Irreversible Fixation of Increased Level of Muscle Type Aldolase Activity Appearing in Rat Liver in the Early Stage of Hepatocarcinogenesis

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SUMMARY

With the use of antimuscle aldolase sera, muscle type aldolase activity in the liver of adult rats was determined during a 60-day interval after stopping the administration of a diet containing 0.06% 3'-methyl-4-dimethylaminoazobenzene for 60 days.

The muscle type aldolase in the livers of rats thus treated was found to be elevated when compared to normal liver, although no appreciable differences were observed in total aldolase activity, mitotic index, histological architecture, and soluble protein, as well as DNA content.

The appearance of this enzymic change was already observable by administrating the azo dye only for 15 days.

The increased level of muscle type aldolase activity observed in the liver of rats fed azo dye for 60 days was maintained during a 300-day observation period.

A similar enzymic alteration could be induced by another hepatocarcinogen, N,N'-2,7-fluorenylenebisacetamide, whereas a noncarcinogen, 2-methyl-4-dimethylaminoazobenzene, failed to induce this change.

INTRODUCTION

In 1949, Clayton and Bauman (4) observed a high incidence of hepatoma in rats when they administered a submanifestational dose of 3'-Me-DAB, which was followed by no carcinogen and then a subsequent administration of the same dose after a 12-week interval.

Similar findings were also obtained by Nakahara and Fukuoka (16) using 2 different carcinogens affecting the same target organ; 20-methylcholanthrene and 4-nitroquinoline 1-oxide. In this case, the administration of submanifestational doses of the respective carcinogens at 200-day intervals resulted in skin cancer production in mice.

The results in both groups seem to suggest that the first exposure to the carcinogenic stimuli causes an irreversible change in the cell, and that this change is maintained in the cell during the long subsequent period during which a carcinogen-free diet is administered.

The present work was undertaken to see whether such an irreversible change involved in the transformation of the cells to the malignant state does indeed exist and, if so, how might it be expressed biochemically. We have investigated the aldolase isozyme pattern during carcinogenesis. Aldolase in the liver has been found to be composed of 2 isoforms, liver type aldolase (EC 4.1.2.7) and muscle type aldolase. The latter was shown to be at least immunologically identical with muscle aldolase (EC 4.1.2.13) (3, 18), which differs from liver type aldolase in substrate specificity (3, 7, 8, 21-23) and in kinetic (26), and immunological properties (3). Especially noteworthy are the findings of Schapira et al. (24), Sugimura et al. (27), and Adelman et al. (1), who found that the activity of liver type aldolase is much higher than that of muscle type aldolase in normal liver and that the converse is true in poorly differentiated malignant liver tumor. These findings led us to presume that the process of hepatocarcinogenesis might eventually be analyzed quantitatively by using the aldolase isozyme pattern.

In analyzing biochemical changes occurring during chemical carcinogenesis, it seems especially desirable to eliminate the problem of nonspecific toxic effects of the carcinogen on the target organ. Otherwise, the possibility cannot be excluded that the results obtained may be a manifestation of the toxic effect of the carcinogen and may be unrelated to transformation of the cells. The toxic effect of carcinogens, similar to the pharmacological effect of drugs in general, disappears after the administration of carcinogens ceased. With this point of view, attention has been given in this study to the performance of the biochemical analysis on the target organ of experimental animals at a sufficient time interval after stopping the administration of the carcinogen so that only irreversible changes would be observed.

MATERIALS AND METHODS

Animals. Male albino rats (Wistar strain, 150 to 200 g) were separated into 5 groups, housed in stainless steel cages (5 rats/cage), and fed diet and water ad libitum.
The basal diet, CE-2 from Nippon Clea Company, Ltd. (Osaka, Japan), consisted of the following parts per 100: water, 60; crude carbohydrate, 56.0; crude protein, 24.0; crude fat, 3.5; crude cellulose, 4.5; ash, 6.0. Vitamin contents of this diet were: vitamin A, 10; D3, 2 i.u.; E, 10; B1, 5.6; B2, 10.0; B6, 4; B12, 0.02; calcium pantothenate, 26; niacin, 44; folic acid, 0.8; and choline chloride, 400 μg/g, respectively. The animals of the first group (control) were maintained on the basal diet and water and sacrificed at the same time as in the second group. Those in the second group were transferred to the basal diet containing 3'-Me-DAB at a concentration of 0.06% for 15, 30, 45, and 60 days. Among these animals, those fed the azo dye diet for 15, 30, and 45 days were sacrificed on the 60th day after stopping the administration of the azo dye diet. Those fed azo dye for 60 days were sacrificed at 60, 90, 210, and 300 days after transferring to the basal diet. Animals in the third group were transferred to the same azo dye diet and maintained for 180 days and then transferred to the basal diet. These animals were sacrificed when tumor development was determined by palpation in the liver region or when the animal showed severe general emaciation. Animals in the fourth and fifth groups were those fed for 60 days with a diet containing 0.06% 2-Me-DAB and 0.025% 2,7-FAA, respectively, and were sacrificed at the 60th day after the respective diet was stopped.

