Deletion of the Cholesterol-negative Feedback System in Liver Tumors*

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SUMMARY

This study represents an attempt to determine whether the cholesterol feedback system, which is characteristic of normal liver, is retained when hepatic tissue becomes malignant. In the mouse hepatoma BW 7756, the Morris rat hepatoma 5123-t.c., and in one relatively well differentiated human hepatoma cholesterol synthesis was found to proceed at significant rates, indicating that the enzymatic mechanism for cholesterogenesis is present in these tumors. Nonetheless, each of these three hepatomas completely lacked the cholesterol-negative feedback control mechanism. The presence of a subcutaneous hepatoma was found to have no influence on the normal feedback system in the liver of the tumor-bearing animal. Finally, it was shown that regenerating livers of both the mouse and the rat are capable of normal feedback response, a finding which suggests that the absence of this feedback system may be secondary to the tissue malignancy per se rather than to rapid cellular proliferation. The observation that cholesterol feedback control is lost in hepatomas derived from three different species offers the first direct experimental support for the suggestion that there may be a relationship between the deletion of feedback control and carcinogenesis.

Although uncontrolled cellular proliferation is considered to be a fundamental property of malignant tumors, the biochemical mechanism by which cancer cells escape from regulated growth characteristic of normal tissue remains unknown. In the past 10 years it has been well established that in bacteria the rates of many key synthetic reactions are rigidly regulated by a feedback mechanism in which characteristically the end-product of the reaction specifically inhibits the first unique step in the reaction sequence (2, 23, 35, 36). A number of negative feedback reactions have likewise been described in higher animals (1, 7, 14, 17); and with the finding in this laboratory (30, 32) that the primary site of the cholesterol feedback system was the first unique reaction in the synthetic pathway it was suggested that certain synthetic reactions of higher organisms might normally be under a regulatory control similar to that which operates in bacteria.

Following the recognition of feedback control mechanisms in animals, several investigators, notably Potter (27, 28), have postulated that the fundamental lesion of malignant cells might consist of a defect in such feedback control leading to uninhibited synthesis of key intermediates and ultimately to uncontrolled growth.

Despite the attractiveness of this hypothesis, direct evidence demonstrating the absence of negative feedback control in a malignant tissue has been lacking. We have, therefore, undertaken studies of the influence of malignancy on the very sensitive cholesterol feedback system which is normally present in the liver cell. The results of these studies, reported previously (33) and described in detail here, clearly demonstrate that in both the mouse hepatoma BW 7756 and the Morris rat hepatoma 5123-t.c. (tissue culture), two experimental tumors that were found capable of active cholesterol synthesis, the cholesterol feedback system is completely absent. That a defect in this feedback system may be a consistent metabolic lesion in malignant liver cells is indicated by the additional finding that in a spontaneous human hepatoma the cholesterol feedback mechanism was likewise missing.

MATERIALS AND METHODS

Experimental animals and patient.—The mice employed in this study were young, adult males of the C57L/J strain purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The spontaneous mouse hepatoma BW 7755 was likewise obtained from the Jackson Memorial Laboratory and was maintained by successive subcutaneous implantation. The tumors were allowed to grow for 3–5 weeks prior to the experiment, at which time they had achieved a size of between 1 and 6.5 gm.

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The rat hepatoma studied was the Morris hepatoma 5125-t.c. which is grown in Buffalo strain rats. We are deeply indebted to Dr. Harold P. Morris of the National Cancer Institute for making these animals available to us. Experiments on the regenerating rat liver made use of rats of the Buffalo strain purchased from the Simonsen Laboratories, Inc., Gilroy, California.

Partial hepatectomies were carried out by removal of the left lateral and the two central lobules of the livers under ether anesthesia as described by Higgins and Anderson (18). At various intervals following surgery the animals were placed on the experimental diets for a 2-day period and the regenerating livers assayed for feedback activity as described below.

The details of the patient and diet used in the study of the human hepatoma are described under “Results.”

