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Dihydrouracil Dehydrogenase Activity in Normal, Differentiating, and Regenerating Liver and in Hepatomas

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SUMMARY

The behavior of dihydrouracil dehydrogenase activity (4,5-dihydrouracil:NADP oxidoreductase, EC 1.3.1.2) was compared in proliferating normal liver (from developing rats and from partially hepatectomized rats) and in neoplastic liver (spectrum of hepatomas of different growth rates). The enzyme activity was determined in the 100,000 × *g* supernatant fluid.

The affinity of the enzyme to the substrate, uracil, was similar in the hepatomas to that in the liver of control normal rats of the same strain. The pH optimum of the control and neoplastic livers was at 7.2.

The activities of dihydrouracil dehydrogenase in rat liver, thymus, intestinal mucosa, spleen, kidney, brain, skeletal muscle, and heart were 2.0, 1.1, 0.9, 0.7, 0.6, 0.4, 0.2, and 0.1 μmoles/hr/g, wet weight, of tissue at 37°, respectively.

In the differentiating liver, the enzyme activity of the average cell increased 2.7-fold from the level observed in the newborn to reach the activity of the liver of adult rat. In the regenerating liver at 24 hr after partial hepatectomy, the enzyme activity decreased to 74% of that of the sham-operated controls.

In the spectrum of hepatomas, dihydrouracil dehydrogenase activity decreased in parallel with the increase in hepatoma growth rate. In the most rapidly growing tumors (9618A2, 3683F), the activities were about 15% or less of those in liver of control rats of the same strain, sex, age, and weight.

Since the regenerating liver proliferates at a rate comparable to that of the rapidly growing hepatoma, the marked decrease observed in the tumor appears to be specific to neoplastic cell growth.

Dihydrouracil dehydrogenase is considered the rate-limiting enzyme in the catabolic pathway of uracil; therefore, the close linking of the decrease in the activity of this enzyme with the increase in hepatoma growth rate provides further evidence in support of the Molecular Correlation Concept.

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INTRODUCTION

Our studies have been concerned with elucidating the pattern in the alteration of gene expression in cancer cells. The Molecular Correlation Concept developed in this laboratory postulated the operation of a pattern of gene expression involving a close linking of the proliferation rate of cancer cells with the imbalance between opposing key enzymes and metabolic pathways (15, 16). As a result of such investigations, it was discovered that the activity of the key glycolytic enzymes increased and concurrently the activity of the key gluconeogenic enzymes decreased in hepatomas in parallel with the increase in tumor growth rate (15, 16).

In our recent work, we recognized that the incorporation of thymidine into DNA increased and concurrently the degradation of thymidine to CO_2 decreased in parallel with the increase in hepatoma growth rate (3). In the catabolic pathway that degrades thymidine or uridine to CO_2, β-amino acid, and ammonia, the enzyme dihydrouracil dehydrogenase (4,5-dihydrouracil:NADP oxidoreductase, EC 1.3.1.2) was identified as the rate-limiting enzyme (1, 2, 4, 6). Since the Molecular Correlation Concept suggests that it is the key enzymes and key metabolic pathways that may be expected to correlate with tumor growth rate (15), the behavior of dihydrouracil dehydrogenase was examined in normal resting and rapidly growing liver and in hepatomas of different growth rates. The results showed that indeed the behavior of the dihydrouracil dehydrogenase activity is linked with hepatoma growth rate and varies reciprocally with tumor proliferation rate.

MATERIALS AND METHODS

The animals were kept in individual cages and illuminated daily from 6 a.m. to 7 p.m. Purina laboratory chow and water were available *ad libitum*.

Studies on Differentiating Liver. Pregnant Wistar rats were purchased from Harlan Industries, Cumberland, Ind. The litters were allowed to remain in the same cage with the mother for 18 days after birth; then each rat was placed in an individual cage.

