Systematic Oscillations in Metabolic Activity in Rat Liver and in Hepatomas
I. Morris Hepatoma No. 7793

Summary

Environmentally induced changes in rat liver were used to determine the capability of a minimal deviation hepatoma (Morris hepatoma 7793) to respond to regulatory influences. Hepatoma-bearing rats were divided among 20 experimental groups testing all possible combinations of 5 levels of dietary protein and 4 different times in the 24-hr day. Casein was fed at levels of 0, 12, 30, 60, and 90%, with glucose as the other variable. Lighting was 6 A.M. to 6 P.M., and animals were killed at 06:00, 12:00, 18:00, and 24:00. Only 2 rats were used in each group, but each group was reenforced by either 4 or 3 other adjacent groups in the experimental pattern. The hepatoma was 1 of the most slowly growing hepatomas available, and the animals were killed 149 days after transplantation, when the hepatomas weighed between 3 and 6 gm in most cases. The animals were adapted to the diet for 44 days before they were killed. Incorporation of thymidine into DNA showed marked cycling in rate with a maximum at 6 A.M. and minimum at 6 P.M. Thymidine kinase also showed daily cycling in activity. A striking generalization could be made in the case of all of the enzymes studied, which included serine dehydrase, ornithine transaminase, tyrosine transaminase, and glucose-6-phosphate dehydrogenase. It was noted that at dietary protein levels that depressed enzyme activity in host liver, enzyme activities in the hepatomas attained values 10-100 times higher than those of livers in the same animals. Since the enhanced enzyme activities in the hepatomas were in the range of the highest values that could be produced in liver under conditions optimal for liver, the phenomenon appears to be some kind of a "feedback deletion" the mechanism of which is as yet unspecified.

Introduction

Jacob Furth has made many contributions to the study of tumor-host relations. In his Clowes lecture (13) he pointed out that, in addition to the frequently studied effects of tumors on their host, it is perhaps even more instructive to consider the effects of the host on the tumors. Whereas the dogma was frequently assumed that tumors are autonomous, Furth emphasized that there are various degrees of autonomy and that in the development of tumors, hormones may play a determining role (Reference 13, p. 27). He developed a "stable transplantable, highly hormone-dependent mammary tumor which grew only in females [rats] highly stimulated with MTH [mammatropic hormone]." Comparing these mammary tumors with the "minimal deviation" hepatomas studied in our laboratories (37) he pointed out that the mammary tumors referred to above are even closer to the normal cell of origin because they are not able to grow in normal hosts unless the mammatropic hormonal level is greatly elevated. Summarizing, he stated, "Thus, the same mammary tumor can be acute and kill the host rapidly, take a slow course of several months, or can be dormant altogether, depending on the mammatropic level of the blood" (Reference 13, p. 26).

Following some of the guidelines established by Furth, we have endeavored to use the minimal deviation hepatomas in comparison with normal liver to study biochemical control mechanisms and have shown that numerous defects occur in the hepatomas in the regulation of various inducible enzymes, some behaving as if they were noninducible and others as if they were derepressed (33, 37), i.e., a "simulated" derepression.6

Pitot suggested (29) that the defective controls of enzyme synthesis characteristic of the hepatomas studied might all be secondary to a more basic molecular lesion in control mechanisms that need not necessarily involve DNA synthesis and cell division. Potter emphasized (37) a concept of multiple defects in
control mechanisms in minimal deviation hepatomas with the available hepatomas representing various possible combinations of essential and nonessential changes and regarded defects in the controls for DNA synthesis and cell division as among the essential changes for the conversion of normal cells to malignant cells.

Casting about for a systematic way to study the controlled variation in rates of DNA synthesis in rat liver in comparison with hepatomas, we were impressed with the demonstrations of “circadian” rhythms in the in vivo labeling of DNA and RNA in normal and regenerating rodent liver as shown by Halberg and Barnum and their coworkers (2, 15). Moreover, it had been shown by Blumenfeld in 1943 (3) that the mitotic count in sections of induced epidermoid cancers in mice was high and constant when observed at 4-hr intervals for 24 hr, whereas normal mouse skin showed a pronounced maximum at noon and a minimum at midnight (approximately 250 and 50 mitoses/500 fields, respectively).

Since the minimal deviation hepatomas all grow very slowly, it seemed likely that the enzymes connected with DNA synthesis might be under partial control in every case but that the degree of control might vary with the growth rate.

In the work to be described, the basic parameter was the rate of incorporation of Tdr into the DNA of Morris hepatoma 7793 during 60 min following a single injection. Similar measurements were made on the livers of the same animals (host liver). In work to be presented subsequently, a variety of other hepatomas were also studied, and samples of normal liver (from nontumorous rats) were included. Autoradiographic determination of the % of cells with labeled nuclei was also carried out. This parameter was used in preference to mitotic counts to determine whether the rate of DNA labeling as measured biochemically correlated with variations in the number of cells engaged in DNA synthesis at the time intervals studied, and this was found to be the case.