Diet and experimental procedure.—The experimental diets in the animal studies consisted of ground Purina pellets, to which was added 20 per cent oleic acid and, where indicated, cholesterol to the extent of 5 per cent by weight. Unless otherwise indicated experimental diets were fed for a period of 2 days prior to assaying the rate of hepatic cholesterogenesis. In the study presented in Table 3 cholesterol was fed for up to 29 days to determine the effect of long-term feeding on the cholesterol feedback system.

Incubation procedure.—Animals were stunned by a blow on the head and their livers and/or tumors promptly removed and placed in chilled Krebs-Ringer bicarbonate buffer pH 7.4. Tissue slices approximately 1 mm. thick were prepared with the aid of a McIlwain Tissue Slicer. In most cases 500 mg. of liver and between 100 and 500 mg. of tumor tissue were placed in 5 cc. of Krebs-Ringer bicarbonate buffer containing 10 μmoles of sodium acetate-2-C\textsuperscript{14} (0.1 μc/μmole). For the study of the human hepatoma between 4.3 and 5.9 mg. of tumor tissue was incubated with 2 μmoles of acetate-2-C\textsuperscript{14} (2 μc/μmole) in 0.4 ml. of the Krebs-Ringer buffer. All incubations were carried out in duplicate in a Dubnoff metabolic shaker at 0.4 ml. of the Krebs-Ringer buffer pH 7.4. Tissue slices approximately 1 mm. thick were prepared with the aid of a McIlwain Tissue Slicer. In most cases 500 mg. of liver and between 100 and 500 mg. of tumor tissue were placed in 5 cc. of Krebs-Ringer bicarbonate buffer containing 10 μmoles of sodium acetate-2-C\textsuperscript{14} (0.1 μc/μmole). For the study of the human hepatoma between 4.3 and 5.9 mg. of tumor tissue was incubated with 2 μmoles of acetate-2-C\textsuperscript{14} (2 μc/μmole) in 0.4 ml. of the Krebs-Ringer buffer. All incubations were carried out in duplicate in a Dubnoff metabolic shaker at 37° C. for 2 hours. The values reported in each case represent the average of the duplicate assays.

Isolation and assay of cholesterol-C\textsuperscript{14}.—At the end of the incubation period cholesterol was isolated as previously described (29). Briefly, 1 cc. of 90 per cent weight per volume of solution, of potassium hydroxide was added to each flask, and saponification was carried out by heating at 125° C. at 15-pound pressure for 1 hour. The non-saponifiable components were obtained by extracting 3 times from a 50 per cent alcohol solution with petroleum ether. Cholesterol was precipitated as the digitonide and washed by the Sperry-Webb procedure (34). An aliquot of the cholesterol digitonide dissolved in methanol was added to 15 ml. of 0.3 per cent diphenyloxazole, 0.015 per cent bis-phenyloxazolyl benzene solution in toluene and the carbon-14 content was assayed in a Packard Liquid Scintillation Counter. C\textsuperscript{14}O\textsubscript{2} and fatty acid-C\textsuperscript{14} were isolated and assayed exactly as described in Reference 32, except that 1 N sodium hydroxide was employed to trap the C\textsuperscript{14}O\textsubscript{2}, and carbon-14 was determined in the solution described by Bray (6).

Assay of mevalonic acid synthesis.—For the estimation of mevalonate synthesis the incubation procedure used was identical to that described above, except that 8 mg. of unlabeled DL-mevalonate was added to the incubation medium to serve as a "\textquoteright"trap" for newly synthesized mevalonate-C\textsuperscript{14}. The mevalonate was isolated as briefly described previously (30). This procedure consists of extracting the mevalonic acid with diethyl ether from the acidified, ammonium sulfate-saturated medium; the dibenzyl ethylenediamine salt of the mevalonate is then formed as described by Hoffman (19); and after regenerating the free acid it is converted to its methyl ester by treatment with diazomethane. Gas-liquid chromatography of the methyl mevalonate is then carried out on a 6-foot column containing 20 per cent diethylene glycol succinate polyester on 60- to 70-mesh Celite. A temperature of 175° C. and a flow rate of argon of 150 ml/min is employed. The C\textsuperscript{14}-labeled mevalonate is collected on anthracene (10) and directly assayed for C\textsuperscript{14} in the Packard Liquid Scintillation Counter.