Each rat was decapitated, and the liver and tumor as well as tumor-free area adjacent to it were subjected to further experiment. Liver tissue and tumor tissue were weighed, cut in pieces with scissors, and homogenized with 9 volumes of cold 0.02 M Tris buffer, pH 7.4, containing 80 mM KCl and 5 mM MgCl2, in a Potter-Elvehjem homogenizer. Each homogenate was centrifuged at 105,000 × g for 1 hour. The supernatant obtained was used as the crude enzyme extract for the aldolase assay and protein estimation.

Enzyme Assay. The aldolase activities were assayed by spectrophotometric measurement of the decrease at 340 mμ, with the use of α-glycerophosphate dehydrogenase, according to slight modification of the method of Racker (19) and Blostein and Rutter (3). Tris-HCl buffer (0.12 M, pH 7.5) was used instead of glycyglycine buffer (3), and all assays were carried out at 30°. The reaction was initiated by the addition of fructose-1,6-di-P (final concentration, 2 mM) or fructose-1-P (final concentration, 10 mM).

For the differential assay of muscle type and liver type aldolase activities, 0.1 ml of the antisera described below (twice diluted) was added to the enzyme extract, and, 10 min after incubation at 30°, the activity remaining was measured in the same manner as described above and without centrifugation for removal of the antigen-antibody complex. The amount of antisera added is enough to neutralize muscle type isozyme in the enzyme extract, and more addition of antisera did not affect the remaining activity. The value obtained by subtracting the activity of antisera alone from the activity remaining was taken as liver type aldolase. The muscle type aldolase activity was calculated as the difference between the total activity (without addition of antisera) and the liver type aldolase activity.

Differential assay of muscle type and liver type aldolase by measurement of the ratio of cleaving velocity of fructose-1,6-di-P to that of fructose-1-P was also carried out according to the method of Schapira (23, 24) and Rutter et al. (21, 22).

Protein Estimation. Protein content of the supernatant of tissues was measured by the method of Lowry et al. (10), with bovine albumin powder (Armour) as the colorimetric standard.

DNA Estimation. DNA content of tissues was determined by the diphenylamine method (25), with calf thymus DNA as the standard.

Preparation and Immunological Property of Antimuscle Aldolase Sera. Crystalline muscle aldolase was prepared from rat muscle according to the method of Warburg and Christian (29) and Taylor et al. (28). Antisera were obtained in hens as follows. Ten mg crystalline muscle aldolase in Freund's complete adjuvant were injected s.c. or i.m. After 2 weeks, 7 mg of the enzyme in 0.9% NaCl solution were injected i.v. as a booster 3 times every other day. The immune sera were obtained 1 week after the last injection and were stored at −15° until used. Neutralization analysis and quantitative precipitation reaction (8) revealed that 1 ml antisera neutralized 0.12 unit crystalline muscle aldolase completely and precipitated 0.32 mg of the enzyme. Double diffusion analysis in agar gel (8) showed that the antisera produced a single precipitation line against crystalline enzyme and also muscle extract as well as liver extract, and these lines fused with each other. The antisera described above were not obtained in each case but only in one-third of the immunized hens.

Materials. 3'-Me-DAB and 2,7-FAA were purchased from Tokyo Kasei Company (Tokyo, Japan); the former was recrystallized from benzene and the latter from alcohol. Their melting points were 119° and 293°, respectively. 2-Me-DAB was synthesized in our laboratory and was recrystallized from benzene. Its melting point was 67°. The carcinogens were sent to Nippon Clea Company, where the rat laboratory chows containing 0.06% 3'-Me-DAB, 0.025% 2,7-FAA, and 0.06% 2-Me-DAB were prepared.