Studies on Regenerating Liver. For studies on the effects of partial hepatectomy and sham operation, male albino Wistar (Harlan Industries) or ACI/N (Laboratory Supply Co., Indianapolis, Ind.) rats, weighing 180 to 200 g, were used. The rats were partially hepatectomized under light ether anesthesia

by removal of 66% of the liver, according to the standard procedure of Higgins and Anderson (7). The remaining liver lobes were examined at different time intervals after operation. Sham-operated animals were used as controls (4, 13).

Tumor-bearing and Control Animals. The hepatomas of different growth rates were transplanted in the inbred strains of male Buffalo or ACI/N rats. The tumors were transplanted bilaterally by Dr. H. P. Morris in Washington, D. C., and the tumor-bearing and control rats were shipped by air express to our laboratories at Indiana University School of Medicine, Indianapolis, Ind. Normal rats of the same strain, sex, age, and weight were sacrificed along with the tumor-bearing rats under the same experimental conditions. We examined a number of tumor lines, including the slowly growing 44, 47-C, 7787, 9618A, and 9618B and the rapidly growing hepatomas 7777, 3924A, 3683F, and 9618A2. The tumors were allowed to grow to a diameter of about 1.5 cm when they were harvested. The biological and growth properties (11) and the biochemical techniques for quantitating proliferation rate of the hepatoma spectrum were described previously (3, 15).

Experimental Procedures, Biochemical Assays, and Cell Counts. The rats were stunned, decapitated, and exsanguinated. Livers and tumors were rapidly removed and placed in beakers which stood on crushed ice. Tissues were carefully dissected free of necrotic, hemorrhagic, and nontumorous material. The careful selection at harvesting of only the viable tumor tissue parts is important in ensuring the comparability of tumors of different ages and of different tumor lines.

Preparation of homogenate was carried out as described previously (17). The homogenizing medium was 0.25 M sucrose containing 1 mM cysteine at pH 7.4. The supernatant fluid was obtained by centrifugation in a Spinco Model L ultracentrifuge for 30 min at 100,000 × *g* (17). The clear, lipid-free supernatant fluid layer was carefully removed by using syringes with long 12-gauge needles.

The dihydrouracil dehydrogenase activity was determined in the supernatant fluid. For the enzyme assay, the method of Fritzson and Spaeren (6) was modified and adapted for the 1-ml volume of the microcuvets and for following the reaction in the Gilford 2000 recording spectrophotometer. Through careful kinetic studies, a standard assay system was developed in which the uracil- or thymine-dependent NADPH oxidation was followed at 37°, and the reaction was recorded. The reaction mixture contained potassium phosphate buffer, pH 7.4, 35 μmoles; NADPH, 0.24 μmole; uracil or thymine, 0.005 μmole; and supernatant fluid, 0.01 to 0.05 ml; distilled water completed the test system to a final volume of 1 ml. The reaction was initiated with the substrate (uracil or thymine), and the rate was recorded at 340 mμ. The blank contained the identical reaction mixture without uracil and the activity was recorded simultaneously. The enzyme activity was given by the difference between the rate observed in the full reaction mixture and that of the blank which contained no added substrate.

Protein determinations were made by the biuret procedure (9). The cell counts were made as described previously (14).

Expression and Evaluation of Results. Dihydrouracil dehydrogenase activity was calculated in μmoles of substrate metabolized per hr at 37° and was expressed per g, wet weight, of tissue, per g of protein, or per average cell. Cell counts were expressed as cellularity calculated in millions of nuclei/g, wet weight, of tissue (14).

The results were subjected to statistical evaluation by means of the *t* test for small samples. Differences between means giving a probability of less than 5% were considered to be significant. For correlation of growth rate and enzyme activity, Spearman's rank correlation coefficients were calculated (10).

RESULTS AND DISCUSSION

Comparison of Kinetic Properties of Normal and Neoplastic Liver

The affinity of the enzyme to the substrate, uracil, to thymine and to the cofactor, NADPH, was examined, and the pH optimum was established in hepatomas and in control livers of the same strain, sex, age, and weight.