RESULTS

Cholesterol synthesis in mouse hepatoma BW 7756.—A number of previous reports have indicated that certain experimental tumors derived from liver cells may have normal (12) and others decreased (12, 13, 24) capacity to synthesize cholesterol. In addition, Popjak et al. has found that ascites cells fail to synthesize cholesterol from acetate (16). It was, therefore, necessary at the outset of this study to obtain a tumor in which the enzymes required for cholesterol synthesis are present and active. The relatively well differentiated mouse hepatoma BW 7756 was initially chosen for study.

As shown in Table 1 it was found that this tumor is capable of active cholesterogenesis, and indeed in this and in subsequent studies it generally synthesized cholesterol at rates greater than that of normal liver. Also indicated in Table 1 is the capacity of this tumor to carry out fatty acid synthesis at least as rapidly as does normal liver. Carbon dioxide production on the other hand, as has been previously noted in other tumors (9), is definitely less than that observed in the liver.

Absence of feedback control in mouse hepatoma.—The demonstration that the mouse hepatoma BW 7756 possesses the enzymes necessary for cholesterogenesis made possible an examination of the cholesterol feedback system in this tumor. The results of six separate such experiments are

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{TABLE 1} & \textbf{CHOLESTEROL SYNTHESIS AND LIPOGENESIS IN LIVER} & \textbf{AND IN MOUSE HEPATOMA BW 7756} \\
\hline
\textbf{ANIMAL} & \textbf{TISSUE} & \textbf{ACETATE-2-C\textsuperscript{14} CONVERTED TO:} \\
& & \textbf{CHOLESTEROL (per cent)} & \textbf{FATTY ACIDS (per cent)} & \textbf{CO\textsubscript{2} (per cent)} \\
\hline
Normal & Liver & 0.71 & 7.0 & 29.4 \\
Tumor-bearing & Liver & 0.15 & 1.0 & 21.6 \\
& Hepatoma & 2.30 & 15.2 & 5.0 \\
\hline
\end{tabular}
\end{table}

\textit{SIPERSTEIN AND FAGAN—Cholesterol Feedback in Liver Tumors}

500 Mg. tissue slices incubated with 1 μc. acetate-2-C\textsuperscript{14} in 5 ml. Krebs-Ringer bicarbonate buffer, 37° C., 2 hr.
shown in Table 2. In each experiment two normal and two tumor-bearing animals were studied, a cholesterol-free diet being fed to one member of each pair and a 5 per cent cholesterol diet to the remaining normal or tumor-bearing mouse. It was observed that in the livers of the normal mice cholesterol synthesis generally proceeded at a slower rate than usually occurs in rat liver. As expected, however, a very active cholesterol feedback system was consistently demonstrated in the livers of the normal mice. The results of such direct measurements of the incorporation of acetate into mevalonate by slices of liver and tumor tissue are presented in Table 4. As previously reported (30, 31), mevalonate synthesis in the livers of cholesterol-fed animals was found to be greatly depressed from one-tenth to one-fortieth of normal. It is noteworthy that the rapid synthesis of mevalonate can be demonstrated in the hepatoma, and in fact this level of mevalonate synthesis is significantly greater than observed in normal liver. The data from this study leave no doubt regarding the presence of a highly active feedback system in the uninvolved liver of the tumor-bearing mouse like wise possesses a normal feedback control mechanism, as indicated by the finding that the feeding of the high cholesterol diet resulted in a depression of cholesterol synthesis which ranged from one-tenth to one-thousandth of that observed in the liver of tumor-bearing animals fed the cholesterol-free diet. These data would indicate that the presence of a hepatoma does not impair the sensitivity of the cholesterol feedback system in the uninvolved liver.

