be replaced by these renewed hepatocytes. Therefore, it seemed of interest to elucidate whether these regenerated hepatocytes possess different characters from normal hepatocytes or not, and how the variation in cell population would reflect on biochemical activities in the liver.

*1 This constitutes Part II of a series entitled “Precancerous Changes in Rat Liver during the Early Stage of 3’-Methyl-4-(dimethylamino)azobenzene Carcinogenesis.” Part I10);
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For this purpose, activities of glucose-6-phosphatase (d-glucose-6-phosphate phosphohydrolase; EC 3.1.3.9), aldolase (ketose-1-phosphate aldehyde-lyase; EC 4.1.2.7), and alkaline phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.1) were examined. As an indicator of the function of hepatocytes, glucose-6-phosphatase activity was determined, since in the liver this enzyme is known to localize exclusively in hepatocytes. The aldolase activity was assayed to examine the precancerous state, because Endo et al. reported that the isozyme pattern of aldolase changes irreversibly in the early stage of 3'-Me-DAB feeding. The activity of alkaline phosphatase was examined because Allard et al. suggested that the change of this enzyme activity in the liver of rats fed 4-(dimethylamino)azobenzene (DAB) is associated with the regenerative process after liver damage by the ingestion of DAB.

Materials and Methods

Male Wistar rats, weighing 170~200 g, were used. They were divided into 4 groups according to the feeding conditions.

Group 1: Control, 15 rats were fed Oriental solid diet (Oriental Yeast Co., Tokyo).
Group 2: About 100 rats, fed Oriental diet containing 0.06% 3'-Me-DAB.
Group 3: 30 rats, fed on the dye-diet for 8 weeks and then on basal diet for 3 days, and 1, 2, 4, or more weeks.
Group 4: 15 rats, fed on the dye-diet for 12 weeks and then on a basal diet for 8 weeks.

Preparation of Homogenates Rats were sacrificed by decapitation. Small pieces of the liver were rapidly cut off for histological examination. The remainder of each liver was placed in an ice-cold 0.25M sucrose, and homogenized with a glass-Teflon homogenizer to make a 20% (w/v) homogenate in 0.25M sucrose solution. The hepatocyte homogenate was prepared from the hepatocyte fraction isolated by the method of Jacob and Bhargava.

Enzyme Assays The activity of glucose-6-phosphatase (G-6-Pase) was measured by essentially the same method as that of Swanson, except for the use of 0.02M Tris-maleate buffer (pH 6.75). The reaction mixture contained glucose 6-phosphate (Sigma) and MgCl₂ at a concentration of 0.025M and 0.005M, respectively. The incubation was made at 37°C for 10 min. Released inorganic phosphorus (Pi) was measured by the method of Fiske and Subbarow.

The activity of alkaline phosphatase was assayed in a medium containing 0.01M Veronal buffer (pH 9.3) and 0.01M β-glycerophosphate as a substrate with and without addition of MgCl₂ (0.005M) as described previously. The reaction mixture was incubated at 37°C for 20 min. The ratio of the activity of fructose diphosphate (FDP) to fructose monophosphate (FMP) was obtained. Electrophoresis of aldolase with the tissue extract on a cellulose acetate membrane (Sepaphore III, Gelman, 2.5 × 17 cm) was carried out using a Veronal buffer (pH 8.6, I = 0.06) containing 10mM β-mercaptoethanol at 250 V for 2 hr. After electrophoresis, the strips were stained for aldolase activity by the method of Masters.
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Matsushima et al. All the reagents for aldolase assay and staining were purchased from the Sigma Chemical Co. (St. Louis).

**Determination of Protein** The protein content was determined according to Lowry et al., using bovine serum albumin (Armour) as a standard.

**RESULTS**

**Change in Glucose-6-phosphatase Activity**

Fig. 1 indicates that the G-6-Pase activity decreased rapidly after 3'-Me-DAB feeding and, by the third week, the activity was about 25% of the control. At the 6th week it increased to 40% of the control and, thereafter, it showed small fluctuation. After cessation of dye feeding at the 8th week (Group 3), the activity increased, but it never recovered to the control value.

![Fig. 1. Glucose-6-phosphatase activity during 3'-Me-DAB feeding](image)

Each point represents average value of 5 rats, and vertical bars indicate standard deviation.