Studies on the Affinity of Dihydrouracil Dehydrogenase to the Substrate in Normal Liver and Hepatomas. The affinity of hepatic dihydrouracil dehydrogenase to uracil was 0.5 μM in adult ACI/N rats. The affinity for thymine was in the same range. An excess of uracil or thymine up to 0.1 mM did not exert an inhibitory effect on this enzyme. The K_m for uracil or thymine in the slowly growing hepatoma 9618A, carried in Buffalo rats, and in the rapidly growing hepatoma 3924A, carried in the ACI/N strain, was in the same range as for the liver in control rats.

The Effect of NADPH on Dihydrouracil Dehydrogenase Activity in Normal Liver and in Hepatomas. The affinity of liver dihydrouracil dehydrogenase to NADPH showed a sigmoid curve which reached a peak at a 0.2 mM concentration of the coenzyme. The apparent K_m was 0.1 mM. Beyond the

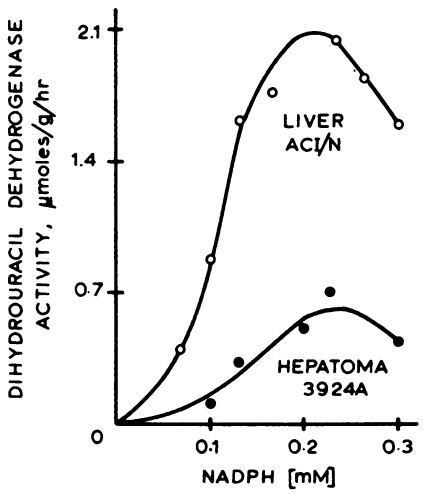

Chart 1. Affinity to NADPH of dihydrouracil dehydrogenase from liver and hepatoma. Assays were performed as described in "Materials and Methods" with NADPH concentrations altered as shown.

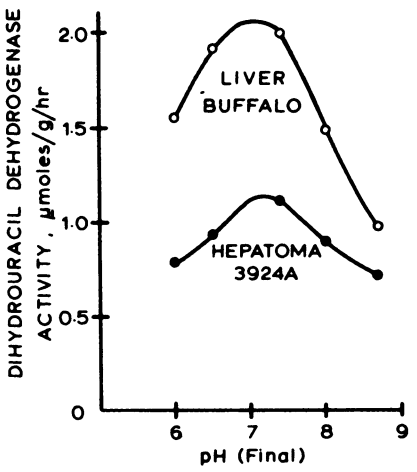

Chart 2. Effect of pH on activity of dihydrouracil dehydrogenase from liver and hepatoma. Assays were performed as described in "Materials and Methods" with the pH of the buffer altered as shown.

Table 1

Dihydrouracil dehydrogenase activity in normal rat organs

Averages of 3 determinations are given for organs from 41-day-old, male Wistar rats, 147 to 150 g. Samples for all organs except for the liver were pooled from 3 animals. Activities are also expressed (in parentheses) as percentages of normal liver values.

Tissues	Dihydrouracil dehydrogenase activity	
	μmoles/g/hr	μmoles/g protein/hr
Liver	2.0 (100)	20.7 (100)
Thymus	1.1 (55)	19.6 (95)
Intestinal mucosa	0.9 (45)	13.6 (66)
Spleen	0.7 (35)	
Kidney	0.6 (30)	7.5 (36)
Brain cortex	0.4 (20)	5.7 (28)
Skeletal muscle	0.2 (10)	4.7 (23)
Heart	0.1 (5)	1.8 (9)

level of 0.22 mM, NADPH became inhibitory. A similar S-shaped curve with a peak, a K_m, and inhibition the same as for the liver enzyme was observed for the enzyme in hepatoma 3924A (Chart 1). Similar results were found for hepatoma 9618A.

