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Specific Effects of a Hepatoma on Metabolic Capabilities of the Liver in Tumor-Bearing Rats (40378)

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Some tissues of tumor-bearing animals, particularly the liver, are known to differ from the corresponding normal tissues in the levels of certain enzyme activities and the concentrations of associated metabolically active components (1–8). Furthermore, the administration of fractions obtained from tumors to normal animals can elicit similar changes (9–11), but it is not yet clear to what degree such changes in host tissues represent the effects of specific directive substances released by the tumor. Some of the reported changes may reflect the pathologic alterations associated with the general deterioration of the host in advanced stages of cancer. Many of the reported changes in host tissues were seen in animals bearing large tumors, so that the metabolic activities of the tumor represented a major part of the host's total metabolism, and the changes in host tissues may have reflected adjustments to the competition and metabolic burdens imposed by the presence of the tumor (12).

We have studied the levels of enzyme activities in the livers of hepatoma-bearing rats during a period when the tumor was still small, to minimize the influence of general tumor metabolism *per se*. The parameters chosen for study included activities that are associated with various cytoplasmic compartments and that in some hepatomas are present at levels different from the levels in normal liver. The relatively slow-growing transplantable Reuber H-35 hepatoma (13) was used so that tumor effects could be studied for an extended period during which the tumor size did not exceed a small percent of the host animal's body weight. The changes from normal liver in host liver and in tumor were determined, and where the host liver showed significant changes from normal liver, the time course of the changes was followed in host liver during the period of early tumor growth. Controls involving transplants of normal liver were also run to further define the specificity of tumor effects on the host livers. Growing animals were used with the expectation that tumor effects might become discernible sooner than in adult animals.

Materials and methods. Female ACl strain rats were obtained from Microbiological Associates at about 4 weeks of age and 70 g weight, and were maintained in our laboratory on a diet of Purina rat pellets and water *ad libitum*. The Reuber H-35 hepatoma, obtained from a tumor-bearing rat kindly supplied to us by Dr. Melvin Reuber, was maintained in our laboratory by serial transplants in female ACl rats. Tumors weighing 4–8g were trimmed free of necrotic tissue, minced in sterile 0.9% NaCl solution, and 0.5 ml of mince, containing 100 mg tumor, was implanted by trochar subcutaneously on the back of rats weighing 100–115 g and about 6 weeks of age. Some animals received similar implants of minced liver taken from a normal female ACl rat of the same age. Normal control animals of the same age and weight were injected with 0.9% NaCl solution. The animals were routinely weighed three times a week and just before killing, and tumor sizes were estimated by the method of Knox (14). Wet weights of livers and tumors were determined at the time of killing.

The rats were killed by decapitation, routinely at 9 AM, and the livers and tumors quickly excised, rinsed, weighed and cooled to 0–2°. The tumors were dissected free of necrotic material. For studies on nonmitochondrial components, liver or hepatoma was minced with scissors and homogenized in 9 vol of cold 0.154 *M* KCl–0.001 *M* EDTA, pH 7.5 in a motor-driven glass homogenizer with a Teflon pestle. For preparation of microsomal fractions, aliquots of the homogenate were centrifuged at 10,000*g* for 15 min, and the resulting supernatant fluid centrifuged at 105,000*g* for 1 hr. The microsome pellet was

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washed by dispersion in 0.154 *M* KCl, recentrifugation at 105,000*g*, and redispersion in 0.154 *M* KCl–0.01 *M* Tris–HCl, pH 7.5. Fresh microsomal fractions were used for assay of demethylase activities. Portions of homogenate and microsomal fractions were frozen for later assay of stable activities, cytochromes and protein content. For preparation of mitochondria, liver or hepatoma was minced with scissors in 9 vol of cold medium containing 0.25 *M* sucrose-0.001 *M* Tris–HCl, 0.001 *M* EDTA, pH 7.5 plus 2 mg defatted bovine serum albumin/ml (STEA medium), then homogenized in a glass homogenizer with a Teflon pestle. Five passes of the pestle were used for liver and four passes were used to produce a similar high degree of cell disruption for hepatoma, as judged by microscopic examination. The mitochondrial fraction was obtained by differential centrifugation of the homogenate (15), washed by dispersion and recentrifugation, and redispersed in STEA medium to a volume of 1 ml per g wet weight of original tissue. Fresh mitochondrial preparations were used for polarographic assays, and aliquots were frozen for later spectrophotometric enzyme assays and protein determinations.