In addition to the studies based upon DNA labeling in vivo, a number of enzyme assays were carried out. These included thymidine kinase, which is the 1st enzyme in the sequence required for DNA synthesis and cell division as among the essential changes for the conversion of normal cells to malignant cells. In the case of the other parameters, marked differences between host liver and hepatoma were also seen and could be generalized as showing a “simulated derepression” of hepatoma enzymes under conditions that resulted in minimal or decreasing levels of host liver enzymes. One of the most significant conclusions is that daily fluctuations in metabolism may need to be considered when comparisons are made between hepatomas and their tissue of origin. Such comparisons may benefit from a range of environmental conditions that can test the adaptive responses of the hepatomas. The present report shows that when this approach was used the hepatoma always showed a systematic response to changes in the hormonal and/or nutritional state of the host.

Materials and Methods

All experiments were carried out with Morris hepatoma 7793 carried by transplantation into Buffalo rats at the National Cancer Institute. The average time between transplantations for this hepatoma has been 4.9 months (41). On arrival in Madison, the animals were shifted to diets containing various amounts of casein made up as previously described (33) except that the diets containing 0, 12, 30, and 60% protein were prepared and pelleted by General Biochemicals, Inc. (Chagrin Falls, Ohio).

The lighting of the windowless animal rooms was automatically regulated to provide an “equinoctial” schedule of 12 hr of light and 12 hr of darkness, corresponding to natural lighting from 6 A.M. to 6 P.M. at the equinoxes. The lights were fluorescent type and provided 110 foot candles at average cage levels.

The normal nocturnal feeding habits of the rat were reinforced in this experiment by removing their food when the lights came on and replacing it just before the lights were turned off. This procedure prevented the rats from eating in the daytime when unavoidable human activity in the room interrupted the animals’ normal period of sleep and may have made the results more uniform than otherwise. Animals were killed at 6 A.M., 12 noon, 6 P.M., and 12 midnight.

It should be emphasized that the animals were maintained on the rigorous lighting schedule and the indicated diet for 44 days before killing. In an earlier report we demonstrated marked changes between 0, 4, and 7 days on a high protein diet (33), but it has not been rigorously established that longer periods of adaptation represent a plateau for all functions measured. Nevertheless, it seems safe to assume that the period of rapid inductive change has been passed and that the changes seen are due to a daily rhythm. For this reason, the 6:00 A.M. averages are plotted at both the beginning and the end of the time sequences. In this way, the trend from 6 A.M. to 6 P.M. is shown by 3 points, and the trend from 6 P.M. to 6 A.M. is shown by 3 points.

DNA LABELING IN VIVO. One hr after the animals were to be killed each rat received 5.97 nmoles of tritiated thymidine s.c. (thymidine-methyl-1-H, with a specific activity of 6.7 c/mmole, purchased from New England Nuclear Corporation, Boston, Mass.) in 1.0 ml of distilled water.

PREPARATION OF HOMOGENATES AND SUPERNATANT FRACTIONS. One hr after the injection of the radioactive precursor each animal was killed by cervical dislocation. The liver and the tumors were removed and immediately dropped into ice-cold buffer solution for rapid cooling. Connective tissue and necrotic tumor

The following abbreviations are used: Tdr, tritium-labeled thymidine; PCA, perchloric acid; DAMP, deoxyadenosine monophosphate; ATP, adenosine triphosphate; Tris, tris (hydroxymethyl) aminomethane; DEAE, diethylaminoethyl; TPN, triphosphopyridine nucleotide.

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were carefully dissected away from the viable neoplastic tissue. A sample of liver, of approximately 600 mg (wet weight), was taken for the determination of glycogen. The rest of the liver and the tumor were homogenized in 4 volumes of 0.2 M potassium phosphate buffer, pH 8.0, containing ethylene diamine tetraacetate (10^{-3} M) and β-mercaptoethanol (10^{-3} M), by means of the Ultra Turrax homogenizer (Janke and Kunkel KG., Staufen, Breisgau, Germany). The homogenates were centrifuged in a Spinco model L2 preparative ultracentrifuge at 104,000 x g for 3 hr. The clear supernatants were stored at -20°C and used for the different enzymatic assays. The residual pellet was frozen at -20°C and subsequently used for the isolation of DNA.

**Radioactivity Measurement in Isolated DNA.** DNA was isolated from the homogenate according to the method of Hecht and Potter (16). The precipitated DNA was resuspended in 0.5 N PCA, hydrolyzed by being heated at 100°C for 15 min, and aliquots of the clear supernatant were assayed in duplicate for DNA content by the Burton modification of the diphenylamine reaction (8) with dAMP used as a standard. For liquid scintillation counting, 0.2-ml aliquots of samples were added to plastic vials to which were then added 10 ml of scintillator solution (naphthalene, 80 gm; 2,5-diphenyloxazole, 5gm; α-naphthylphenyloxazole, 50 mg; dissolved in 1 liter of a mixture of 5 parts xylene, 5 parts dioxane, and 3 parts ethanol) (20). The sample radioactivity was measured with a 3-channel liquid scintillation spectrometer (Automatic Tri-Carb liquid scintillation spectrometer, Packard Instrument Co., La Grange, Illinois). The data were corrected for quenching by the channels ratio method.