Fructose-1,6-di-P and α-glycerophosphate dehydrogenase-triose phosphate isomerase mixture were purchased from C. F. Boehringer and Soehne (Mannheim, Germany). NAD was obtained from the Sigma Chemical Company (St. Louis, Mo.) and was reduced by sodium dithionite to prepare NADH (2) shortly before the enzyme assay.

RESULTS

Inhibition by Antimuscle Aldolase Sera for the Activity of Crude Liver Enzyme. For determination of muscle type and liver type isozyme activities in the livers of control and rats treated with azo dye and in liver cancer, differential
assays were carried out primarily with antimuscle aldolase sera as described in "Materials and Methods." For this purpose, it is an absolute requirement that the antisera neutralize only the muscle type isozyme and not cross-react with the liver type isozyme. The specificity of antisera was scrutinized by double diffusion analysis, the quantitative precipitation test, and the neutralization of enzyme activity. These results, however, do not tell us the quantitative relationship between the antisera and the enzyme in the crude extract. In order to determine this, the equal volumes of the crude liver enzyme and the crystalline muscle aldolase solution were mixed, and the fructose-1,6-di-P-cleaving activities of the mixture were determined before and after the treatment of antimuscle aldolase sera. As shown in Table 1, the mixture of crude liver extract and crystalline muscle aldolase was found to show the same fructose-1,6-di-P-cleaving activity as the sum of the two premixed components, before and after the treatment with antisera. These results indicate clearly that the substance mentioned above and immunologically identical with muscle aldolase is neutralized certainly by antimuscle aldolase sera in the same manner as crystalline muscle aldolase and that the remaining activity is never affected by antisera in the experimental condition used.

**Examination of the Change in Histological Architecture.**

In this study, biochemical analysis was attempted on the livers of rats at a sufficient time interval after the stopping of the administration of 3'-Me-DAB to avoid the non-specific toxic effects of the carcinogen. We therefore needed to know the time interval required for the reversal of the toxic effects of 3'-Me-DAB. For this purpose, histological and cytological examinations were performed on the livers at various time intervals after azo dye administration was stopped.

In comparison with normal liver (Fig. 1), severe damage was observed in the specimen prepared from the livers of rats fed azo dye for 60 days as shown in Fig. 2. This damage was still observed in the specimens prepared 14 days after the cessation of dye feeding (Fig. 3), but considerable recovery was seen in the specimen at 30 days after the cessation of dye treatment (Fig. 4). The specimens prepared at 60 days after the end of dye treatment (Fig. 5) was practically indistinguishable from that of normal liver.

The percentage distribution of the 5 types of cells composing liver tissues was measured according to the classification of Daoust et al. (5). As shown in Table 2, a remarkable decrease of parenchymal cells as well as a concomitant increase in bile duct and connective tissue cells was observed immediately after administration of azo dye for 60 days. These changes also seem to be reversed during the recovery period after the stopping of azo dye feeding, and the percentage distribution of the cells in the liver measured at the 60th to 90th day after the termination of dye feeding was found to be practically the same as that of normal liver.

**Examination of Change in Mitotic Rate.** As shown in Table 2, the mitotic rate which is normally 0 to 3/4000 parenchymal cells in liver was found to be elevated to 12 to 20/4000 cells immediately after the cessation of 60 days of azo dye administration. This change, however, seems to be reversed during the interval after the feeding of azo dye was stopped. Thus the mitotic rate in the liver was found to be practically at the same level as that of normal liver when measured at 60th to 90th day after the end of dye feeding.

**Examination of Change in DNA and Soluble Protein Content.** The content of DNA and soluble protein per wet weight of liver were measured in normal liver and in liver from rats 60 days subsequent to the end of azo dye administration. As shown in Table 2, no appreciable difference could be seen in the livers of rats fed with azo dye for 60 days and then with normal diet for 60 days as compared with that of normal rats.

These results indicate that the non-specific toxic effect of 3'-Me-DAB can be eliminated by the feeding of a normal diet for 60 days after 2 months of azo dye administration.