Glucose-6-phosphatase activity was determined in aliquots of fresh liver homogenate by assay of the inorganic phosphate released from 20 m*M* glucose-6-phosphate, as previously described (16), and fructose-1,6-diphosphatase activity on aliquots of homogenates stored at −10° by a similar procedure (16), with 5 m*M* fructose-1,6-diphosphate as substrate. For cathepsin determinations, frozen homogenates were thawed and rehomogenized after addition of 0.2% (v/v) Triton X-100, and centrifuged for 15 min at 15,000*g*. The supernatant fluid was assayed by the method of Anson (17) and results expressed as equivalents of nmoles tyrosine liberated/min/mg protein.

Fresh microsomal fractions were assayed for *N*-demethylation activity with aminopyrine as substrate and for *O*-demethylation with *o*-nitroanisole as substrate, by the general procedure previously described (18); activities were calculated as nmoles/min/mg microsomal protein. The cytochrome P-450 and cytochrome b_5 contents of microsomal fractions that had been stored at −10° were determined from difference spectra (18). Cytochrome c reductase activities, with NADPH, NADH or succinate as electron donors, were determined spectrophotometrically by increase in absorbance at 550 nm (18), using microsomal fractions stored at −10° and mitochondrial fractions that had been frozen and thawed three times.

Respiratory activities of freshly prepared mitochondrial fractions were assayed polarographically at 25° by adaptation of the procedures of Sordahl (19) and Pedersen (20), using a closed 1.7 ml reaction vessel with a Clark oxygen electrode. The assay medium routinely contained 250 m*M* sucrose, 10 m*M* Tris–HCl, pH 7.5, 10 m*M* KPO_4 buffer, pH 7.5, 5 m*M* $MgCl_2$ and 1 m*M* EDTA, plus 5 m*M* substrate, which was added after the endogenous respiration rate was determined. After the "State 4" respiration rate was established, with substrate but without added ADP, 250 nmoles of ADP were added to give a brief period of "State 3" respiration. The respiratory control ratio (RCR) was determined as the ratio of State 3 respiration to State 4 respiration. The Q_{O_2} values are reported as natoms oxygen consumed/min/mg mitochondrial protein during State 3 respiration. Mitochondrial fractions were stored at 0–2° until assayed, routinely 2 hr after the animals were killed.

Protein content of homogenates and fractions was determined by the micromethod of Lowry (21), using bovine serum albumin as standard. Differences between assay results for normal liver and either host liver or tumor were analyzed for statistical significance by Student's *t* test. In the discussion of the data, differences with *P* values of <0.05 are considered significant.

Results. Tumor growth. At 7–8 days after implantation, a small mass was usually palpable at the site of implantation, and the wet weight of the tumor mass was 0.2–0.5 g. As shown in Fig. 1, the tumors weighed about 3.5 g at 17 days after implantation, and then grew rapidly to about 30 g in 30 days and to a maximum of approximately 60 g at 40 days, when the animals became moribund and usually died within a few days.