The most striking finding of this study is that the feedback regulation of cholesterol synthesis is consistently absent in this mouse hepatoma. As shown by the data in the last line of Table 2, in none of the six separate experiments was a significant depression of cholesterol synthesis produced in the tumor tissue by the feeding of the high cholesterol diet (average added acetate-2-C14 incorporated into cholesterol: normal diet, 0.88 per cent; 5 per cent cholesterol diet, 0.97 per cent); as a result, cholesterologenesis in the tumors of these mice proceeded at a rate which averaged 160 times that of the livers of the same mice.

Effect of long-term cholesterol feeding.—In the previous experiments cholesterol was fed to the mice for 2 days, a period which is adequate to cause marked suppression of cholesterol synthesis in normal liver (32). The possibility existed that, despite the failure to demonstrate any evidence of feedback regulation in the tumor during this interval, the hepatoma possesses a feedback system which is less sensitive to cholesterol feeding than is that of normal liver, and which might therefore be detected by longer periods of cholesterol feeding. For this reason the previous experiments were repeated with groups of normal and tumor-bearing mice fed the 5 per cent cholesterol diet for 12, 22, and 29 days. The results of this experiment (Table 3) demonstrate that, even after 29 days on the high cholesterol diet, no indication of feedback control in the tumor can be detected. It should be emphasized that this 29-day interval represented almost the entire growth period of the tumor so that feedback inhibition was absent despite the fact that a large percentage of the malignant cells had been continuously exposed to the potential feedback inhibitor throughout their development.

Mevalonate synthesis in mouse hepatoma BW 7756.—It is conceivable that the apparent absence of the cholesterol feedback system in the hepatoma could be due to the utilization by the tumor of an alternate biochemical pathway of cholesterol synthesis which would by-pass the site of feedback control. Although of interest, such a mechanism would not constitute deletion of the normal feedback mechanism for controlling cholesterogenesis in the tumor. Since we have previously demonstrated that the actual site of feedback regulation of cholesterogenesis is specifically at the reaction responsible for mevalonate synthesis (30, 31) (Chart 1), a critical test of the integrity of the cholesterol feedback system in tumors would be the determination of whether mevalonate synthesis itself is inhibited by exogenous cholesterol.

The results of such direct measurements of the incorporation of acetate into mevalonate by slices of liver and tumor tissue are presented in Table 4. As previously reported (30, 31), mevalonate synthesis in the livers of cholesterol-fed animals was found to be greatly depressed from that observed in mice fed a normal diet. This is consistent with the fact that a large percentage of the malignant cells had been continuously exposed to the potential feedback inhibitor throughout their development.
were thoroughly washed to free them of blood, and the strate that penetration of the dietary cholesterol into both determined. The results presented in Table 5 demons

the possibility remained that the absence of the cholesterol feedback system in the mouse hepatoma suggests that the loss of feedback control is the result of the malignant change per se, it seemed possible that such a defect could simply be the consequence of the rapid proliferation of the tumor cells. To rule out this possibility two-thirds of the livers were removed from a group of normal C57L/J mice, and either 2½ or 8 days later, after 2 days on the experimental diet, feedback regulation of cholesterol synthesis was examined in the regenerating liver. As indicated by the data presented in Table 6, by 2½ days after the partial hepatectomy, the earliest interval at which liver regeneration was adequate to permit an in vitro study, a normal degree of feedback control was present.

Feedback regulation in regenerating mouse liver.—Although the observation that the cholesterol feedback system is absent in the mouse hepatoma suggests that the loss of feedback control is the result of the malignant change per se, it seemed possible that such a defect could simply be the consequence of the rapid proliferation of the tumor cells. To rule out this possibility two-thirds of the livers were removed from a group of normal C57L/J mice, and either 2½ or 8 days later, after 2 days on the experimental diet, feedback regulation of cholesterol synthesis was examined in the regenerating liver. As indicated by the data presented in Table 6, by 2½ days after the partial hepatectomy, the earliest interval at which liver regeneration was adequate to permit an in vitro study, a normal degree of feedback control was present.

It is apparent, therefore, that in nonmalignant tissue, even during rapid cellular proliferation, the synthesis of enzymes involves the concomitant incorporation of a normally sensitive feedback control mechanism into the newly synthesized protein.