**Change in Aldolase Activity**

The aldolase activity was measured using both FDP and FMP as a substrate. As shown in Fig. 2, the fluctuation pattern of FMP activity was similar to that of G-6-Pase activity, but the FMP activity scarcely increased by cessation of the dye feeding in contrast to the G-6-Pase activity. The degree of inhibition of FDP activity by dye feeding was less than that of FMP activity and, when dye feeding was stopped, the FDP activity rose to the control level. Consequently, the ratio of FDP/FMP increased during dye feeding as shown in Fig. 3. Three peaks of activity ratio occurred at 3, 6, and 12 weeks of dye feeding.

Electrophoretic pattern showed that the muscle-type aldolase in the liver of rats fed the dye for 4 weeks exhibited a stronger band than that of normal liver (Fig. 4). This strong activity of muscle-type aldolase persisted even when dye feeding was stopped after 8 weeks. When the cellulose membrane was stained by FMP as a substrate, only one band was detected at the site of liver-type aldolase which was stained by FDP.
Fig. 2. Change in aldolase activity

- - - - and - - FDP activity of aldolase;  and - - FMP activity.

Fig. 3. Activity ratio of aldolase (FDP/FMP)

Values were calculated from the data in Fig. 2.
PRE CANCEROUS CHANGES IN ENZYME ACTIVITY

Change in Alkaline Phosphatase Activity

The activity of alkaline phosphatase was measured both with and without addition of MgCl₂ in the medium. As shown in Fig. 5, the activity with MgCl₂ fluctuated in almost the same pattern as that of FDP activity of aldolase, while the activity without MgCl₂ increased and maintained a higher level than the control. The activity ratio of that without Mg²⁺ to that with Mg²⁺ elevated during dye feeding, with peaks at 2, 6, and 9 weeks (Fig. 6).

![Fig. 4. Electrophoretic pattern of aldolase](image)

The liver extracts were subjected to electrophoresis on cellulose acetate membrane.

- a: Normal liver of rat, b: Group 2, 2 weeks, c: Group 2, 4 weeks, d: Group 2, 12 weeks, e: Group 3, 4 weeks after cessation of dye, f: The muscle-type aldolase prepared from a normal rat, g: Normal liver whose activity was stained by FMP. a–f show FDP-activity stained by FDP as a substrate.

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![Fig. 5. Activity of alkaline phosphatase](image)

- - - and ○—○ Alkaline phosphatase activity with addition of MgCl₂ (5mM).
- - - - and ○—○ Activity without addition of MgCl₂.

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Activities of Glucose-6-phosphatase and Alkaline Phosphatase in the Liver of Immature Rats and in Hepatic Tumors Induced by 3'-Me-DAB

Table I indicates that the G-6-Pase activity in the fetal liver on the 20th day of gestation was lower than that of the normal adult liver, while the alkaline phosphatase activity

<table>
<thead>
<tr>
<th>Liver</th>
<th>No. of rats</th>
<th>Protein (mg/g liver)</th>
<th>G-6-Pase μmoles</th>
<th>Alkaline phosphatase with MgCl₂ (5 mM)</th>
<th>Alkaline phosphatase without MgCl₂</th>
<th>Ratio of activity, −Mg²⁺/+Mg²⁺ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal adult</td>
<td>5</td>
<td>201 ± 18.3a</td>
<td>56.6 ± 3.93</td>
<td>8.7 ± 0.54</td>
<td>2.2 ± 0.21</td>
<td>25.4</td>
</tr>
<tr>
<td>Fetal (1 day before birth) (pooled)</td>
<td>9</td>
<td>137</td>
<td>26.9</td>
<td>23.1</td>
<td>33.7</td>
<td>145.5</td>
</tr>
<tr>
<td>Newborn (5 days)</td>
<td>5</td>
<td>155</td>
<td>30.7</td>
<td>16.9</td>
<td>8.5</td>
<td>50.4</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2, 23 weeks</td>
<td>1</td>
<td>211</td>
<td>25.9</td>
<td>6.5</td>
<td>2.8</td>
<td>43.1</td>
</tr>
<tr>
<td>Tumor-free area</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td></td>
<td>127</td>
<td>5.4</td>
<td>7.7</td>
<td>8.2</td>
<td>106.5</td>
</tr>
<tr>
<td>Group 2, 25 weeks</td>
<td>1</td>
<td>104</td>
<td>2.7</td>
<td>7.9</td>
<td>9.2</td>
<td>116.5</td>
</tr>
<tr>
<td>Tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2, 33 weeks</td>
<td>1</td>
<td>207</td>
<td>26.1</td>
<td>7.9</td>
<td>1.9</td>
<td>24.1</td>
</tr>
<tr>
<td>Tumor-free area</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td></td>
<td>151</td>
<td>1.9</td>
<td>3.7</td>
<td>3.7</td>
<td>100.0</td>
</tr>
</tbody>
</table>

a) Mean ± standard deviation.
was higher, especially the Mg$^{2+}$-insensitive activity showing a higher value. Newborn liver showed an intermediate activity between those of the adult and fetal liver.