Tumor effects on body weight and liver weight. As in shown in Fig. 1 for a typical pair of rats, the body weight of the tumor-

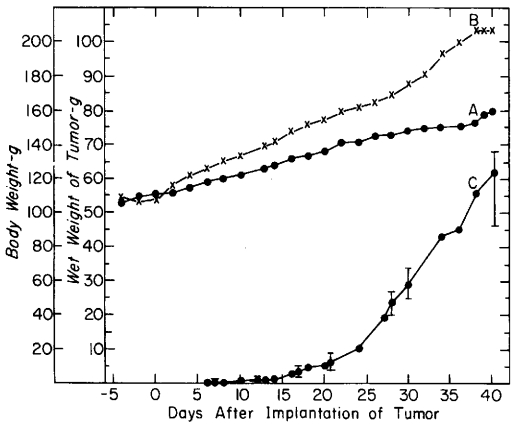

FIG. 1. Rate of growth of Reuber H-35 hepatoma and effect of tumor implantation on body weight. Curves A and B—Change of total body weight with time in a typical pair of rats. A—Normal saline-injected control; B—Tumor implanted at day 0 as described under Materials and Methods. Curve C—Weight of tumors harvested at various times after implantation. Points plotted are single values or the mean and range of weights for 3–6 tumors at a given time after implantation.

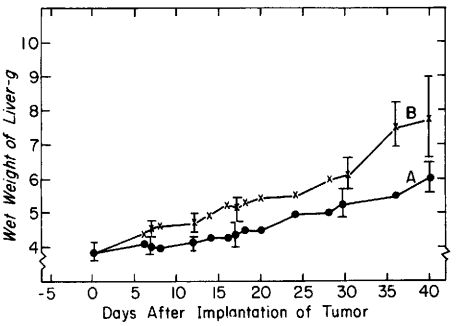

FIG. 2. Effect of tumor implantation on wet weight of liver. Points plotted are single values or the mean and range of values from 3 to 6 rats at given time after implantation of Reuber H-35 hepatoma or control saline injection by procedures described under Materials and Methods. Curve A—Liver of normal, saline-injected control rats. Curve B—Liver of hepatoma-bearing rats.

bearing animal increased at a more rapid rate than did that of the normal animal; the excess in body weight was greater than the typical tumor weight for about three weeks after tumor implantation. Later, the non-tumor body weight of the tumor-bearing animals actually decreased. Therefore, to study possible specific effects of the tumor on host tissue metabolism, minimizing general effects of tumor metabolism and avoiding degenerative changes associated with the terminal stages of cancer, livers and tumors were obtained at 17 days after tumor implantation. At this time the tumor is well established but still is less than 3% of body weight, and the non-tumor body weight of the host animals is continuing to increase at an essentially normal rate. Within 1 week of tumor implantation, the wet weight of the liver in tumor-bearing animals also began to increase at a more rapid rate than that of the normal animals (Fig. 2), and the increased liver weight persisted during the 40-day period of observation.

Comparison of host liver and hepatoma with normal liver. Table I summarizes characteristics of host liver from rats 17 days after tumor implantation, 17-day hepatomas from these host animals, and liver from normal rats of the same age. The wet weight of the host livers, compared to normal livers, showed a significant increase ($P < 0.01$), but the total protein content per g wet weight and microsomal protein and mitochondrial protein contents per g wet weight were not significantly lower in the host livers. In contrast, the protein content of the hepatoma per g wet weight was significantly lower ($P < 0.001$) than in normal liver. Concentrations of cell components and of enzyme activities have been expressed on a protein basis rather than a wet weight basis, because the protein content provides a better measure of metabolically active cellular material and because this procedure eliminates some misleading appearent decreases of activity levels in the hepatoma that are seen when the data are expressed on wet weight basis. As example, glucose-6-phosphatase activities, per mg total protein, in host liver and in the hepatoma were not significantly different from the level in normal liver, but if the activities are expressed per g wet weight, the activity in the hepatoma would appear to be significantly lower than in normal liver. Fructose-1,6-diphosphatase was significantly decreased ($P < 0.001$) in both host liver and hepatoma, with the host liver value intermediate between normal liver and hepatoma. In contrast, there was a significant increase of cathepsin activity in the host liver, and a smaller but still significant increase in the hepatoma. The microsomal drug-metabolizing activities and the associated cytochrome P-450 and NADPH-cytochrome c reductase activity