**Total Aldolase and Muscle Type Aldolase Activity in the Livers and Liver Cancers of Rats Fed 3'-Methyl-DAB.** The liver tissues of rats fed azo dye for 15, 30, 45, and 60 days were assayed for total aldolase and muscle type aldolase activity on the 60th day after stopping the azo dye administration. The total aldolase and muscle type aldolase activity were assayed with fructose-1,6-di-P as substrate and were compared with the activity found in normal liver, azo dye-induced liver cancer, and a tumor-free area adjacent to the cancer. Both activities in normal liver were fairly constant during a period of 180 days of feeding the standard diet.

With respect to the total activity, no appreciable difference could be seen in the various tissues tested, as shown in Table 3. On the contrary, the muscle type aldolase activity was found to be greatly elevated in liver cancer as compared with normal liver, and the increase in muscle type aldolase was already seen in the livers of rats

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**Table 1**

*Differential assay of muscle type and liver type aldolase with antimuscle aldolase sera*

<table>
<thead>
<tr>
<th>Activity found without antisera (total activity)*</th>
<th>1. Crude liver</th>
<th>2. Muscle aldolase</th>
<th>1 + 2 (expected)</th>
<th>3. Mixture of 1 and 2 (observed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity remaining after neutralization with antisera (liver type activity)**</td>
<td>0.060</td>
<td>0.062</td>
<td>0.122</td>
<td>0.123</td>
</tr>
<tr>
<td>Activity neutralized with antisera (muscle type activity)**</td>
<td>0.052</td>
<td>0.052</td>
<td>0.070</td>
<td>0.073</td>
</tr>
</tbody>
</table>

* Aldolase activity was expressed as the decrease of absorbance at 340 mμm/min at 30°C (3). Fructose-1,6-di-P (2 mm) was used as substrate.
Table 2

Changes in histological architecture and mitotic rate and in content of DNA and soluble protein in liver of rats fed on 3'-Me-DAB diet for 60 days and further on basal diet for various periods

<table>
<thead>
<tr>
<th>Days of feeding on 3'-Me-DAB diet*</th>
<th>Distribution of cell types (%)</th>
<th>Content (mg/g wet liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>0</td>
<td>64.6</td>
<td>27.9</td>
</tr>
<tr>
<td>60</td>
<td>26.5</td>
<td>32.0</td>
</tr>
<tr>
<td>60</td>
<td>44.0</td>
<td>36.0</td>
</tr>
<tr>
<td>60</td>
<td>54.0</td>
<td>31.0</td>
</tr>
<tr>
<td>60</td>
<td>60-90</td>
<td>62.0</td>
</tr>
<tr>
<td>60</td>
<td>150</td>
<td>58.8</td>
</tr>
</tbody>
</table>

*3'-Me-DAB diet contained 0.06% 3'-Me-DAB in basal diet.
†Cell types composing liver tissue: I, parenchymal cells; II, littoral cells; III, cells of bile ducts; IV, cells of connective tissue; V, cells of blood vessel walls.
‡Mitotic rate was expressed as the no. of mitotic figures per 4000 parenchymal cells.
§Data were expressed as the mean value ± S.D.

Table 3

Changes in total and muscle type isozyme activities of liver aldolase of rats fed on the diet containing carcinogenic and noncarcinogenic compounds and fed afterward on basal diet

<table>
<thead>
<tr>
<th>Days of Feeding on 3'-Me-DAB Diet*</th>
<th>No. of Rats</th>
<th>Aldolase Activity (μ mole fructose-1,6-di-P cleaved/min/mg protein at 30°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Muscle Type</td>
</tr>
<tr>
<td>3'-Me-DAB Diet (Basal Diet)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>25</td>
<td>0.094 ± 0.014</td>
</tr>
<tr>
<td>15</td>
<td>60</td>
<td>0.088 ± 0.013</td>
</tr>
<tr>
<td>30</td>
<td>60</td>
<td>0.104 ± 0.014</td>
</tr>
<tr>
<td>45</td>
<td>60</td>
<td>0.113 ± 0.017</td>
</tr>
<tr>
<td>60</td>
<td>60-90</td>
<td>0.092 ± 0.015</td>
</tr>
<tr>
<td>60 (interrupted)*</td>
<td>60</td>
<td>0.109 ± 0.007</td>
</tr>
<tr>
<td>60</td>
<td>150</td>
<td>0.128 ± 0.009</td>
</tr>
<tr>
<td>60</td>
<td>210</td>
<td>0.112 ± 0.004</td>
</tr>
<tr>
<td>60</td>
<td>300</td>
<td>0.108 ± 0.012</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>10</td>
<td>0.090 ± 0.029</td>
</tr>
<tr>
<td>Tumor-free area adjacent to cancer</td>
<td>4</td>
<td>0.080 ± 0.022</td>
</tr>
<tr>
<td>3'-Me-DAB Diet (Basal Diet)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td>0.095 ± 0.007</td>
</tr>
<tr>
<td>3'-Me-DAB Diet (2-Me-DAB Diet)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>0.091 ± 0.0092</td>
</tr>
<tr>
<td>2,7-FAA Diet (Basal Diet)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13</td>
<td>0.093 ± 0.0134</td>
</tr>
<tr>
<td>2,7-FAA Diet (Basal Diet)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>0.097 ± 0.0102</td>
</tr>
</tbody>
</table>