Each of the tumors induced by 3'-Me-DAB showed a negligible G-6-Pase activity and their alkaline phosphatase activity was the Mg$^{2+}$-insensitive type. On the other hand, the non-cancerous portion of the tumor-bearing liver possessed a lower, but appreciable activity of G-6-Pase, and a normal level of alkaline phosphatase activity. These results show that the higher activity of Mg$^{2+}$-insensitive alkaline phosphatase is a common character of both immature liver and liver tumor, while the deficiency of G-6-Pase is one of the characteristics of a liver tumor.

**Enzyme Activities in the Separated Hepatocyte Fraction**

The hepatic tumor was not observed in rats of Group 3, while in Group 4 the tumors were found in 3 out of 9 rats. The hepatocyte fractions were prepared from the liver of rats in these two groups. The G-6-Pase activity in hepatocytes still persisted at the lower level than that of normal hepatocytes, in spite of withdrawal of the dye from the diet. The total alkaline phosphatase activity in hepatocyte fraction, which was measured with the addition of MgCl$_2$, hardly changed, while the Mg$^{2+}$-insensitive activity increased gradually (Table II). Moreover, the activity ratio of aldolase increased in the hepatocytes.

<table>
<thead>
<tr>
<th>Weeks fed on 3'-Me-DAB diet</th>
<th>nmoles G-6-Pase/min/mg protein</th>
<th>Alkaline phosphatase with MgCl$_2$ (5mM$^2$)</th>
<th>Alkaline phosphatase without MgCl$_2$</th>
<th>Ratio of activity (Mg$^2+$/Mg$^+$)</th>
<th>Aldolase FDP/FMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (4)</td>
<td>286±16</td>
<td>36.2±4.8</td>
<td>9.4±2.6</td>
<td>25.9±1.1</td>
<td>1.24±0.11</td>
</tr>
<tr>
<td>8-4 (3)</td>
<td>232±22</td>
<td>40.8±3.0</td>
<td>18.9±4.9</td>
<td>45.4±3.6</td>
<td>1.82±0.28</td>
</tr>
<tr>
<td>12-8 (4)</td>
<td>219±19</td>
<td>33.4±2.8</td>
<td>12.1±1.0</td>
<td>35.8±4.7</td>
<td>1.54±0.27</td>
</tr>
<tr>
<td>Hepatocyte fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (4)</td>
<td>436±49</td>
<td>13.5±2.6</td>
<td>7.4±2.2</td>
<td>55.0±3.6</td>
<td>1.02*</td>
</tr>
<tr>
<td>8-4 (3)</td>
<td>403±13</td>
<td>16.1±4.1</td>
<td>10.2±3.3</td>
<td>62.0±1.5</td>
<td>—</td>
</tr>
<tr>
<td>12-8 (4)</td>
<td>368±30</td>
<td>14.3±1.1</td>
<td>12.2±4.3</td>
<td>84.7±1.2</td>
<td>1.51*</td>
</tr>
</tbody>
</table>

The results are given as mean±standard deviation. Figures in parentheses indicate number of animals.

* Hepatocyte homogenates were pooled and centrifuged to obtain the soluble fraction for aldolase assay.

Photos 1 and 2 show the size of hepatocytes separated from normal liver and the liver of a rat in Group 4. Compared with normal cells (Photo 1), variously sized hepatocytes were observed and small hepatocytes having a diploid nucleus were seen in the treated liver (Photo 2). The histochemical staining of G-6-Pase of these fractions showed that the small hepatocytes possessed less G-6-Pase activity.

**Discussion**

During 3'-Me-DAB feeding for 13 weeks, the alteration of hepatocytes showed three stages as regards their histological behavior and enzyme activities.
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The first stage was up to 4 weeks after starting of dye feeding. Many hepatocytes degenerated markedly and some of them disappeared. Subsequently, the proliferated cholangiolar cells took the place of the degenerated hepatocytes. Such quantitative changes in hepatocytes were represented by the decrease of G-6-Pase activity and aldolase activity which was measured with FMP, because these activities are mainly localized in hepatocytes.

