Fatty Acid Metabolism

VIII. Acetate Metabolism in Vitro during Hepatocarcinogenesis by \( p \)-Dimethylaminoazobenzene*

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Previous studies have revealed that transplanted tumors, including the hepatoma, display a distinctive pattern of fatty acid metabolism which bears little resemblance to that of liver, being more characteristic of nonhepatic tissue. Thus, tumor slices oxidize fatty acids but produce little or no ketone bodies (19); they utilize acetoacetate more readily than do liver slices (19); and they synthesize relatively little fatty acid from acetate as compared with liver slices (7, 8). With regard to the metabolism of glucose, Olson (11) has found that the carcinogen-induced primary liver tumor also differs markedly from its tissue of origin, since it oxidizes glucose about 10 times more rapidly, does not store glycogen appreciably, and synthesizes fatty acid from glucose much more rapidly than does liver. As an extension of our previous studies in transplanted tumors, the present work was undertaken to learn whether the pattern of metabolism of fatty acids in a primary liver tumor also differs from its tissue of origin as does the transplanted hepatoma, and, if so, whether such metabolic changes appear during the “preneoplastic” period.

Many differences in activities of individual enzymes between the normal and “preneoplastic” liver, and between these and the primary liver neoplasm, have been reported (3); as yet, however, few data are available concerning the metabolism of foodstuffs by these tissues. The possibility that changes in fatty acid metabolism in the preneoplastic livers of carcinogen-fed rats might precede the formation of the tumor can be anticipated from the marked changes which have been reported in mitochondrial content (13-15), in the levels of coenzymes (17), and in enzymatic activities associated with protein synthesis (15).

Because of its central position in metabolism, acetic acid was selected as a substrate for this study. Acetic acid in the form of its coenzyme-A ester is established as a common intermediate in the metabolism of both glucose and fatty acids and is also an immediate precursor of the carbon skeletons of fatty acids, cholesterol, and ketone bodies. These experiments were conducted according to the following plan. Rats on an 18 per cent casein, low-riboflavin diet containing 0.06 per cent \( p \)-dimethylaminobenzene (DAB), were sacrificed at approximately semimonthly intervals during the course of about 400 days. The livers were sliced and incubated in Warburg vessels with acetate-\( 14 \). Where tumors developed, these were studied in the same manner. Control animals, on the same diet without DAB, were studied concomitantly. The following metabolic transformations of acetate were studied: its oxidation to \( \text{CO}_2 \) and its conversions to acetoacetic acid, to fatty acids, and to cholesterol. A similar investigation of glucose metabolism is being conducted, and the results will be published separately.

MATERIALS AND METHODS

\( p \)-Dimethylaminobenzene was selected as the carcinogen in this study, because its mild carcinogenic action and low toxicity should result in a minimum of other pathological changes and should permit a long induction period for observations of preneoplastic metabolic changes. The rats were 37- to 56-day-old males from our stock colony Carworth Farms strain. The carcinogen was present in the amount of 0.06 per cent in a diet of the following composition: General Biochemicals vitamin-free casein, 18 per cent; Crisco, 9 per cent; cod-liver oil (one-fourth of which was a halibut-liver concentrate), 1 per cent; cystine, 0.2 per cent;...
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rial or nontumorous liver tissue. Five gm. were
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relatively few in number in each liver. In these in-
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period when tumors were expected, the rats were ex-
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the 18 per cent casein diet were large, discrete, and
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malignant tumors. When these animals were interspersed with those on the
casein diet. Rats of both control groups gained
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mal, nourished rats (6). To discover whether non-
pathology has any bearing on the behavior
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placed in a large Warburg flask. In a few instances
which amount of liver or tumor was not
able, the amounts of saline medium and sub-
strate were reduced in proportion, and the re-
resulting analytical and metabolic data were cor-
correct to correspond to values for 5 gm. of tissue.