*3'-Me-DAB diet and 2-Me-DAB diet contained 0.06% 3'-Me-DAB or 2-Me-DAB, respectively, in basal diet.
†Differences from the control value were statistically significant (p < 0.001).
‡Rats were fed on 3'-Me-DAB diet for 30 days, basal diet for 30 days, and 3'-Me-DAB diet for 30 days.
§Differences in muscle type aldolase activities between the 3'-Me-DAB group and their control, and the 2,7-FAA group and their control were statistically significant (p < 0.01 and p < 0.02, respectively).
**2,7-FAA diet contained 0.025% 2,7-FAA in basal diet.
were chosen. 2-Me-DAB is known to bind with protein (12) despite its inability to produce cancer (11). Thus the effect of 2-Me-DAB was examined with the same experimental procedure as that of 3′-Me-DAB. 2,7-FAA is known to be a potent hepatocarcinogen, although this compound induces malignant tumors in various organs other than liver in low frequency (13–15).

The results are shown in Table 3. Compared with normal liver, no appreciable increase of muscle type aldolase was seen in the livers when 60 days of 2-Me-DAB administration were followed by the subsequent administration of standard diet for another 60 days.

On the other hand, an increase of muscle type aldolase activity in the liver was observed in the 2,7-FAA-fed animals. The degree of this increase was found to be of the same order as that observed with 3′-Me-DAB-fed animals.

Relation between Muscle Type Aldolase Activities Measured by Antisera and Fructose-1,6-di-P:Fructose-1-P Ratio. The activities of aldolase isozymes in crude liver extract have been determined by measurement of the ratio of cleaving velocity of the substrate fructose-1,6-di-P to that of the substrate fructose-1-P (21–24). The fructose-1,6-di-P:fructose-1-P ratio, however, changes not linearly but hyperbolically, responding to percentage of increase of muscle type aldolase activity, and varies slightly even by the considerable change of the muscle type aldolase content, especially in its low level. This property is not considered to be adequate for the purpose of the present study in which only slight increases of muscle type aldolase activity were observed. On the other hand, changes in the muscle type aldolase activity measured by the antisera method are expressed linearly. This is why the antisera method was used in this experiment.

To confirm further the reliability of the results obtained in the present study, an attempt was made to see whether the same result may be obtained by both the antisera and the fructose-1,6-di-P:fructose-1-P ratio methods. For this purpose, a theoretical relation between the ratio of muscle type aldolase activity to the total activity and the fructose-1,6-di-P:fructose-1-P ratio was calculated. In this case, the following experimental values were used; fructose-1,6-di-P:fructose-1-P ratio is 1 for liver type isozyme (18, 20) and 50 for muscle type isozyme (unpublished data), just as for muscle aldolase (18, 20). Considering now the mixture of muscle type and liver type isozymes showing the different activities against the 2 substrates fructose-1,6-di-P and fructose-1-P, where muscle type aldolase activity for fructose-1-P is a and for fructose-1,6-di-P is 50a and where liver type aldolase for fructose-1-P is b and for fructose-1,6-di-P is b, the fructose-1,6-di-P:fructose-1-P ratio is expressed as (50a + b)/(a + b); thus the following equation can be given,

\[ b = a \frac{(50 - R)}{(R - 1)} \]  

where \( R \) denotes fructose-1,6-di-P:fructose-1-P ratio. Since the percentage, \( P \), of muscle type activity to total activity is shown to be

\[ P = \frac{50a}{(50a + b)} \times 100 \]  

the following equation can be derived from Equations A and B:

\[ P = \frac{50}{49} \frac{1}{1 + \frac{1}{FDP/FIP}} \times 100 \]  