Examination of feedback regulation in a rat hepatoma.—The possibility remained that the absence of the cholesterol feedback system in the mouse hepatoma BW 7756 might represent an isolated defect which fortuitously accompanies the malignancy in this specific hepatoma. To obtain evidence indicating whether feedback deletion might be a general characteristic of malignant tumors a hepatoma from another animal species was next examined. The Morris rat hepatoma 5123-t.c. is known to be a relatively

that, in contrast to the liver, the tumor continues to synthesize mevalonate at normal levels in the presence of exogenous cholesterol. This experiment, therefore, provides a direct demonstration that the cholesterol feedback reaction is no longer functioning in the tumor tissue.

Equilibrium between serum and tumor cholesterol.—One possible explanation for the absence of feedback regulation in the hepatoma could be simply a complete failure of dietary cholesterol to penetrate the tumor cell. Although it seemed unlikely that cholesterol would not enter the tumor after periods of exposure of up to 29 days, this possibility was directly examined by feeding cholesterol-4-C\textsuperscript{14} to a group of tumor-bearing mice. At various intervals mice were killed, slices of liver and tumor 1 mm. thick were thoroughly washed to free them of blood, and the specific activities of the cholesterol in the two tissues were determined. The results presented in Table 5 demonstrate that penetration of the dietary cholesterol into both

the tumor and the liver does promptly occur. The specific activities of the tumor and liver cholesterol both increased significantly by 2 days. The value in the tumor averaging approximately one-half that of the liver. Thereafter relative equilibrium was apparently reached in both tissues, since, despite the variation observed in data obtained from different individual animals, it is clear that no further significant increase in specific activity was observed.

The ratio of tumor cholesterol-C\textsuperscript{14} to liver cholesterol-C\textsuperscript{14} remained at approximately 1:2 throughout this period of feeding, a finding which would indicate that the actual rates of penetration of the dietary cholesterol into the tumor and liver are approximately the same. The difference in the specific activities between two tissues is best explained by the fact that on this diet the liver does not synthesize significant amounts of cholesterol whereas the active cholesterol synthesis in the tumor continues to dilute the dietary cholesterol with unlabeled cholesterol. It should be emphasized that a marked inhibition of penetration of cholesterol into the tumor would be required in order for this factor to play a role in the failure of the tumor to respond to cholesterol feeding. On a 5 per cent cholesterol diet a rate of penetration of even one-half that into the liver would be adequate to suppress almost completely a normal feedback system. Since previous studies have demonstrated that even a 0.5 per cent cholesterol diet will inhibit cholesterol synthesis to less than 1 per cent of normal in a 2-day period, the failure of the tumor to respond to cholesterol feeding cannot, therefore, be explained on the basis of an inability of dietary cholesterol to enter the tumor.

Feedback regulation in regenerating mouse liver.—Although the observation that the cholesterol feedback system is absent in the mouse hepatoma suggests that the loss of feedback control is the result of the malignant change per se, it seemed possible that such a defect could simply be the consequence of the rapid proliferation of the tumor cells. To rule out this possibility two-thirds of the livers were removed from a group of normal C57L/J mice, and either 2½ or 8 days later, after 2 days on the experimental diet, feedback regulation of cholesterol synthesis was examined in the regenerating liver. As indicated by the data presented in Table 6, by 2½ days after the partial hepatectomy, the earliest interval at which liver regeneration was adequate to permit an in vitro study, a normal degree of feedback control was present.

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1 M. D. Siperstein, and V. M. Fagan, unpublished observation.