TABLE I. CHARACTERISTICS OF NORMAL LIVER, HOST LIVERS AND HEPATOMA 17 DAYS AFTER IMPLANTATION OF HEPATOMA OR LIVER[a]

Assay	Normal liver	Host liver [tumor implant]	Host liver [liver implant]	Hepatoma
1. Wet weight (g)	4.41 ± .32	5.12 ± .28[c]	4.58 ± .35	3.41 ± .96
2. Total protein (mg/g)	188.4 ± 2.5	180.2 ± 2.2	185.2 ± 3.2	142.5 ± 3.0[b]
3. Microsomal protein (mg/g)	16.7 ± 0.6	15.7 ± 0.6	17.0 ± 0.8	11.5 ± 0.9[b]
4. Mitochondrial protein (mg/g)	25.2 ± 1.0	26.0 ± 0.8	26.2 ± 1.1	5.2 ± 0.4[b]
5. Glucose-6-phosphatase	18.1 ± 1.6	18.0 ± 2.3	—	15.4 ± 3.1
6. Fructose-1,6-diphosphatase	12.6 ± 0.8	9.2 ± 1.0[b]	12.8 ± 0.9	3.9 ± 0.6[b]
7. Cathepsin	12.3 ± 1.8	21.2 ± 2.3[b]	14.8 ± 2.3	16.6 ± 2.3[c]
8. Aminopyrine demethylation	.278 ± .033	.189 ± .027[c]	—	.077 ± .015[b]
9. O-Nitroanisole demethylation	.508 ± .060	.356 ± .050[c]	.524 ± .078	.112 ± .021[b]
10. Mic. cytochrome P-450	.550 ± .062	.412 ± .053[c]	.528 ± .073	.185 ± .035[b]
11. Mic. cytochrome b_5	.413 ± .056	.365 ± .052	—	.335 ± .052[d]
12. Mic. NADPH-cyt c reductase	185 ± 30	146 ± 27[c]	176 ± 30	74 ± 15[b]
13. Mit. Q_{O_2}, succinate	83.5 ± 9.8	77.5 ± 8.7	—	44.2 ± 10.5[b]
14. Mit. RCR, succinate	6.05 ± .84	5.58 ± .82	—	2.7 ± .74[b]
15. Mit. succinate-cyt c reductase	124 ± 9	113 ± 12	—	41 ± 8[b]
16. Mit. NADH-cyt c reductase	174 ± 15	168 ± 15	—	138 ± 19[c]

[a] Data from normal rat liver, from H-35 hepatoma and from host liver of rats 17 days after implantation either of H-35 hepatoma (Host Liver [Tumor Implant]) or of liver mince (Host Liver [Liver Implant]) as described in Materials and Methods. Values reported are mean ± SD from 6 to 8 animals. Assays are as described under Materials and Methods. Units are, for 5, 6 and 7-nmoles/min/mg protein; 8, 9 and 12-nmoles/min/mg microsomal protein; 10, 11-nmoles/mg microsomal protein; 13-natoms oxygen/min/mg mitochondrial protein with succinate as substrate; 15, 16-nmoles cyt c reduced/min/mg mitochondrial protein.

[b] $P < 0.001$, compared to normal liver.

[c] $P < 0.01$, compared to normal liver.

[d] $P < 0.05$, compared to normal liver.

showed significant decreases, compared to normal liver; all four of these microsomal components showed very significant decreases in the tumor. The microsomal cytochrome b_5 concentration showed a decrease of marginal significance ($P < 0.05$) in the tumor, but not in host liver. For all of the mitochondrial oxidative activities presented in Table 1, the slightly lower numerical values for host liver mitochondria did not represent statistically significant differences from normal liver, but the decreases in the hepatoma mitochondria were significant.

To determine whether the lower respiratory control ratio in hepatoma mitochondria might reflect an increased rate of deterioration in the period between killing the animal and assay of the mitochondria, mitochondrial preparations were stored at 0–2° and assayed at intervals. As indicated by the similar slopes for the plots in Fig. 3, mitochondrial preparations from normal liver, host liver and hepatoma have essentially the same value for the first order rate constant for decrease of the respiratory control ratio with time.