This implies that the percentage of muscle type aldolase in total aldolase can be calculated directly from the fructose-1,6-di-P:fructose-1-P ratio. Indicating \( P \) (%) on the ordinate and \( 1/FDP/FIP \) on the abscissa, Formula C can be expressed in a line as shown in Chart 1. Next, fructose-1,6-di-P:fructose-1-P ratio and the percentage of muscle type aldolase in the total aldolase measured on normal liver, liver of rats fed azo dye for 60 days, azo dye-induced liver cancer, and tumor-free areas adjacent to liver cancer, and all values obtained on each tissue sample were plotted. As shown in Chart 1, the measured values were found to coincide well with the theoretical curve in the cases showing low-level muscle type aldolase content, whereas the deviation from ideality, although small, increases with appearance of muscle type aldolase, presumably because of the use of an insuf-
sufficient amount of fructose-1-P, as discussed below. This
implies that, at least on the liver tissues in the early stage of hepatocarcinogenesis, the similar results can be ob-
tained even in the present experimental condition by ei-
ther the use of the fructose-1,6-di-P: fructose-1-P ratio
or the antimuscle aldolase sera, with respect to the per-
centage of muscle type aldolase in the total activity.

DISCUSSION

This study provides evidence that an enzymic change
appears in the liver in the early stage of azo dye hepatocarcinogenesis, at which time no appreciable morphologi-

cal change can be seen. This early event is expressed as
an increase of muscle type aldolase activity and is fixed
irreversibly for at least 300 days. Moreover, it was also
demonstrated that the increase of muscle type aldolase
was seen by feeding the other hepatocarcinogen, 2,7-
FAA, whereas the noncarcinogen, 2-Me-DAB, failed to
induce this enzymic change, indicating the carcino-
genic-specific property of this event. Thus it seems likely that
the carcinogenic stimuli, directing the cell to cancer for-
mation, is fixed in the cell as an irreversible change.

In the present experiment, the muscle type aldolase ac-
tivity of normal rat liver was shown to be about 13% in
average. This value is considerably higher than that re-
ported by Rutter et al. (3), 7%, and is rather near to that
of normal human liver (17). The discrepancy of both val-
ues may be mainly due to the difference of animals spe-
cies and strains.

It is well known that 3'-Me-DAB induces not only hepa-
toma but also cholangiocarcinoma in rat liver. In the
present study, however, both malignant tumors were sub-
jected to the experiment unclassified. According to the
recent study of Adelman et al. (1), the fructose-1,6-di-
P: fructose-1-P activity ratio of 3'-Me-DAB-induced hepa-
toma was about 2, and that of cholangiocarcinoma was
about 20. It therefore seems likely that the fluctuation of
the muscle type aldolase activities of 3'-Me-DAB-induced
malignant tumors observed in the present work may be
due to the unseparate handling of both tumors. It must be
pointed out, however, that the fructose-1,6-di-P: fruc-
tose-1-P ratio, 2, when expressed with percentage of
muscle type aldolase to total aldolase activity, is 51.0%.
Thus, the muscle type aldolase activity of 3'-Me-DAB-
induced hepatoma is much higher than those of normal
liver as well as the liver of rats fed 3'-Me-DAB for 60
days and then fed normal diet for further 60 days.

As illustrated in Chart 1, the deviation of experimental
values from theoretical line, although small, increases
with appearance of muscle type aldolase in tissues. This
is partly due to the fluctuation in the values of azo dye-in-
duced liver tumors as described above. However, the
main cause of the deviation seems to be the use of 10 mM
fructose-1-P, since this concentration is very close to the
Km value for rat muscle aldolase, whereas the use of 2
mM fructose-1,6-di-P in this experiment is sufficient to
saturate either muscle type or liver type aldolase of rat.

Thus, a higher maximal velocity with fructose-1-P would
result in a higher fructose-1-P: fructose-1,6-di-P ratio
for any given percentage of muscle type aldolase, in bet-
ter agreement with the theoretical line. In this sense, the
use of a large quantity of fructose-1-P is needed at least
for the determination of fructose-1,6-di-P: fructose-1-P
ratio in tissue extracts showing high activity of muscle type
aldolase.