**Assay for Thymidine Kinase.** Thymidine kinase was assayed by a modification of the method of Ives et al. (18). The standard assay mixture contained the following components: thymidine-2-14C, 0.10 μM (10 mc/mmole); ATP, 9.0 μM; MgCl₂, 9.0 μM; potassium 3-phosphoglycerate, 7.5 μM; Tris-HCl, pH 7.8, 0.04 M; KCl, 0.031 M; and 0.4 ml of the supernatant enzyme mixture, to give a total volume of 1.0 ml. The reaction was run at 37°C for 40 min; aliquots were withdrawn every 10 min, placed into centrifuge tubes, and heated to 100°C for 2 min. The denatured protein was removed by centrifugation, and a 25-μl portion of supernatant was applied to strips of DEAE-cellulose paper (Whatman No. DE20). The thymidine and thymine were eluted from the paper with distilled water for 4 hr, the strips dried overnight, and the origin containing the anionic phosphates was cut out, immersed in a scintillation solution, and counted in a Packard Tri-Carb liquid scintillation counter (12, 40). From these data and from the total counts obtained from a noneluted strip it is possible to obtain an initial reaction rate.

**Determination of Liver Glycogen.** Glycogen was determined by use of the anthrone reagent according to the method of Roe and co-workers (9, 39).

**Ornithine Transaminase and Automated Enzyme Assays.** Ornithine transaminase was assayed by the method of Peraino and Pitot (26). Tyrosine transaminase, serine dehydrase, and glucose-6-phosphate dehydrogenase were assayed automatically with a combination unit previously described (34). The methods of assay were as follows: tyrosine transaminase by the method of Lin and Knox (21) as modified by Pitot, Priess, and Poirier (to be published); serine dehydrase by the method of Holzer (17) as modified for automation (34). Glucose-6-phosphate dehydrogenase was determined by Assay No. 1 of Bottomley et al. (5) with the following solutions in the automated unit (34):

Reservoir: 370 mg KCl, 105 mg magnesium acetate, 61 mg nicotinamide, 184 mg glucose-6-phosphate (potassium salt), made up to 100 ml volume with 0.10 M Tris buffer, pH 8.0; 40 mg TPN added just prior to use.

**TABLE 1**

<table>
<thead>
<tr>
<th>TIME OF DAY</th>
<th>0%</th>
<th>12%</th>
<th>30%</th>
<th>60%</th>
<th>90%</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Body wt. (gm)</td>
<td>Liver (gm)</td>
<td>Body wt. (gm)</td>
<td>Liver (gm)</td>
<td>Body wt. (gm)</td>
</tr>
<tr>
<td>06:00</td>
<td>136</td>
<td>4.61</td>
<td>0.61</td>
<td>208</td>
<td>6.87</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>4.39</td>
<td>1.70</td>
<td>193</td>
<td>6.31</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>3.56</td>
<td>4.33</td>
<td>176</td>
<td>6.26</td>
</tr>
<tr>
<td>12:00</td>
<td>127</td>
<td>3.68</td>
<td>2.87</td>
<td>204</td>
<td>5.95</td>
</tr>
<tr>
<td></td>
<td>218</td>
<td>5.45</td>
<td>2.08</td>
<td>202</td>
<td>7.03</td>
</tr>
<tr>
<td>18:00</td>
<td>126</td>
<td>3.61</td>
<td>2.81</td>
<td>200</td>
<td>6.00</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.34</td>
<td>3.15</td>
<td>210</td>
<td>6.11</td>
</tr>
<tr>
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<td>3.04</td>
<td>3.36</td>
<td>108</td>
<td>4.54</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>4.08</td>
<td>2.27</td>
<td>172</td>
<td>4.56</td>
</tr>
<tr>
<td></td>
<td>126</td>
<td>3.66</td>
<td>2.63</td>
<td>191</td>
<td>5.78</td>
</tr>
<tr>
<td>± S.E.</td>
<td>±13.4</td>
<td>±0.73</td>
<td>±1.12</td>
<td>±15.9</td>
<td>±0.81</td>
</tr>
</tbody>
</table>

*The heptatomas were inoculated on May 13, 1964, received in Madison on September 14, 1964, and placed on experimental diets on arrival. The animals were killed on October 28, 1964. This was Generation 9 and the hosts were females. Two of the groups that were killed at 6:00 a.m. had an extra animal per group.*
Sample tubes contained 0.05 ml 20% Sj, 2.0 ml 0.1 M Tris buffer, pH 8.0, containing 10 mg TPN/100 ml buffer. The sample time was 1 min at the × 4 speed and total cycle time was 7 min.

Results

Table 1 records the body weight, liver weight, and tumor weight for each individual rat among the various groups and also shows the average values as a function of the protein content of the diet. It is clear that the protein level of the diet has a significant effect on all 3 measurements with tumor weight slightly higher at 30% and 60% protein than at the other levels. Variations in these parameters due to time of day are probably small compared to the individual variations, and thus in Table 1 all of the data from animals on a given level of protein were averaged. In all other measurements, the averages and individual values are shown as a function of time of day as well as of protein.