Liver implants. As indicated in Table I, implantation of liver mince instead of tumor mince did not result in any statistically significant changes in host liver 17 days after implantation for those parameters that showed significant changes in host liver 17 days after tumor implantation.

Time course of changes in host liver. Table II shows the time course of changes in host liver for those activities that showed significant changes 17 days after tumor implantation. In each case, a substantial portion of the change seen at 17 days had already occurred by 7 days after implantation of the tumor, when the tumor is less than 0.5% of body weight. The maximum changes apparently were approached by 17 days, with only small changes between day 17 and day 30, a period during which the size of the tumor increased almost tenfold to represent about 20% of body weight. The table indicates also the changes in the parameters measured over the 30-day period in normal rats of the same age; adjusting the data for host liver for these changes would not alter qualitatively the pattern of the time courses of the changes in host liver.

Discussion. The significant changes that were seen in host liver 17 days after tumor implantation, when the tumor was still quite small, are suggestive of specific directive effects of the tumor on host liver metabolism. The fact that implants of normal liver did not produce such changes in the host liver (Table I) is further indication of a tumor-specific basis for the changes. It may be significant that the activities or parameters whose levels were changed in host liver also showed changes in the tumor, compared to normal liver, and the changes were in the same direction but usually of lesser magnitude than in the tumor. The wide diversity in the direction and magnitude of changes that have been reported previously for host tissues appears to reflect the varying effects of different tumor types and period of tumor growth, particularly when the quantitatively major effects of the general metabolism of a large tumor may obscure tumor-specific effects. Most of the findings in the present study are generally consistent with those observations in other studies on host liver in which the tumors, often with rapid growth rates, were apparently not yet of large size. These findings include the increase in host liver wet weight (1, 3), increase of non-tumor body weight during the early period of tumor growth (8), lack of change in glucose-6-phosphatase (8), and decreases in microsomal drug-metabolizing activities and the associated cytochrome P-450 and NADPH-cytochrome c reductase activity (6, 22, 23). Decreases of the microsomal components were also observed in liver of rats administered extracts of the rapidly growing Walker 256 carcinosarcoma (11). Our finding that cathespsin activity in host liver increased more than in the tumor is not a consistent pattern for hepatomas, but was found previously in rats bearing the Reuber H-35 hepatoma in a study that used a cathepsin assay with different substrate specificity (7). A decrease in fructose-1,6-diphosphatase activity of host livers was not seen in a previous study with the Reuber H-35 hepatoma (24), and results were variable with rats bearing the Walker 256 carcinosarcoma (4, 5).

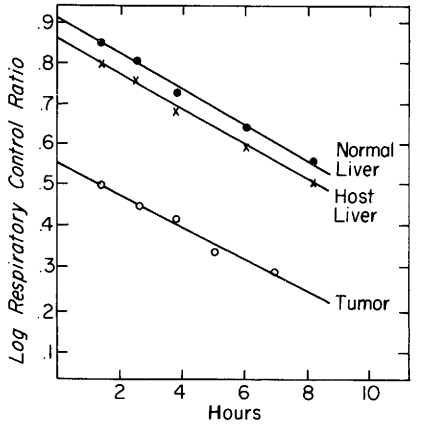

FIG. 3. Decrease of respiratory control ratios with time in mitochondria from normal rat liver, host liver of tumor-bearing rats and tumor. Mitochondria from H-35 hepatoma, liver from rats 17 days after implantation of H-35 hepatoma and normal liver from saline-injected rats of the same age were prepared and assayed as described under Materials and Methods. Values plotted are the mean of values from three preparations. Hours indicate time elapsed since killing the animals, with mitochondria being stored at 0–2° until assay.

The data on the time course of the changes in host liver (Table II) indicate that the liver can respond rapidly to the presence of even a very small tumor, which further supports the concept that the changes involve release

TABLE II. TIME COURSE OF CHANGES IN HOST LIVER OF RATS IMPLANTED WITH HEPATOMA.