In the present study, we did not measure the actual con-
centration of aldolase isozymes, but merely their activi-

ties. It should, however, be mentioned that the actual
concentration of muscle type aldolase in tissues contain-

ing more than one molecular species of aldolase is exag-
ergerated greatly by the high turnover of muscle type iso-

yzme relative to liver type aldolase. In this respect, we
are studying now whether the observed increase of mus-
cle type aldolase activity appearing in rat liver in the early
stage of hepatocarcinogenesis is actually caused by its net
synthesis.

Recently, Penhoet et al. (18) demonstrated that a mole-
cule of aldolase is composed of a tetramer, like that of
lactic dehydrogenase, and, in addition to muscle type and
liver type isozymes, 3 kinds of hybrids exist in the liver,
which consist of subunits of both isozymes. In the present
study, it seems to be very important to solve whether the
inhibition by antimuscle aldolase sera for the activity of
each hybrid is parallel to the content of muscle type sub-
units. The antimuscle aldolase sera were shown to inhibit
the activity of muscle type aldolase completely and, in
decreasing fashion, the 3 hybrids, but they had no effect on
the liver type isozyme (18). The same results were also
obtained in our laboratory with hybrids that were recon-
stituted artificially and isolated by isoelectric focusing
electrophoresis (unpublished data). These results imply
that the antimuscle aldolase sera block the activity of mus-
cle type subunit of each hybrid selectively without inter-
fering with liver type subunit, and, thus, the muscle type
aldolase activity measured by the use of antiserum is the
sum of the activities of pure muscle type isozyme and
muscle type subunit in each hybrid.

Regarding the noncarcinogen, 2-Me-DAB, Warwick
reported recently that this azo dye produced hepatomas
in partially hepatectomized rats (30). For confirmation of
whether the increase in muscle type aldolase activity ob-
served in precancerous liver has any correlation to hepa-
tocarcinogenesis, it may be necessary to assay muscle type
aldolase activity in the liver of rats partially hepatecto-
mized during administration of 2-Me-DAB diet.

In 1948, Druckrey and Kupfmüller (6) advocated a
"summation theory" based on their findings that the total
dose necessary for producing cancer is practically con-
stant when the daily doses are varied. From this point of
view, an attempt was made in the present experiment to
determine whether the increase of muscle type aldolase
is summed up (Table 3). From the obtained results we
could not, however, reach any conclusion, since statis-
tically significant differences could not be gained in the
respective increased level of muscle type aldolase activity
induced by the administration of azo dye for 15, 30, 45,
and 60 days, including interrupted feeding. In this connection, it is noteworthy that the increased level of muscle type aldolase in adjacent liver tissue of the tumor is at most 30% of total activity, in spite of the long-term feeding of azo dye. This led us to consider that the summation of the observed irreversible change, if existing, might occur within a definite limit, and the increase beyond the limit might be due to the growth of the cell which acquired autonomy, that is, of the cancerized cell.

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Fig. 1. Section of normal rat liver. H & E, × 50 (A); × 125 (B).

Fig. 2. Section of rat liver prepared immediately after stopping 60-day administration of diet containing 0.06% 3'-Me-DAB. Severe damages caused by the azo dye are seen in the specimen. H & E, × 50 (A); × 125 (B).

Fig. 3. Section of rat liver prepared at 14-day intervals after stopping 60-day administration of 3'-Me-DAB diet. The damages by the azo dye are still retained in the liver. H & E, × 50 (A); × 125 (B).

Fig. 4. Section of rat liver prepared at 30-day intervals after stopping the 60-day administration of 3'-Me-DAB diet. Considerable recovery from the damages can be seen in the specimen. H & E, × 125.

Fig. 5. Section of rat liver prepared at 60-day intervals after stopping the 60-day administration of 3'-Me-DAB diet. This specimen is practically indistinguishable from that of normal liver (Fig. 1). H & E, × 50 (A); × 125 (B).
Fixation of Muscle Type Aldolase in Hepatocarcinogenesis
Irreversible Fixation of Increased Level of Muscle Type Aldolase Activity Appearing in Rat Liver in the Early Stage of Hepatocarcinogenesis

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