The second stage occurred in 4~8 weeks with a peak at the 6th week, at which the small hepatocytes proliferated markedly. Although the enzyme activities changed after variation in the cell population of the hepatic tissue, qualitative change of enzymes in the hepatocytes seems to be more significant than their quantitative change. The patterns of aldolase and alkaline phosphatase altered in the hepatocyte fractions.

As for aldolase, both activities with FDP and FMP decreased in the first stage and, when activities elevated in the second stage, the electrophoretic pattern altered to increase the muscle-type aldolase. Although the change in aldolase activity resembled that of G-6-Pase activity, FMPase activity did not recover like G-6-Pase activity when the dye was withdrawn from the diet. The irreversible alteration of aldolase pattern in the precancerous stage which has been reported by Endo et al. seems to occur in these renewed hepatocytes.

Concerning alkaline phosphatase, two peaks of Mg\textsuperscript{2+}-insensitive activity appeared after 2 and 6 weeks of dye feeding. The first peak appears to be derived from the increase of this activity in the proliferating cholangiolar cells or biliary epithelium. In this connection, we have reported the increase of Mg\textsuperscript{2+}-insensitive activity when cholangiolar cells proliferated after biliary ligation. The time of second peak corresponded to that of G-6-Pase and aldolase, and elevated ratio of the activity of Mg\textsuperscript{2+}-insensitive to that of Mg\textsuperscript{2+}-sensitive (= total alkaline phosphatase activity) persisted irreversibly until later, like aldolase pattern. As mentioned in the Introduction, Allard et al. suggested the activity of Mg\textsuperscript{2+}-insensitive alkaline phosphatase to increase in neoplastic liver. They studied the liver of rats fed DAB and Mg\textsuperscript{2+}-insensitive activity increased after 80 days of dye feeding, while in our study the Mg\textsuperscript{2+}-insensitive activity increased in much earlier period of 3'-Me-DAB feeding. However, both results are in agreement with the suggestion that Mg\textsuperscript{2+}-insensitive activity increases during the regenerative process after liver degeneration by dye feeding.

Variation in the isozyme pattern of acid phosphatase which showed a similar change in this second stage will be described elsewhere. As shown in Table I, the liver in this stage resembled both fetal liver and hepatoma as regards the pattern of aldolase, alkaline phosphatase, and also acid phosphatase, while the deficiency of G-6-Pase activity was more conspicuous in tumor than in the fetal liver. On the other hand, the renewed small hepatocytes seem to differentiate and to mature toward the normal adult cells as a part of cell functions, because their growth was accompanied by the increase of G-6-Pase activity and the ability of dye binding, whose peaks appeared in the 6th week.

Although Hugues reported the development of hepatic tumors in the rats fed 3'-Me-DAB for 5 weeks, we did not find any tumor in the rats fed the dye for 5~8 weeks. It appears that the discrepancy between these results is due to the difference in the strain of rats and the diet used.
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The third stage of changes was seen after 9 weeks. Under our conditions, hepatic tumors developed in rats fed normal diet for 8 weeks after cessation of the dye feeding in this stage (Group 4). According to Berenblum and Boutwell, it seems that the cells in the second stage correspond to the so-called "initiated cells" and some promoting factors are needed for the conversion of initiated cells into the tumor cells, and this step may occur in this stage.

Therefore, it is expected that the cells in the second and third stages possess different characters in the process of carcinogenesis. However, it is difficult to study this phenomenon biochemically at the tissue level, because it is considered histologically that only a few cells in the hyperplastic area acquire an ability for autonomous propagation to become cancer cells. Further experiment on the level of separated cells is necessary to clarify this point.

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EXPLANATION OF PLATE IV

Photo 1. The hepatocyte fraction separated from the normal liver. The hepatocyte fraction consists of binucleated tetraploid cells, mononucleated tetraploid cells, and binucleated diploid cells. The size of these cells appears to be similar. Giemsa stain. ×250.

Photo 2. The hepatocyte fraction separated from the liver of a rat fed normal diet for 8 weeks after 12 weeks of 3'-Me-DAB feeding. Various sized hepatocytes are seen. Compared with the normal, very small mononucleated diploid cells and also enlarged hyperploid cells are conspicuous. Almost all of hepatocytes appear to be mononucleated. Giemsa stain. ×250.