In general this experiment represents a very mild host effect because the hepatomas grew very slowly and were small in size, averaging from 2.63 to 5.85 gm in the various dietary groups (Table 1). There were only 5 hepatomas out of 42 which weighed over 7 gm, and these were evenly distributed with 1 in the 12% protein group, 2 in the 30% protein group, and 2 in the 60% protein group. The largest tumor weighed only 10.3 gm, and it was carried in the 4th largest rat in the series, suggesting that gross disturbances in host metabolism had not yet occurred, even after 149 days of hepatoma bearing.

Measurements of DNA labeling, glycogen content, and enzyme activity show marked variations according to time of day. The data can be visualized as a graph in 3 dimensions, in which the average value from every pair of rats falls on a curve between 2 other pairs of measurements in the time dimension, which is cyclic, and between 2 additional pairs of measurements in the protein dimension for groups receiving 12, 30, or 60% protein (12 groups). In the case of animals receiving 0 or 90% protein, the data fall at the ends of the curves. Although supported by 2 other pairs of measurements in the time dimension they are supported by only 1 other pair of measurements in the protein dimension (8 groups).

These relationships are illustrated in Charts 1, 2, and 3, which are 3-dimensional representations of the findings for ornithine transaminase, tyrosine transaminase, and serine dehydrase. Although useful in showing the general results for these 3 enzymes, Charts 1, 2, and 3 do not yield the detailed information that can be obtained from a standard 2-dimensional graph, and it is therefore desirable to examine the data plotted in 2-dimensional charts with each measurement 1st as a function of time of day and then as a function of the protein content of the diet. In each case the data for each individual rat is shown as well as the average for each group of 2 or 3 rats. Although larger numbers of animals per group would have increased the significance of the averages, inspection of the curves to follow shows how each average is reinforced by data from 6 or 8 other animals.

THYMIDINE INCORPORATION INTO DNA. In Chart 4, A–E, the daily change in the rate of DNA labeling in the hepatoma is remarkably consistent. Similar data were obtained with several
Chart 4, A–I. The incorporation of labeled thymidine into DNA of Morris hepatoma 7793, and into DNA of host liver during 60 min in vivo as a function of time of day and of dietary protein. In Chart 4, A–E, the individual charts are for rats receiving 0, 12, 30, 60, and 90% protein in the diet, respectively, and the abscissa in each case is the time of day. In Chart 4, F–I, the individual charts are for rats killed at 00:00, 12:00, 18:00, and 24:00, respectively, and the abscissa in each case is the level of protein in the diet. The averages at the end of the dark period in Charts A–E are repetitions of the averages at the beginning of the day period to show the trends for both the light period and the dark period. The curves that are thus extended are shown by dashed lines. The solid circles represent hepatoma data, and the open circles represent host liver. When such points coincide they are shown half black, and when they fall on an average, a point is placed in the center of the circle.

Each open or closed circle represents an individual rat, except when data from 2 or 3 rats fall on the same point. Such points are indicated by 2 or 3 small bars respectively on the appropriate symbol.

other strains of minimal deviation hepatoma. The rate is only slightly influenced by the protein content of the diet (Chart 4, F–I), and the 5 curves showing the daily oscillation in rate could obviously be superimposed to obtain a S.D. calculated with 10 or more rats at each time point. It can be seen that in every part of the chart the rate at 6 a.m. is about 10-fold higher than at 6 p.m. with the intermediate points falling on an approximately straight line between the maximum and the minimum values.

In comparing the hepatoma values with host liver it is clear from Chart 4, A–E, that the livers incorporated thymidine to an extent that was about 0.1 as great as the hepatoma rate. It appears from other studies that host liver in animals bearing other minimal deviation hepatomas (e.g., Morris 5123) was similarly low, and in fact lower than normal liver from non-tumor-bearing animals.

The daily oscillation in DNA labeling by thymidine could conceivably reflect a variety of phenomena, and it would be most difficult to predict growth rate from an attempted integration of rates that vary as much as 10-fold in 24 hr. There is no assurance that labeling by thymidine reflects growth rate, nor is it known to what extent competing pathways of thymidylic synthesis may dominate the over-all synthetic rate, or to what extent pyrimidine pools may vary during 24 hr. However, what can be done is to see what % of cells are actually labeled during the 60-min exposure to thymidine. This has been done by means of the autoradiographic technic in a large series of other hepatomas to be presented later wherein it was found that the hepatoma data such as are present in Chart 4, A–E, were paralleled by very large oscillations in % of cells labeled. Thus it may be inferred that the oscillations must reflect the effect of factors acting in individual hepatoma cells and not a simple oscillation of circulating thymidine levels acting on all cells.

THYMIDINE KINASE. In Chart 5, A–I, it may be seen that the thymidine kinase activity of the hepatomas was strongly influenced by both the time of day and the protein content of the diet, while the activity in host liver was not very responsive to
The activity of thymidine kinase in Morris hepatoma 7793 and in host liver as a function of time of day and of dietary protein. Separate charts and symbols as in Chart 4.

either factor. The hepatomas clearly showed a maximum at 12:00 noon and a minimum at 6 P.M. which was very noticeable and consistent at all levels of protein above 0%. At the time of peak activity the protein effect was very marked (Chart 5G), rising from a value of about 170 to a value of about 750, while host livers in general showed an activity of less than 100 at all levels of protein with closer agreement between individual samples.