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**TABLE 3**

**EFFECT OF LONG-TERM CHOLESTEROL FEEDING ON FEEDBACK REGULATION**

<table>
<thead>
<tr>
<th>Time on diet (days)</th>
<th>Animal</th>
<th>Tissue</th>
<th>Cholesterol in diet (per cent)</th>
<th>Cholesterol synthesis (per cent added acetate-2-C\textsuperscript{14})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>Liver</td>
<td>0</td>
<td>0.477</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Liver</td>
<td>5</td>
<td>0.007</td>
</tr>
<tr>
<td>6</td>
<td>Tumor-bearing</td>
<td>Liver</td>
<td>0</td>
<td>0.095</td>
</tr>
<tr>
<td></td>
<td>Tumor-bearing</td>
<td>Liver</td>
<td>5</td>
<td>0.002</td>
</tr>
<tr>
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<td>Tumor-bearing</td>
<td>Hepatoma</td>
<td>0</td>
<td>0.436</td>
</tr>
<tr>
<td></td>
<td>Tumor-bearing</td>
<td>Hepatoma</td>
<td>5</td>
<td>0.363</td>
</tr>
<tr>
<td>21</td>
<td>Normal</td>
<td>Liver</td>
<td>0</td>
<td>0.190</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Liver</td>
<td>5</td>
<td>0.034</td>
</tr>
<tr>
<td>29</td>
<td>Tumor-bearing</td>
<td>Liver</td>
<td>0</td>
<td>0.726</td>
</tr>
<tr>
<td></td>
<td>Tumor-bearing</td>
<td>Liver</td>
<td>5</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>Tumor-bearing</td>
<td>Hepatoma</td>
<td>0</td>
<td>0.162</td>
</tr>
<tr>
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<td>Hepatoma</td>
<td>5</td>
<td>0.208</td>
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<tr>
<td>29</td>
<td>Normal</td>
<td>Liver</td>
<td>0</td>
<td>1.729</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Liver</td>
<td>5</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Liver</td>
<td>0</td>
<td>0.886</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Liver</td>
<td>5</td>
<td>0.006</td>
</tr>
<tr>
<td>29</td>
<td>Tumor-bearing</td>
<td>Liver</td>
<td>0</td>
<td>0.495</td>
</tr>
<tr>
<td></td>
<td>Tumor-bearing</td>
<td>Liver</td>
<td>5</td>
<td>0.008</td>
</tr>
<tr>
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<td>Hepatoma</td>
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<td>0.581</td>
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<tr>
<td></td>
<td>Tumor-bearing</td>
<td>Hepatoma</td>
<td>5</td>
<td>0.368</td>
</tr>
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</table>

200 Mg. tissue slices incubated with 1 m\textmu. acetate-2-C\textsuperscript{14} in 2 ml. Krebs-Ringer bicarbonate buffer, 37°C., 2 hr.
well differentiated tumor (22), and, as indicated by the results presented in Table 7, this tumor proved capable of rapid cholesterol synthesis. In the three experiments carried out in this study normal feedback regulation was demonstrated in the livers of both the normal and tumor-bearing Buffalo strain rats. Again, however, in striking contrast to the response obtained in liver, no detectable feedback regulation was present in any of the three hepatomas examined.

It is of incidental interest that, as these rats grew older—Experiment 3 was carried out 3 months after Experiment 1—cholesterol synthesis both in the liver and the tumor progressively decreased; however, the presence of feedback control in the liver and its absence in the tumor is still clearly demonstrable at least 4 months after implantation of the tumor.

Feedback control in the regenerating rat liver.—An attempt was next made to confirm in the rat the finding that feedback control is present in the proliferating cells of regenerating liver. The results of the three experiments shown in Table 8 demonstrate that, as was the case in the mouse, regenerating rat liver possesses a normal feedback mechanism for the control of cholesterol synthesis.

Absence of the cholesterol feedback system in one human hepatoma.—In an attempt to determine whether the cholesterol feedback system might also be absent in a spontaneously human hepatoma, a number of such tumors were examined for their capacity to synthesize cholesterol. Menghini needle biopsies were employed to obtain the necessary tissue, and cholesterol synthesis was assayed by the microtechnic previously described (3). In several hepatomas studied no synthesis of cholesterol could be detected, and presumably, therefore, the tumors in these cases were sufficiently undifferentiated that one or more of the enzymes necessary for cholesterol synthesis were absent.

The human hepatoma that was studied for the presence of the cholesterol feedback system was derived from a 65-year-old man who had a long-standing history of Lennec's cirrhosis. Three weeks prior to the study a mass was noted in his liver, and biopsy indicated that this represented a relatively well differentiated hepatoma with large cells containing an abundant, very pink-staining (H. & E.), cytoplasm and large clear nuclei. The cells tended to form liver cords, and acini containing secretions were noted. It may be significant that the patient had a hypercholesterolemia of 463 mg/100 ml.

