Distribution and Turnover of Cholesterol in Rats Fed 3'-Methyl-4-dimethylaminoazobenzene*

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Little comparative work has been done on the effect of liver damage and neoplasia, induced by p-dimethylaminoazobenzene derivatives, on the cholesterol metabolism and turnover in rats. Data collected by Greenstein (9) demonstrated that rat hepatoma contained significantly more cholesterol than did normal liver. Baker and Greenberg (1) showed that the in vivo conversion of acetate to cholesterol proceeded at the same rate/gm of tissue in the normal as in the whole tumorous liver.

This communication presents data comparing the distribution of cholesterol in the normal and tumorous liver and the effect of tumor growth on the cholesterol distribution in the total animal. A determination of the turnover rate of the total blood cholesterol was made in these two groups. To obtain this information the total cholesterol* and the digitonin-precipitable activity in the various fractions of normal and tumor-bearing rats were followed after injection of radioactive cholesterol.

METHODS

Young male Wistar rats averaging approximately 180 gm. were employed. Liver tumors were induced by the addition of 0.06 per cent of 3'-Me-DAB to the stock diet of MacDonald et al. (12). Our analysis indicated approximately 2 μg of dietary cholesterol/gm of food. Tumorous animals, after injection of radioactive cholesterol, were maintained on the stock diet.

Cholesterol labeled at carbon-4 with C₁⁴ was prepared according to Turner's preparation of the enol-lactone of unlabeled cholesterol/gm of food. Tumorous animals, after injection of radiocholesterol, and control animals, at all times, were maintained on the stock diet.

Cholesterol labeled at carbon-4 with C₁⁴ was prepared according to Turner's preparation of the enol-lactone of unlabeled cholesterol (15), the synthesis of labeled methyl iodide of Cox et al. (6), Heard and Ziegler's preparation of cholesteneone labeled at C-4 (10), and Belleau and Gallagher's reduction of the enol-acetate of cholesteneone to cholesterol (2). Its specific activity was approximately 7.0 × 10⁴ cpm/mg in our thin-window Geiger counter mentioned above. Self-absorption corrections were calculated according to conventional methods. Digitonides were made and quantitatively transferred to fritted glass discs and counted as described by Van Slyke et al. (16) under the same thickness-window Geiger counter mentioned above. Self-absorption corrections were calculated according to the method of Gora and Hickey (7). Animals were sacrificed by exsanguination under anesthesia. When whole portions of the rat were analyzed, the hydrolysis and extraction were conducted without carrier. Suitably sized aliquots were taken for digitonin precipitation.

RESULTS

Series A.—Preliminary investigations were begun with four male rats averaging 250 gm. Two animals were on the carcinogenic diet for 90 days; the others on the stock diet for the same length of time. To each of these rats was administered 5.0 mg. (2.44 × 10⁴ c.p.m.) of radioactive cholesterol by stomach tube. At seven intervals during the next 200 hours, small tail blood samples were taken. The turnover rates in the normal and pre-tumorous animals were approximately equal. However, since the isolation method was not specific for cholesterol, another was adopted for Series B.

At 200 hours after intubation, the rats were sacrificed and dissected. The livers of the animals on the carcinogenic diet were firm, slightly enlarged, and presented a hob-nailed appearance. Microscopically, they showed bile duct carcinoma.

*Preparative work indicated that 16 hours after injection of radioactive cholesterol little activity was present in the peritoneal fluid absorbed by filter paper discs. At 94 hours, the abdominal viscera and the peritoneal cavity were rinsed several times with ether. The pooled residues from the evaporated ether showed no significant activity.

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zonal fatty degeneration, and cirrhosis. Each rat was separated into the following four fractions: liver, decapitated carcass (including the thoracic viscera, urogenital system, and skin), the "remainder" (including the rest of the abdominal viscera and the head), and the blood. The cholesterol weight and activity of each of these fractions were determined, and the more important data are summarized in Table 1.