The strong effect of dietary protein on hepatoma thymidine kinase (Chart 5, F–I) and the relatively weak effect on hepatoma DNA labeling in vivo (Chart 4, F–I) again raise doubt as to whether the thymidine kinase is rate limiting or is proportional to DNA labeling. Nevertheless there is clearly a correlation in that both parameters are lower in host liver than in hepatoma.

glycogen. The glycogen data for the host livers are shown in Chart 6. The host livers contained glycogen in inverse proportion to the amount of dietary protein and in proportion to the dietary glucose at all time points studied (Chart 6). The hepatomas contained too little glycogen to be measured, and therefore no amount is shown. The maximum glycogen values in host livers were all seen at 6:00 A.M. at the end of the feeding period, and ranged from 3.1% on the 90% protein diet to about 8.7% on the 0% protein diet. The 6:00 A.M. values tended to be maintained until noon even though the food had been removed from the cages, and then dropped rapidly to a minimal value at 6 P.M. or midnight. It was noted that the midnight value showed a sharp increase only in the case of the 0% protein diet, and there was a
marked lag in the glycogen response to feeding in the case of all the other diets. These data on glycogen are for host livers in rats bearing Morris hepatoma 7793, and do not necessarily depict the response of normal liver or host liver in rats bearing other tumors or larger tumors.

The glycogen data demonstrate that the rats were systematically responding to both the protein content of the diet and the time of day. This demonstration involves an in vivo parameter that is not subject to the controversial interpretations that may be placed upon measurements of enzyme activity, and hence may be helpful in guiding the interpretation of the remaining enzyme data, which will now be presented. It should also be mentioned that convincing data are in the literature regarding the fluctuations in rat plasma corticosterone, which apparently rises to a maximum during the late hours of daylight (14, 23), possibly triggered by diminishing carbohydrate reserves.

**GLUCOSE-6-PHOSPHATE DEHYDROGENASE.** Chart 7, A–E, shows the rather interesting result that the host liver exhibited essentially no daily variation on the 0% protein diet or on the 60 and 90% protein diets, but showed marked variation on 12% protein (445–755, Chart 7B) and on 30% protein (665–1140, Chart 7C), which are in the optimum range for this enzyme except for the 6:00 A.M. points (Chart 7F). The maximum activity was reached at 30% protein, and the values declined smoothly from the maximum as the protein content of the diet was increased to 60 and 90% (Chart 7, G–I). These data do not stand alone. In the acute experiments by Potter and Ono (38), it was shown that the synthesis of liver glucose-6-phosphate dehydrogenase after a 3-day fast was optimum at 30% protein with lower values at 20, 40, and 60% protein and almost zero at 0 or 90% protein (Fig. 4 in Reference 38). Moreover, in the study by Ono et al. Reference 25, Charts 3a, 3b, and 5, a marked synthesis of this enzyme was seen in 4–6 days after male or female rats were shifted to 30 or 90% protein without previous fasting.

In the Morris hepatoma 5123 there was an increase in glucose-6-phosphate dehydrogenase when the diet was changed from Purina Chow to a high protein diet for 1 week (25) but no increase when the animals were previously fasted for 3 days (38). In the present study, we are not dealing with a “metabolic transition” (38) that occurs during a few days when animals are shifted from 1 diet to another. Rather we are dealing with the metabolic transition that occurs every 12 hr in animals that have been on various diets for 44 days.

The 7793 hepatomas did not exhibit a clear pattern of daily variation in glucose-6-phosphate dehydrogenase (Chart 7, A–E) but in Chart 7, F–I, which shows enzyme levels as a function of dietary protein, it is seen that the hepatoma parallels the host liver quite closely up to 30% protein, but whereas host liver values break above 30% protein at 12:00, 18:00, and 24:00, the hepatoma values continue to rise to a high plateau value at 90% protein. It is as if a repression function that set in for host liver was inoperative in the case of the hepatoma. This phenomenon will be recalled when it is seen in another circumstance to be reported below.

The assay for glucose-6-phosphate dehydrogenase was not corrected for the further reaction of the product. Further work with the automated system will be required to learn whether the initial rates of TPN reduction are mainly due to glucose-6-phosphate dehydrogenase per se or whether Assay No. 2 (5) will be preferable.

**SERINE DEHYDRASE.** This enzyme in the rat appears to be the same as threonine dehydrase (24) and although both substrates were used in earlier studies (33, 38) it was considered sufficient to employ serine alone in the present work. The previous studies did not include measurements at different times of day but did show a linear increase in normal rat liver from very low levels to much higher levels at 4 and 7 days after shifting to a 91% protein diet. Various studies have shown an increase in the enzyme during fasting, e.g. Pilot (27) and Potter and Ono (Reference 38, Fig. 15). It was also shown that hepatoma 5123 contained very high levels of the enzyme and produced a marked reduction in the enzyme activity in host livers (33). Hepatoma 7793 was shown to have moderately high levels of threonine dehydrogenase on a chow diet, which gave low values in host liver, and to have similar high values after 1 week on 91% protein (4).