Metabolic experiments.—From the time rats
were placed on the diets, experiments were run pe-
periodically, usually at intervals of a month in the
early stages, and oftener thereafter. On each occa-
sion, experiments with a control and a DAB-fed
animal were run simultaneously. The animals were
prepared for the metabolic experiments by being
fasted for 24–48 hours and refed a high-carbohy-
drate diet (6) for 24 hours. This procedure ensured
adequate nutrition of the animal and thus optimal
conditions for fatty acid synthesis. During the pe-
period when tumors were expected, the rats were ex-
ained regularly, and, when a tumor was evident
on abdominal palpation, the animal was used
promptly for an experiment.

The rats were decapitated, and samples of liver
or tumor were removed for glycogen determination
and histologic examination. In contrast with the
diffuse, multifocal tumors usually obtained on a
low-protein diet, many of the tumors obtained on
the 18 per cent casein diet were large, discrete, and
relatively few in number in each liver. In these in-
stances it was possible to dissect out sufficient
"pure" tumor tissue for metabolic studies without
including appreciable quantities of necrotic mate-
rial or nontumorous liver tissue. Five gm. were
then sliced with the Stadie slicer (16) and were
placed in a large Warburg flask. In a few instances
in which this amount of liver or tumor was not
available, the amounts of saline medium and sub-
strate were reduced in proportion, and the re-
resulting analytical and metabolic data were cor-
correct to correspond to values for 5 gm. of tissue.

The methyl-labeled sodium acetate was obtained
from Tracerlab, Inc., on allocation by the Atomic
Energy Commission. It was diluted with inactive
acetate to a specific activity of between 28,000
and 35,000 counts/min. and made up in 0.4 m
concentration. One ml. of this preparation was added
to 39 ml. of buffer, giving a final concentration of
0.01 m. Specific activities and conversions were
calculated by our standard procedures described
previously (7). After incubation and collection of
the respiratory CO2, the slices and medium were
transferred to large centrifuge tubes, in which the
slices were separated from the supernatant fluid
and washed 3 times with water. The medium and
washings were combined and immediately frozen
until acetoacetate could be determined (18). The
washed tissues were then treated for recovery of
fatty acids and cholesterol. Extraction of these
substances and procedures for their isolation and
purification were given in a previous publica-
tion (7).

RESULTS

Analytical and metabolic data are reported for
seventeen control rats on the 18 per cent casein
diet, 21 control rats on the Checkers diet, nineteen
DAB-fed rats in which no tumors had yet ap-
peared, and six rats which developed tumors in
sufficient amount for metabolic studies.

Analytical data.—As shown in Table 1, livers of
control rats on the 18 per cent casein diet (Group 1)
had essentially the same content of glycogen,
fatty acids, and cholesterol as did those on the
Checkers diet (Group 6). These values are in close
agreement with those reported previously for nor-
mal, nourished rats (6). To discover whether non-
tumor pathology has any bearing on the behavior
of the preneoplastic liver, the nontumorous livers
of the DAB-fed rats were divided into two groups.
The first, Group 2 in the table, consisted of eleven
livers which showed no gross or microscopic ab-
normalities, and is designated as "normal." The
second group of livers, Group 3, was from eight
rats generally on the diet a long time; these dis-
payed various degrees of pathological change,
ri., fibrosis, slight to severe cirrhosis, and irregu-
larities in cell size and shape. Neither of these
groups deviated significantly from the normal in
glycogen, fatty acid, or cholesterol content.

Of the tumors obtained on DAB-feeding, some
did not yield sufficient tissue for the metabolic ex-
periments, and some were used for other studies.
In all, six were used in the present investigation;

The term "preneoplastic" is used merely to indicate the
liver of the DAB-fed rat. It does not connote any gross or
microscopic pathology.

1 The authors are indebted to Dr. A. J. Donnelly and the
Department of Pathology of this Institute for preparation and
examination of histological material.