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Pretumorous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per cent of total</td>
<td>4.2</td>
<td>2.6</td>
</tr>
<tr>
<td>body cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wet wt of liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cholesterol in liver</td>
<td>7.4</td>
<td>6.2</td>
</tr>
<tr>
<td>activity of liver</td>
<td>1.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Ratio of specific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>activity of liver</td>
<td>1.4</td>
<td>1.1</td>
</tr>
<tr>
<td>to specific activity of carcass</td>
<td></td>
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</tbody>
</table>

The average percentage of total activity recovered from the enlarged livers of the pretumorous animals is significantly greater than that of the normal animals. A fairer measure, the ratio of the specific activity of the liver cholesterol to that of the carcass, indicates that the pretumorous group have only a slight advantage in the degree of labeling of the cholesterol present in the liver.

**Series B.**—Six carefully selected male rats, three on the carcinogenic diet for 180 days and three on the stock diet for 100 days, were given injections of radioactive cholesterol. Tail blood samples were periodically taken and analyzed as described under “Methods.” As indicated in Chart 1, the decay of the specific activity in the whole blood was exponential, with a half-life of 164 ± 10 hours for the normal rats and 212 ± 10 hours for the tumorous animals. The intercepts at zero time indicate that the specific activity of the normal group was approximately twice that of the tumorous.

After 390 hours the animals were sacrificed and dissected. The tumorous livers were greatly enlarged, slightly firmer than normal, and presented large, white, hard, tumorous masses, easily separable from the adjoining liver tissue. Histologically, the liver was characterized by zonal fat infiltration and mild cirrhosis. The tumors were identified as bile duct carcinoma, partially necrotic.

The rats were dissected into fractions similar to those of Series A, except that the skins of the normal rats and the skins, tumors, and heads of the tumorous group were treated separately. These portions were analyzed for cholesterol content and radioactivity. The results are presented in Table 2.

The total cholesterol of the tumorous rats averaged 38 per cent higher than that of the control group, with only a 12 per cent difference in body weight. The greater part of this difference in cholesterol content was accounted for in the enlarged liver and tumors and, to a lesser degree, in the skin. The livers of the tumor-bearing animals contained approximately 3 times as much cholesterol as the livers of the control animals. A comparison of the values of the cholesterol content of the carcasses indicated no change whatsoever. The other fractions of the tumorous animals also showed a moderate increase in cholesterol content, expressed as mg/gm of wet tissue, especially the blood.

No significant difference in the recovery of injected radioactive cholesterol from total body sterol was observed between the control and tumorous animals. The average recovery from all animals was 45.9 ± 6.2 per cent. The specific activities in all fractions of the tumorous group were lower than in the corresponding fractions of the controls. The specific activities of the enlarged liver and tumor, however, were identical. The ratios of the specific activities of liver to carcass were 1.4 for the normals and 1.1 for the tumorous (a reversal of the trend observed in the comparison of these ratios for Series A).

**Series C.**—Two tumor-bearing rats, comparable to those in Series B, were injected with radioactive cholesterol. They were sacrificed at 23 and 42 hours, respectively, and analyzed as above. Table

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3 We are indebted to Leo F. Bleyer, M.D., Chief Pathologist of St. Joseph's Hospital, Providence, R.I., who generously made the histological examinations of the tissues described in this paper.

4 Standard deviation, throughout this paper.
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shows, in every case, that the cholesterol of the tumors, ranging in size from 1.2 to 11.6 gm., had a significantly lower specific activity than that of the liver, and that the specific activity ratio of tumor to liver cholesterol tended to increase with time. The specific activity of the blood cholesterol, in each case, was slightly higher than that of the liver.

The analyses of the cholesterol-injected rats of Series C indicate that two important phenomena occur concerning initial radiocholesterol distribution. First, tumorous animals rapidly degrade the injected cholesterol, losing approximately one-third within the first $\theta$ days after injection. This observation closely parallels that of Chaikoff et al. (5), who find that, in normal rats, as much as 31