The effect of the protein content of the diet on host liver serine dehydrase is strikingly revealed both in Chart 8, A–E, and Chart 8, F–I. There appears to be a linear increase in enzyme after the 30% protein level is reached, which is in contrast to data (27) with normal liver showing a linear increase above 12% protein in the diet. This comparison suggests that the tumors depress serine dehydrase activity in host livers by a rather small fixed amount, but further experiments with paired controls will be needed. Chart 8, A–E, shows that the time of day had very little effect on the serine dehydrase activity of host liver, although some of the shifts at 60% and 90% protein may be significant.

When we turn to the hepatoma data in Chart 8, A–I, the results show very high levels of activity even at low levels of protein in the diet (in comparison with host liver or normal liver) while at the same time revealing marked shifts in activity at different times of day. The tendency to show 2 peaks of activity, at noon and midnight respectively, seems to constitute a clear pattern, while the occurrence of a minimum value at 6:00 A.M. is unquestionable. The total impression gained from Chart 8, A–I, is one in which the hepatoma is responsive to the positive effects of protein both in the long time adaptation basis and the daily positive effects of carbohydrate depletion or high protein intake, but is indifferent to the negative or repressive effects of dietary carbohydrate, which have been clearly demonstrated in normal liver in short-time experiments by Pilot and Persaino (31, 32).

**TYROSINE TRANSMANASE.** The value of the multivariable approach to the comparison of liver and hepatoma is especially well illustrated in Chart 9, A–I. The strong linear response of this enzyme to dietary protein in host liver with increments beginning between 0 and 12% protein (in contrast to serine dehydrase, see above, Chart 8, F–I) would have been missed if the midnight samplings had been omitted (Chart 8F). Unpublished studies have repeatedly shown this marked peak at midnight in normal liver, comparable to the peaks in host liver at high levels of protein (Chart 9, D and E).

The midnight peaks in host liver are paralleled by peaks in the hepatoma, but only at the high levels of protein (Chart 9, D and E). In strong contrast are the results at 0 or 12% protein, wherein the hepatomas develop a new maximum of their own at 6:00 A.M., which is the normal time for a falling level of this enzyme in liver. The shape of the hepatoma curves in Chart 9, A and B, may be compared with their counterparts in Chart 8, A and B, which show reciprocal trends for the daily cycles of serine dehydrase compared with tyrosine transaminase.
Chart 7, A–I. The activity of glucose-6-phosphate dehydrogenase in Morris hepatoma 7793 and in host liver as a function of time of day and of dietary protein. Separate charts and symbols as in Chart 4.
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Chart 8, A-I. The activity of serine dehydrase in Morris hepatoma 7793 and in host liver as a function of time of day and of dietary protein. Separate charts and symbols as in Chart 4.
The striking differences in metabolic responsiveness between the hepatoma and liver would obviously have been missed if the comparisons had been limited to a "normal" diet such as the 30% protein with samplings during a "normal" working day (Chart 9E).

The inclusion of the 12% protein diet revealed a clear maximum in enzyme activity in the hepatoma that was more marked at 6:00 A.M. and decreased progressively as the day advanced (Chart 9, F-H) only to be obliterated at midnight as the high protein maximum was reached (Chart 9F). The peak in tyrosine transaminase at 12% protein and the resulting marked deviation from the linear increase with dietary protein in normal and host liver was specific for tyrosine transaminase, as can be seen by comparison with serine dehydrase and ornithine transaminase, each of which had their own specific responses to dietary protein (see below).

ORNITHINE TRANSAMINASE. This enzyme occurs in rat liver mitochondria (26) and probably has a slower turnover than the preceding 2 enzymes (31, 32). Like tyrosine transaminase, it is very sensitive to dietary protein and increases linearly from 0% protein to 90% (Chart 10, F-I). Unlike tyrosine transaminase it does not fall to low levels at noon and 6 P.M., but like tyrosine transaminase the most marked change during the 24-hr cycle is at midnight (Chart 10, A-E). However, whereas tyrosine transaminase at high protein levels increased at midnight (Chart 9, D and E), the level of ornithine transaminase decreased in host liver (Chart 10, D and E). This enzyme is completely unresponsive to steroid hormones (32), whereas tyrosine transaminase is highly responsive to the corticosteroids (19).

In general the ornithine transaminase values in the hepatoma were fairly steady during the 24-hr cycle, and the most noteworthy phenomenon is the tendency for the hepatoma curves in Chart 10, F-I, to cut across the host liver values which are linear and proportional to dietary protein, and average out to a peak at 30% protein, in contrast to the tyrosine transaminase values which peaked at 12% protein.
Comparisons among Charts 6–10 make it quite clear that over-all changes in % protein in the soluble supernatant fraction per gm of wet weight could not possibly have accounted for the results, since in many cases the enzymes changed in opposite directions.

Discussion

The Morris hepatoma No. 7793 is one of the slowly growing minimal deviation hepatomas and was developed with the same carcinogen that was used to induce the 5123 series. The growth rate expressed as average transplantation times has recently been tabulated with other minimal deviation hepatomas and rapidly growing hepatomas by Shonk et al. (41), who studied the activity of 11 glycolytic enzymes. These workers reported the transplantation time for this hepatoma as 4.9 months, which is much slower than the Morris 5123 hepatomas A, C, and D, which were all about 2.1 months. Only the Morris 7787 hepatoma grew more slowly and was reported as 9.8 months.