Component	Initial level in normal liver[a]	% Change in normal liver in 30 days[b]	Relative activity in host liver after[c]			
			7 days	12 days	17 days	30 days
Fructose-1,6-diphosphatase	12.1 ± 0.9	+5	.92	.80	.69	.70
Cathepsin	11.3 ± 0.9	+12	1.41	1.65	1.77	1.80
O-Nitroanisole demethylation	.485 ± .061	+6	.91	.82	.73	.75
Mic. cytochrome P-450	.520 ± .068	+8	.93	.85	.81	.80
Mic. NADPH-cyt c reductase	180 ± 24	−1	.94	.85	.80	.77

[a] Values reported are mean ± SD from 6 control normal rats at time of tumor implantation. Assays and units are as for Table 1.

[b] Means of values from 3 to 5 normal rats 30 days older than initial normal rats.

[c] Means of ratio of values from 3 to 6 rats at each of indicated times after tumor implantation to mean value for initial control normal rats.

of a potent directive agent(s) from the tumor rather than adjustment to quantitative major metabolic imbalances. While it appeared that maximum changes were approached by 17 days, the rapid growth of the tumor in the subsequent two weeks makes uncertain the interpretation of the changes during the latter period. Changes in enzyme levels have also been reported in host liver within 4–7 days after implantation of the rapidly growing Walker 256 carcinosarcoma (6, 8), when the tumors were also small, or of a lymphoma (25).

The metabolic characteristics of the Reuber H-35 hepatoma reported in this study in general follow the pattern of moderate decreases of specialized functions, compared to normal liver, that is commonly seen with hepatomas of moderate growth rate (26–29). The enzymatic activities on a protein basis presented here are consistent with previous reports that this tumor has significantly lower levels than liver of fructose-1,6-diphosphatase (24) and drug-metabolizing activities (29) and a moderately higher cathepsin activity (7).

From the enzyme activity and protein content data of Table I and from morphometric studies (27, 30), it appears that the markedly lower mitochondrial enzyme activities of the hepatoma, per unit wet weight of tumor, reflect primarily a reduced mitochondrial mass per g tissue. The decrease in tumor, compared to liver, of the respiratory rate per mg mitochondrial protein with certain substrates may result in part from selective deficiency of a specific dehydrogenase in the tumor. Thus, the low rate of succinate oxidation and the markedly greater reduction of succinate–cytochrome c reductase activity than of NADH-cytochrome c reductase activity in the tumor (Table I) may reflect a selective deficiency of succinate dehydrogenase (31). The plots of Fig. 3 suggest that the mitochondrial fraction from the tumor has a lower respiratory control ratio (RCR) at the time of preparation than does the fraction from normal liver. The lower RCR value for tumor mitochrondria may not represent an intrinsic difference in tumor mitochondria, but may in part result from a higher content of mitochondria damaged *in vivo* because of an unfavorable environment in the tumor or, as suggested by the results of Kaschnitz (32), from an increased content of fragile mitochondria that are damaged by the procedure used to isolate the mitochondria. To varying degree, a reduced mitochondria mass, or either intrinsic deficiency or damage to fragile tumor mitochondria may be responsible for the many observations of reduced respiratory activity and decreased respiratory control which have been cited in support of Warburg's theory of oncogenesis (33).

Summary. The presence of a transplantable hepatoma led to changes in host liver metabolic capabilities during the early stages of tumor growth. The changes included increases of liver wet weight and cathepsin activity and decreases of fructose-1,6-diphosphatase activity, microsomal drug-metabolizing activities, cytochrome P-450 and NADPH-cytochrome c reductase activity. The nature and early appearance of these changes and the failure of liver implants to produce similar changes suggest that there may be specific directive effects of the tumor on host tissues. The low respiratory control ratio seen with hepatoma mitochondria preparations did not reflect an increased rate of deterioration with storage.

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