| TABLE 2

DISTRIBUTION OF CHOLESTEROL AND CHOLESTEROL RADIOACTIVITY FOR NORMAL AND TUMOROUS RATS OF SERIES B

<table>
<thead>
<tr>
<th>Organ</th>
<th>Per cent total weight</th>
<th>CPM/mg cholesterol</th>
<th>CPM/mg cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>31.1 ± 5.8</td>
<td>16.6 ± 59.1</td>
<td>5.17 ± 3.19</td>
</tr>
<tr>
<td>Tumor</td>
<td>16.0 ± 6.6</td>
<td>14.9 ± 29.0</td>
<td>5.58 ± 3.14</td>
</tr>
<tr>
<td>Carcass</td>
<td>0.5 ± 0.5</td>
<td>109 ± 5.9</td>
<td>1.55 ± 1.56</td>
</tr>
<tr>
<td>Skin</td>
<td>10.3 ± 1.1</td>
<td>12.0 ± 57.9</td>
<td>2.28 ± 3.27</td>
</tr>
<tr>
<td>&quot;Remainder&quot;</td>
<td>1.3 ± 0.3</td>
<td>0.9 ± 3.3</td>
<td>0.28 ± 0.22</td>
</tr>
<tr>
<td>Blood†</td>
<td>6.0 ± 0.5</td>
<td>15.0 ± 27.3</td>
<td>0.99 ± 1.48</td>
</tr>
<tr>
<td>Total</td>
<td>501 ± 691</td>
<td>1.13 ± 2.46</td>
<td>1.0 ± 0.40</td>
</tr>
</tbody>
</table>

N* = Normal rats.  T = Tumor-bearing rats.  † Blood weight calculated as 6 per cent of animal.

In addition, two tumorous rats were injected with 4.0 mg. of radioacetate and sacrificed at 14 and 90 hours. Three of the four tumors analyzed had a specific activity ratio of tumor to liver cholesterol of 0.5 or less. The smallest of the tumors had the highest specific activity. Schwenk and Werthessen (14) found that when more than 4 hours had elapsed from the time of injection with radioacetate the specific activity of the crude cholesterol digi177 from the liver was not altered by purification of the cholesterol through the dibromide.

DISCUSSION

Previous investigators have demonstrated that, in the rat, the liver is the chief site of cholesterol metabolism and also that the liver and blood plasma cholesterol are in equilibrium. Measurements of the radiocholesterol in the blood should be a fairly accurate index of the specific activity of liver cholesterol, and periodic analyses should be of value in comparing cholesterol turnover in both control and experimental groups. The cholesterol of whole blood can be used, since the specific activity of erythrocyte, free plasma, and esterified plasma cholesterol are all equal in a relatively brief time after the injection of radioactive cholesterol (4, 8).

The rapid degradation levels off after 2 days, as indicated both by the exponential decay of activity observed in the blood—an average half-life of 7.8 ± 1.0 days in all animals—and by the retention in the animal of slightly less than half of the total activity after 380 hours. Thus, the injected

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| TABLE 2

RATIOS OF SPECIFIC ACTIVITIES* OF VARIOUS FRACTIONS TO SPECIFIC ACTIVITY OF LIVER FOR CHOLESTEROL-INJECTED RATS OF SERIES C

<table>
<thead>
<tr>
<th>Time after injection (hours)</th>
<th>Liver</th>
<th>Tumor</th>
<th>Carcass</th>
<th>Skin</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>0.98</td>
<td>0.50</td>
<td>0.46</td>
<td>0.09</td>
<td>0.64</td>
</tr>
<tr>
<td>42</td>
<td>1.0</td>
<td>0.45</td>
<td>0.48</td>
<td>0.21</td>
<td>1.08</td>
</tr>
</tbody>
</table>

* Specific activity = cpm/mg of cholesterol.

† Four tumors in each liver were analyzed, and the specific activities of their cholesterol are reported separately.
cholesterol constitutes a fraction, at first subject to rapid degradation but then becoming gradually mixed into a larger pool, which dilutes and spares it. Besides the well known inert brain cholesterol compartment, at least two others can be distinguished in the rat. One includes the liver and blood and has a rapid turnover; the other includes, chiefly, the carcass, with a very slow turnover. Pihl et al. (13) have determined the half-lives of liver and carcass cholesterol to be 6 and 31-32 days respectively, with complete equilibration taking more than a month.