2 The term "preneoplastic" is used merely to indicate the
liver of the DAB-fed rat. It does not connote any gross or
microscopic pathology.
two were classified histologically as cholangiomas and four as hepatocarcinomas. Their content of cholesterol, fatty acids, and glycogen (Group 5) may be compared with the adjacent nontumorous liver lobes, Group 4. All the livers in Group 4 were moderately to severely cirrhotic; nevertheless, the levels of these components were essentially those of normal liver. The glycogen values were somewhat below normal; however, if two livers, containing only 25 and 45 mg. are omitted, the average would have been 301 mg/gm, which is not far from the normal level. Since these animals, in which large tumors are present, do not eat with the appetite of normal animals, the somewhat lower glycogen levels can probably be attributed to this factor rather than to a loss in the ability to store glycogen. In contrast, it appears that the liver tumor has largely lost this capacity. In none was the glycogen content higher than 20 mg/gm, and the average was only 11 mg. The cholesterol and fatty acid levels were a little higher than, but in the same range as, their levels in liver.

**Metabolic data.**—In Table 2 there are presented data on the various metabolic transformations of acetate-2-C14 in the tissues under study. In this table the groups are the same ones listed in Table 1.

Originally, a series of experiments was performed on a group of rats maintained on a 12 per cent casein diet with 0.06 per cent DAB, on which Kline et al. (5) obtained a tumor incidence of 100 per cent after 4 months. These were abandoned, however, when it was found that livers of control rats on this diet, owing to a deficiency of protein or lipotropic factors or both, displayed a marked impairment in lipogenesis. However, the impairment was no greater in the preneoplastic livers of the DAB-fed than in the control animals, and except for a relatively high fat content these livers were not singularly different in other metabolic behavior from those of animals on the 18 per cent casein diet.

![Table 1](image)

**TABLE 1**

**ANALYTICAL DATA FROM LIVERS AND TUMORS OF RATS DURING CARCINOGENESIS WITH p-DIMETHYLAMINOAZOBENZENE**

<table>
<thead>
<tr>
<th>Group no.</th>
<th>DAB</th>
<th>No. rats</th>
<th>Days on diet</th>
<th>Condition</th>
<th>Glycogen (mg.)</th>
<th>Fatty acid (mg.)</th>
<th>Cholesterol (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>17</td>
<td>53-565</td>
<td>Normal</td>
<td>(265-545)</td>
<td>(59-134)</td>
<td>(2.6-9.0)</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>11</td>
<td>53-203</td>
<td>Normal</td>
<td>(260-510)</td>
<td>(81-140)</td>
<td>(2.1-9.9)</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>8</td>
<td>133-456</td>
<td>Pathological</td>
<td>360</td>
<td>96</td>
<td>10.4</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>6</td>
<td>211-367</td>
<td>Nontumor lobes of tumorous liver</td>
<td>210</td>
<td>90</td>
<td>10.4</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>6</td>
<td>211-367</td>
<td>Tumors</td>
<td>11</td>
<td>127</td>
<td>15.0</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>21</td>
<td>32-363</td>
<td>Normal</td>
<td>(250-685)</td>
<td>(65-165)</td>
<td>7.0</td>
</tr>
</tbody>
</table>

*Allied Mills Rat Checkers diet.

![Table 2](image)