Despite the growth rates mentioned, the 7793 hepatomas ap-
peared to be closer to normal liver than the 7787 on the basis of the 11 glycolytic enzymes (41) although it was not claimed that either of these hepatomas differed significantly from normal liver in the instances studied.

With respect to tryptophan pyrrolase, Cho et al. (10) found that hepatoma 7793 normally contained more of this enzyme than any other hepatoma studied and responded to both tryptophan and cortisone to a greater degree. Values of 4.2 units were reached in each of the latter cases starting from 1.2 units, while host livers started at 2.7 and reached values of 27.0 and 13.4 with tryptophan and cortisone injection, respectively. Pitot (28) has shown that in the 7793 hepatoma, as in the Reuber H 35 hepatoma, the response to tryptophan was dependent on the presence of sufficient levels of corticosteroids in the host. Adrenalectomy of the tumor-bearing host abolished the substrate response of this enzyme in the tumors but not in the liver. Dyer et al. (11) also studied hepatoma 7793 and reported nearly normal values for tryptophan pyrrolase and good responses to both tryptophan and cortisone. No data on this enzyme have been published for hepatoma 7787, and although in the study just cited hepatoma 7794 and 7795 appeared to resemble 7793, these tumors were not employed in the other studies (10, 41).

Recent studies by Wu et al. (45) have shown that 3 out of 3 rapidly growing hepatomas and 2 out of 3 minimal deviation hepatomas contained less than 10 units of glutamine synthetase while the remaining minimal deviation hepatoma contained more than was found in normal liver. Thus the Morris 7800 hepatoma contained 276.8 units while control livers contained only 202.4 units of the enzyme. These findings are particularly interesting in relation to the present work with hepatoma 7793 because the growth rates (transplantation times) of the minimal deviation hepatomas were reported as 9.8, 4.9, 3.8, 3.1, and 2.1 months for hepatomas numbered 7777, 7793, 7316A, 7800, and 5123D respectively (41). Since Wu et al. studied only the last 3 hepatomas, it becomes of great interest to study the glutamine synthetase of hepatomas 7777 and 7793 and to determine the effect of the protein content of the diet and the 24-hr variation, if any, particularly in view of the earlier data by Wu (44) showing the effect of dietary protein on this enzyme in normal liver. He found low values on 0% protein, rising to values around 200 at 25% protein with no further increase or a slight decrease at 75% protein. Such a pattern resembles the pattern for glucose-6-phosphate dehydrogenase (Chart 7) rather than the patterns for the serine, tyrosine, or ornithine enzymes (Charts 8-10), and the fact that hepatoma 7800 had higher than normal values raises the question of whether the glutamine synthetase in this tumor follows a pattern of simulated derepression\(^7\) that seems to characterize hepatoma 7793 for the enzymes studied in the present instance.

If we examine the effect of dietary protein on the serine, tyrosine, and ornithine enzymes (Charts 8-10) we see that in all cases the liver has a smooth linear response, modulated by the 24-hr cycle, but in no case and at no time of day exhibiting a reversal in the trend toward lower enzyme level at lower levels of dietary protein. When we examine the hepatoma data we see a completely deviated but systematic pattern that includes the generalized result of high values at low levels of dietary protein plus the individual features of a peak value for enzyme activity at some value other than the highest dietary protein as seen in the host liver. Thus in the hepatoma, serine dehydrase showed a peak at 30-60% protein (Chart 8, F-J), tyrosine transaminase showed a peak at 12% protein (Chart 9, F-J), and ornithine transaminase showed a peak at 30% protein (Chart 10, F-J). It was as if the host liver was repressed at low levels of protein by the high glucose content of the diet (31, 32), while this repression failed in the hepatoma.

Equally impressive is the same phenomenon on the other end of the scale. In the case of glucose-6-phosphate dehydrogenase, some kind of a repression occurs the host liver at dietary protein levels above 30% (Chart 7, F-J), a phenomenon not seen in the case of the amino acid enzymes studied. But now the hepatoma rises to higher and higher values as the protein level is increased, again as if a failure in repression had occurred.

The phenomena of "simulated" derepression\(^7\) seen in Charts 7-10 are quite in keeping with the data on DNA labeling and on thymidine kinase (Charts 4, 5) which can be interpreted as derepression at all levels of dietary protein, while in Chart 7 the derepression is at the high end of the protein scale and in Charts 8-10 the derepression is at the low end of the protein scale. It should be noted that in Charts 9 and 10 the tumor values are also depressed at the high end of the protein scale in comparison with normal liver. Whether these phenomena represent capabilities inherent in the hepatoma cells or whether they are artifactual and result from the absence of a portal circulation and a bile drainage system in the hepatomas cannot be easily proved, but in experiments with liver lobes in which the portal circulation was ligated (5), the level of induced enzyme was lower than in the nonligated lobes of the same animals. This finding suggests that the increased enzyme levels seen in Charts 5 and 7-10 seem unlikely to be explained by the absence of input from the portal vein.