A comparison of the decay curves for the specific activity of the blood in normal and tumorous animals shows that the initial specific activity of the blood cholesterol in the normal is approximately twice that in the tumorous. This difference could be attributed either to more rapid initial and sustained destruction and elimination of cholesterol in the tumorous animals or to a more extensive dilution of the injected cholesterol in the tumorous animals due to the high level in the blood and liver. The former explanation, more rapid destruction, seems precluded by the fact that statistically equal recoveries of total activity were obtained in the two groups after 330 hours. Keeping in mind that the tumorous livers have 3 times as much cholesterol as the normal, this difference of specific activities in the blood must be due to dilution.

In seeming contradiction to the fact that the recovery of total activities is not different, is the observation that the half-life of the radiocholesterol of the blood in normal animals is significantly shorter than that in the tumorous animals. It should be stressed, however, that a difference in rate of loss of blood activity reflects, solely, the difference in the rate of lowering of the specific activity of the liver cholesterol and not a difference in the rate of loss of activity from the total animal. One immediate explanation for this difference in half-lives would be a more rapid exchange of carcass cholesterol (low initial specific activity) with liver cholesterol (higher specific activity), thereby presenting a lower liver cholesterol specific activity for subsequent degradation and elimination. A second source of dilution of liver cholesterol activity is the faster net synthesis of cholesterol in tumorous animals, evidenced by the fact that they contain approximately one-third more than the normals. A third explanation, which best fits the facts, is the progressive dilution of the liver cholesterol by the less highly labeled tumor cholesterol. The animals of Series C which were injected with radioactive cholesterol exhibited a specific activity ratio of tumor to liver cholesterol of 0.35 at 23 hours, and 0.5 at 42 hours.

At 330 hours the tumorous animals of Series B showed a ratio of 1.0. The average total cholesterol content of the livers of these animals was 58.1 mg., while that of the tumors was 98.8 mg., so that a resulting dilution of the specific activity of liver cholesterol seems considerable.

The cholesterol/gm of tumor, in most cases, is at least equal to that of the liver of the same animal. When the acetate-injected rats of Series C were analyzed at 14 and 20 hours after injection, the specific activity of the tumor was lower than that of the liver cholesterol. Yet, by 330 hours, in the animals of Series B injected with radiocholesterol, the specific activities of the two were invariably equal. While the initially low activities in tumor tissue may reflect inferior circulation rather than impaired synthetic ability, they indicate that in practically all cases the bulk of tumor cholesterol is not synthesized in tumor tissue but elsewhere, particularly in the enlarged liver.

SUMMARY

1. Normal and pretumorous liver were compared and found similar in their ability to take up and degrade radiocholesterol.

2. The analysis of tumors from rats injected with radiocholesterol and sacrificed after 1 and 2 days, indicated that, in every case, the cholesterol of the tumor had a lower specific activity than that of the liver, and that the specific activity ratio of tumor to liver cholesterol tended to increase with time.

3. Tumor-bearing rats contained 38 per cent more cholesterol than did normals, the excess being chiefly concentrated in the enlarged liver and tumors. The amount of cholesterol/gm of tissue was greater in all fractions of the tumorous group, except in the carcass, where it remained constant.

4. In all animals, 330 hours after injection of radiocholesterol, the ratio of specific activities of liver and blood cholesterol was 1.0. The specific activities of tumor and liver cholesterol were identical. The recovery of activity from total body cholesterol, at this time, averaged 46 per cent and was not statistically different in the tumorous and control groups.

5. Periodic tail blood samples indicated that the half-life of the blood cholesterol activity was 212 hours in the tumorous rats and 164 hours in the controls. The initial specific activity of the blood cholesterol in the controls was approximately twice.
that of the tumorous group. This difference in specific activities is probably due to dilution rather than to differential destruction. The difference in half-lives is probably due to progressive dilution from the initially weakly labeled tumor cholesterol, and possibly to a more rapid carcass-liver exchange of cholesterol.

6. In animals injected with radioacetate, the specific activity of the tumor cholesterol was much lower than that of the liver. This fact, in conjunction with the observations made on cholesterol-injected animals, indicates that the bulk of tumor cholesterol is not synthesized in tumor tissue but is transferred there from the more active liver.

REFERENCES
Distribution and Turnover of Cholesterol in Rats Fed 3′-Methyl-4-dimethylaminoazobenzene

William A. Fish, William M. Stokes and Frederick C. Hickey