**TABLE 2**

**CONVERSIONS OF ACETATE-2-C14 IN LIVERS OF RATS DURING CARCINOGENESIS WITH p-DIMETHYLAMINOAZOBENZENE**

<table>
<thead>
<tr>
<th>Group no.</th>
<th>DAB</th>
<th>No. rats</th>
<th>OXYGEN CONSUMPTION (µl)</th>
<th>Amount (µl)</th>
<th>R.S.A. (Per cent)</th>
<th>Acetate incorporated (µatoms C)</th>
<th>Amount (µl)</th>
<th>R.S.A. (Per cent)</th>
<th>Acetate incorporated (µatoms C)</th>
<th>CHOLESTEROL (µatoms C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>17</td>
<td>(990-1990)</td>
<td>1130</td>
<td>6.94</td>
<td>(48.0-109.4)</td>
<td>140</td>
<td>98.4</td>
<td>(10.3-15.0)</td>
<td>19.8</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>11</td>
<td>(990-1910)</td>
<td>1060</td>
<td>5.08</td>
<td>55.0</td>
<td>83</td>
<td>77.5</td>
<td>99.0</td>
<td>91.0</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>8</td>
<td>(960-1970)</td>
<td>950</td>
<td>3.99</td>
<td>57.2</td>
<td>60</td>
<td>87.5</td>
<td>89.0</td>
<td>90.4</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>6</td>
<td>(1140-1479)</td>
<td>1370</td>
<td>7.40</td>
<td>48.8</td>
<td>76</td>
<td>87.0</td>
<td>94.0</td>
<td>99.0</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>6</td>
<td>(990-1190)</td>
<td>990</td>
<td>6.37</td>
<td>54.8</td>
<td>82</td>
<td>14</td>
<td>13.4</td>
<td>19.9</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>81</td>
<td>(710-1550)</td>
<td>1000</td>
<td>6.88</td>
<td>70.4</td>
<td>54</td>
<td>11.1</td>
<td>70.4</td>
<td>17.9</td>
</tr>
</tbody>
</table>
tumor, the gatoms, was less than any value given. The average incorporation was only 13.7 micro-
toacetate in the tumors was far below that in liver.

The persistence of certain biochemical features of differentiated tissue throughout the neoplastic transformation has been well documented (8, p. 358). As examples, one may cite the production of melanin in melanomas, bone formation in osteo-
genic sarcomas, and hormone secretion in certain glandular tumors. It is thus of unusual interest that such specialized functions of the normal liver cell as the accumulation of glycogen and the formation of acetoacetate and fatty acids are largely lost in the primary liver tumor. No trend was apparent which would indicate that these changes, observable in neoplastic cells, had occurred generally throughout the mass of liver cells prior to the appearance of the tumor. The possibility remains, of course, that metabolic changes may have taken place in a relatively small number of cells destined ultimately to become neo-
plastic; if so, their number was too small to affect the essentially normal pattern of fatty acid metabolism of the preneoplastic liver.

Other investigators have also observed abrupt changes in metabolism on initiation of primary, in-
duced cancer, with little or no prior deviations during the preneoplastic period. Hayashi and Tomita (4) observed that liver tumors in rats, caused by feeding of o-aminoazotoluene, displayed a marked increase in glycolysis, and similar observations were made during liver tumorigenesis with DAB by Nakatani et al. (10). The latter investigators, however, also observed an increase in anaerobic glycolysis in the preneoplastic liver, and essentially similar observations were made by Burk et al. (1). In a subsequent study, Orr and Stickland (19) found that glycolysis in liver is in reality glyco-
genolysis and is variable in normal liver, being de-
pendent on the glycogen content. They found that

Table 1. Both control groups showed essentially the same metabolic behavior. During the 4-hour incubation period, about 1,000 μM of oxygen were consumed, and approximately the same quantities of CO₂ were produced. The relative specific activities of the carbon dioxide indicate that less than 7 per cent of the total represents carbon from the substrate; the major portion must have been derived from endogenous metabolites of the tissue. Despite a considerable range in the total quantity of substrate carbon converted to CO₂, the average of each control group was exactly the same, 70.4 μatoms. This represents the complete oxidation of approximately 15 per cent of the substrate. Yields of acetoacetate as well as its relative specific activity varied widely in the different livers. On the average, about one-half as much was produced in livers of rats on the Checker diet as from those on the casein diet. An observation of interest was the high relative specific activity of the acetoacetate, which, on the average, was about 30 per cent, or more than 4 times that of the CO₂.

Incorporation of acetate carbon into the lipids followed the pattern previously observed (6). A wide range of incorporation was displayed but was generally high in the control livers; and the incorporation of acetate into fatty acids was about one-half to one-third that of the CO₂.

Comparison of Groups 2 and 3 with 1 and 6 makes it evident that the feeding of DAB over long periods does not affect the utilization by liver of acetate for oxidation to CO₂ or for conversion to acetoacetate, fatty acids, or cholesterol. Even the liver tissue of Group 4, adjacent to well developed tumors, did not differ appreciably from the control livers in these respects. In contrast, the tumors (Group 5), though displaying almost unimpaired capacity for oxidation of acetate to CO₂ or for conversion to cholesterol, had a markedly lower capacity for ketogenesis and for fatty acid formation from acetate.