### TABLE 4

<table>
<thead>
<tr>
<th>ANIMAL</th>
<th>TISSUE</th>
<th>CHOLESTEROL IN DIET PER CENT</th>
<th>ACETATE-2-C¹⁴ CONVERTED TO:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mevalonate (per cent)</td>
</tr>
<tr>
<td>Normal</td>
<td>Liver</td>
<td>0</td>
<td>0.55</td>
</tr>
<tr>
<td>Normal</td>
<td>Liver</td>
<td>5</td>
<td>0.03</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Liver</td>
<td>0</td>
<td>0.54</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Liver</td>
<td>5</td>
<td>0.03</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Hepatoma</td>
<td>0</td>
<td>1.08</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Hepatoma</td>
<td>5</td>
<td>1.24</td>
</tr>
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</table>

200 Mg. tissue slices incubated in 2 ml. Krebs-Ringer bicarbonate buffer containing acetate-2-C¹⁴ (1 μc. for cholesterol synthesis or 4 μc. for mevalonate synthesis). Nonradioactive mevalonate, 8 mg., was added to the flasks used for mevalonate determinations. Incubations carried out for 2 hr. at 37°C.

### TABLE 5

Penetration of Dietary Cholesterol-4-C¹⁴ into Liver and Tumor

<table>
<thead>
<tr>
<th>TIME ON DIET (DAYS)</th>
<th>SPECIFIC ACTIVITY (S.A.) OF CHOLESTEROL-C¹⁴ IN</th>
<th>TUMOR S.A. X 100</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Liver S.A. (PER CENT)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1983</td>
<td>708 (39)</td>
</tr>
<tr>
<td></td>
<td>1884</td>
<td>1432 (63)</td>
</tr>
<tr>
<td></td>
<td>1984</td>
<td>492 (36)</td>
</tr>
<tr>
<td>4</td>
<td>1526</td>
<td>652</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>43</td>
</tr>
<tr>
<td>10</td>
<td>1960</td>
<td>1064</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

* Data from three individual animals.
For details of procedure, see text.

A relatively slow but significant rate of cholesterol synthesis was found in the biopsy of this tumor (Table 9). The patient was, therefore, placed for 3 days on a diet containing 3–4 grams of cholesterol per day—approximately 1 per cent of the total diet—an amount of cholesterol which has been previously shown to cause an almost complete suppression of cholesterol synthesis in human
TABLE 6
CHOLESTEROL FEEDBACK REGULATION IN REGENERATING MOUSE LIVER

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Cholesterol in diet (per cent)</th>
<th>Cholesterol synthesis (per cent added acetate-2-C^14)</th>
</tr>
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<tbody>
<tr>
<td>1*</td>
<td>0</td>
<td>0.270</td>
</tr>
<tr>
<td></td>
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<td>0.140</td>
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</tbody>
</table>

500 Mg. liver slices incubated with 1 μc. acetate-2-C^14 in 5 ml. Krebs-Ringer bicarbonate buffer, 37° C., 2 hr.
* 8 days after partial hepatectomy; on diet 2 days.
† 2½ days after partial hepatectomy; on diet 2 days.

TABLE 7
CHOLESTEROL FEEDBACK CONTROL IN RAT LIVER AND MORRIS HEPATOMA 5123 t.c.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tissue</th>
<th>Cholesterol in diet (per cent)</th>
<th>Cholesterol synthesis (per cent added acetate-2-C^14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Liver</td>
<td>0</td>
<td>2.40 0.79 0.25</td>
</tr>
<tr>
<td>Normal</td>
<td>Liver</td>
<td>5</td>
<td>0.01 0.005 0.006</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Liver</td>
<td>0</td>
<td>0.31 0.29 0.018</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Liver</td>
<td>5</td>
<td>0.004 0.006 0.003</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Hepatoma</td>
<td>0</td>
<td>4.20 1.04 0.28</td>
</tr>
<tr>
<td>Tumor-bearing</td>
<td>Hepatoma</td>
<td>5</td>
<td>2.30 1.25 0.67</td>
</tr>
</tbody>
</table>

500 Mg. tissue was incubated in 5 ml. Krebs-Ringer bicarbonate buffer containing 1 μc. acetate-2-C^14 for 2 hr. at 37° C. All animals were on diet for 3 days.