The generalized phenomenon of derepression or, in general terms, feedback deletion, (36, 37) can only be studied in the case of enzymes that are present in adequate amounts to be studied. In the case of the minimal deviation hepatomas many enzymes that characterize liver are present and seem to demonstrate something akin to derepression. However, some enzymes such as tryptophan pyrrolase (30) and glutamine synthetase (45) appear to be deleted or present in extremely low amounts in some of the minimal deviation hepatomas and from all of the rapidly growing hepatomas, and apparently cannot be derepressed in such hepatomas. Whether these facts should be interpreted along the lines of multiple mutations (36, 37) or in terms of a basic nonmutational event (29) cannot be decided at this time, but further studies are clearly suggested by the present data. Moreover the question of how to attack the biochemistry of cancer is clearly raised, and it appears that the further study of the phenomenon of "simulated" derepression in molecular terms appropriate to the mammalian cell will be furthered by a realization that the phenomenon can only be studied in the case of enzymes that are present and capable of responding to environmental change.

In previous discussions the term "feedback deletion" has been used in its most general sense (36, 37) and in the present discussion the term "simulated derepression" has been used because it appears that the activity (concentration?) of a considerable number of enzymes is greatly increased under conditions which produce a decreased activity (concentration?) in the correspond-
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ing normal tissues. Whether this increased activity is due to a derepression at the level of gene transcription, at the level of the enzyme-forming system, or at the level of preformed enzyme remains for further research. With conditions described for attaining simulated derepression, it should now be possible to learn more about the molecular mechanisms of repression in mammalian liver, in the same sense that Loomis and Magasanik (22) envision the advantages that accrue from their development of E. coli mutants that are catabolite repression negative (22), although there is no assurance that repression in the mammal operates at the same level as in E. coli. Elsewhere the demonstration of the repression of serine dehydrase and ornithine transaminase in normal liver by glucose has been described (31, 32) and evidence in favor of repression at the level of the enzyme-forming system was provided. The possible role of altered template, i.e., messenger RNA, stability has been discussed in terms of the endoplasmic reticulum membranes (29), and sedimentation studies have demonstrated quantitative decreases in the binding of the polysomes to the membrane in minimal deviation hepatomas as compared to normal liver (42, 43).

It seems possible that the increased glycolysis seen in advanced cancers is a manifestation of the same general phenomena reported in the present work; Burk (6) regards the increased glycolysis as a result of a loss of hormonal regulation of glucokinase activity and has published many reports dealing with this phenomenon. He has questioned the validity of the publications reporting that the glycolytic rate in certain minimal deviation hepatomas is not significantly different from normal liver and in fact has claimed that a direct relationship exists between growth rate and anaerobic glycolysis, with the concomitant claim that there are no minimal deviation hepatomas that possess an anaerobic glycolysis that is not significantly greater than that of normal liver (7). This claim hinges on whether the glycolytic rate in certain minimal deviation hepatomas, particularly No. 7787 and No. 7793, is in fact significantly different from normal liver, and in the data he presented the claim rested on the assignment of Q values below 0.5 to normal liver in comparison with a composite value for hepatomas 8624, 7794B, 7787, and 7793 at around 0.7. The hepatoma values were all assigned a growth rate of 10 months per generation despite the fact that the average rate for No. 7793 is reported to be 4.9 months, with a range of 4.1-6.2 months, while for No. 7787 it is reported as 9.8 months, with a range of 5.9-12.6 months (41).

It appears that the prediction "that a tumor that is now minimal may be replaced by a tumor that represents a new minimal" (35) has been substantiated by subsequent events. Hepatoma 7793 now must be considered as the new minimal standard, and hepatoma 5123 is no longer the hepatoma that most resembles liver. Further detailed studies are needed on hepatoma 7787.

The present report has emphasized the importance of recognizing and utilizing the existence of daily oscillations in metabolic activity in the study of the biochemistry of cancer. We have avoided the use of the term "circadian rhythm" not because we minimize its importance but because we have not attempted to prove that the observations are due to circadian rhythms. Our studies involved the reinforcement of the normal light and dark feeding patterns by actually removing the food during the light periods, a procedure that has proved very helpful in standardizing the metabolic control systems so that hepatomas could be compared with normal liver. H. A. Krebs (personal communication) has also found that fortuitous feeding by experimental rats during the day can lead to uncontrolled variation in metabolic studies. Our studies emphasizing the need for better control of environmental conditions leads to complete agreement with the conclusions of Andrews and Folk (1), who observed cyclic deviations as high as 80% in the $Q_O$ of adrenal glands and commented "workers should be aware of the influence of circadian profiles when they measure metabolic quotients for isolated tissues."

As noted in the introduction, Jacob Furth remarked that tumor-host relations could be divided into effects of the tumor on the host and effects of the host on the tumor (13). The present report has dealt with the effects of the host on the tumor and has utilized knowledge of the effect of the host and the host environment on its own liver in order to make comparisons between hepatoma and liver that otherwise would not have been possible. It is a pleasure to dedicate this report to Professor Furth, and we feel that the subject matter is well within his sphere of influence and interest. Future work in this area will involve the interaction between the hormones of the host and the receptor systems in the hepatomas, and here the systems described by Furth will surely be helpful.

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