The average of 990 μM oxygen consumed by the tumor slices, as well as the range of variation of from 690 to 1130 μM, was well within the range exhibited by the normal liver slices. The average CO₂ production of 54.8 μM in the tumor was somewhat lower than that of 70.4 in liver slices, but the ranges overlapped considerably. The values for cholesterol formation were likewise within the ranges of both the normal and the preneoplastic livers. However, incorporation of acetate into acetoacetate in the tumors was far below that in liver. The average incorporation was only 13.7 micro-
atoms, which was one-sixth of the average for the adjacent liver tissues; and the highest value for a tumor, 26.8 μatoms, was less than any value given by liver. Incorporation of acetate into fatty acids in the tumor slices was on the average less than one-eighth that of liver slices, and again was considerably below the lowest value found for adjacent liver tissue.

DISCUSSION

The abrupt change in the metabolic pattern on the advent of the tumor is clearly displayed in Chart 1, which also shows graphically how glycogen content, ketogenesis, and lipogenesis remain essentially constant throughout the period of tu-
more induction. The glycogen levels in livers of the DAB-fed rats are as high, throughout the whole induction period of over a year, as in the controls, and this was, on the whole, true for the incorpora-
tion of acetate into acetoacetate and fatty acids. Only in the tumor is there almost complete failure in glycogenesis, ketogenesis, and lipogenesis.

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genolysis and is variable in normal liver, being de-
pendent on the glycogen content. They found that
the ability to glycolyze exogenous glucose was very low in liver, whether normal or preneoplastic up to incipient tumor formation. This characteristic property of tumor cells appeared only with onset of tumor formation. In confirmation of these results, Dickens and Weil-Malherbe (2) also observed a high aerobic and anaerobic glycolysis in the DAB-induced hepatoma, with little or no change from normal in the adjacent nontumorous portions of liver tissue. Among other changes, they observed in the tumor marked decreases in such specialized liver functions as urea, acetoacetate, and sugar synthesis, and in the oxidation of uric acid. These changes were unaccompanied by appreciable deviations from normal in the surrounding liver tissues.

From previous studies on tumor induction by carcinogens it is evident that the cellular transformation leading to carcinogenesis occurs early. The Millers (9) have shown that maximum dye binding to liver proteins occurs at 4–5 weeks following introduction of DAB into the diet. It is well established that feeding of certain azo dyes for periods of about 3–4 months results inevitably in 100 per cent tumor induction whether or not carcinogen administration is continued thereafter. Thus, an irreversible change in the liver cell must have occurred by this time, which leads inevitably to a

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**Chart 1.**—Glycogen content and acetoacetate and fatty acid formation in livers of normal and DAB-fed rats during carcinogenesis, and in tumors so derived. Open circles represent the control livers; lined circles, the DAB-fed rat livers; and solid circles, the tumors.
neoplasm. Whatever may be the ultimate relationship of this change to the carcinogenic process, and thus far no such relationship is evident, apparently it does not involve and is not associated with deviations in the normal hepatic pattern of fatty acid metabolism.

SUMMARY
The following conversions of acetate-$\text{C}^{14}$ were studied in normal and preneoplastic liver slices of rats fed $p$-dimethylaminoazobenzene and in tumors arising thereby: oxidation to $\text{CO}_2$ and formation of acetoacetate, fatty acids, and cholesterol.

Conversion to $\text{CO}_2$ and cholesterol were essentially similar in all three tissue types, whereas acetoacetate and fatty acid formation were greatly decreased in the primary tumor as compared with either normal or preneoplastic liver. Glycogen content was also extremely low in the tumor, whereas it was essentially normal in preneoplastic liver.

Thus, it appears that the deviations in pattern of acetate metabolism displayed by the primary tumor from that of the normal liver occur abruptly with the onset of tumor formation and are not evident in the bulk of the preneoplastic liver cells.

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