The dates on which the experiments were conducted were as follows: Exp. 1, 10/17/62; Exp. 2, 11/9/62; Exp. 3, 1/14/63. The hepatomas were implanted either on 7/18/62 or 9/17/62; the exact date in each case is not known.

liver (3). After the 3-day period on this diet a second biopsy showed no depression of cholesterol synthesis. Finally, a third determination of the rate of cholesterol synthesis made after the patient had been returned to a low cholesterol diet for 3 days confirmed the accuracy of the initial measurement of cholesterogenesis. These studies on a single tumor provided tentative evidence that the cholesterol feedback system may be absent from the spontaneous hepatoma of man as well as from the hepatoma of the mouse and rat.

DISCUSSION

The studies presented here were designed to determine whether the negative feedback control of synthetic reactions is retained when a tissue becomes malignant. For this purpose use was made of the very sensitive and relatively well characterized cholesterol feedback system which is normally present in the livers of all animal species so far studied.

2 This diet has been described previously (3) and contains 68 mg. of cholesterol.
in preliminary form previously (33), therefore, provide the first direct experimental support for Potter's theory that there may exist a relationship between deranged negative feedback control and carcinogenesis.

It should be emphasized, however, that an examination of many more feedback systems must be carried out before an etiologic relationship between failure of feedback control and the development of cellular malignancy can be adequately evaluated. Indeed, evidence that not all feedback systems are absent in tumor tissue is indicated by the recent demonstration that in several hepatomas the enzyme responsible for regulating pyridine synthesis is under normal feedback control (8). Obviously, the failure to control the synthesis of certain key metabolites may play an important role in the regulation of growth, whereas the uncontrolled production of other normal end-products could have minor pathologic consequences. It should be pointed out that feedback control of cholesterol synthesis is normally not present in many nonmalignant tissues such as the intestine (32). At the present time, therefore, the role which the deletion of this specific feedback mechanism in liver tumors and the consequent overproduction of cholesterol or its precursors may play in carcinogenesis is not apparent.

On an enzymatic level the finding of a deletion of a negative feedback system implies several possible changes in protein structure. The fact that the three hepatomas examined in this study retain the capacity to synthesize cholesterol, of course, indicates that they are capable of synthesizing each of the approximately 26 enzymes necessary for the complete process of cholesterol synthesis. Since we have previously demonstrated that the primary enzymatic site of feedback regulation of cholesterol synthesis is at the conversion of 3-hydroxy-3-methylglutaric acid to mevalonic acid (HMG reductase) (Chart 1) (30–32), it would follow that the defective cholesterol feedback regulation of tumor tissue demonstrated in this study must involve a deranged synthesis of this specific enzyme. Current evidence would suggest that the normal cholesterol feedback mechanism utilizes direct end-product inhibition rather than enzyme repression (31), and in such systems in bacteria Changeux (10) and Gerhart and Pardee (15) have shown that the end-product inhibiting the feedback system is bound to a specific and unique (allosteric) (21) site on the enzyme surface. If, as seems likely (21), a comparable situation applies to animal feedback systems, a possible explanation for our observations is that the malignant liver cell synthesizes adequate amounts of HMG reductase but that in the abnormal enzyme produced by this cell the allosteric site is either absent or incapable of binding the feedback inhibitor. Alternately, normal binding of the cholesterol inhibitor may take place, but the enzyme may be incapable of responding with the conformational change required to inhibit the synthesis of mevalonic acid. A theoretical model of the normal enzyme and these proposed defects in the HMG reductase of the hepatoma are illustrated in Chart 2. Experiments designed to differentiate between these possible defects in the tumor enzyme are currently in progress.

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