The Effect of pH on Dihydrouracil Dehydrogenase Activity in Normal Liver and in Hepatomas. The pH optimum for the enzyme from the liver of Buffalo and ACI/N strains of rats and from both slowly and rapidly growing hepatomas was the same: pH 7.2 (Chart 2).

The data presented show that the affinity to the substrate, uracil or thymine, to the coenzyme, NADPH, and the pH optimum were similar in liver and hepatomas.

As a result of careful kinetic work a routine assay was arrived at which is described in "Materials and Methods." With this method, proportionality with amount of enzyme added and with time elapsed was obtained at 37° for an assay period of 5 min.

Comparison of Activities of Dihydrouracil Dehydrogenase in Different Organs of the Adult Rat

Table 1 shows that the enzyme activity was highest in the liver, thymus, and intestinal mucosa. The activities in spleen, kidney, and brain were 35, 30, and 20%, respectively, of the activity observed in the liver. In the skeletal muscle and the heart, the activities were 10% or lower and near the limit of accuracy of this assay.

The behavior of the activity of this enzyme, apart from liver, has not been studied in detail. Special interest would lie in exploring the behavior of this enzyme in the thymus and the intestinal mucosa which exhibit active DNA synthesis.

Behavior of Activity of Dihydrouracil Dehydrogenase in Differentiating Liver

The enzyme activity, high in the liver of newborn rats, in subsequent development increased about 2.5-fold, reaching the level observed in the adult rat liver (Table 2). Expressed on a

Table 2

Behavior of dihydrouracil dehydrogenase activity in differentiating rat liver

Values given show means ± S.E. for 3 or more determinations. Data in parentheses express the enzyme activities as percentages of values observed in liver of adult rats. For rats 7 days old and younger, 2 to 4 samples were prepared, each containing pooled livers of 10 to 12 animals. Beyond this age group, individual livers were used.

Age (days)	Body weight (g)	Liver weight (g)	Cellularity[a]	Total liver cellularity[b]	Dihydrouracil dehydrogenase activity (μmoles/cell/hr × 10^{-10}[c])	
1	7.2 ± 0.1	0.26 ± 0.01	650 ± 11	168 ± 7	39 ± 3	(36)
3	7.5 ± 0.1	0.29 ± 0.01	647 ± 12	189 ± 7	43 ± 2	(40)
7	17.0 ± 0.3	0.47 ± 0.01	590 ± 40	274 ± 3	53 ± 10	(50)
30	90.0 ± 4.0	4.3 ± 0.3	322 ± 2	1376 ± 120	139 ± 13	(130)
50	213.0 ± 4.0	8.8 ± 0.2	220 ± 5	1936 ± 58	107 ± 10	(100)

[a] Millions of cells/g, wet weight, of tissue.
[b] Total number of liver cells in millions (liver weight × cellularity).
[c] Data are to be multiplied by the exponential given to arrive at the actual values.

Table 3

Correlation of dihydrouracil dehydrogenase activity with hepatoma growth rate

The data are given as means ± S.E. with percentages of corresponding control liver values in parentheses. The activities per cell are to be multiplied by the exponential given to arrive at the actual values. Assay conditions are described in "Materials and Methods." Growth rate is expressed as the mean transplantation time given in months between inoculation and growth to a size of approximately 1.5 cm diameter.

Tissues	No. of observations	Transplant generation	Growth rate	Dihydrouracil dehydrogenase activity	
				μmoles/g/hr	μmoles/cell/hr × 10^{-10}
Normal liver (ACI/N)					
Control for 3924A	15			1.9 ± 0.2	95 ± 13
Control for 3683F	3			2.9 ± 0.1	129 ± 5
Normal liver (Buffalo)					
Control for 44	4			2.8 ± 0.4	118 ± 19
Control for 47-C	2			2.9 ± 0.1	137 ± 3
Control for 7787	2			2.4 ± 0.4	123 ± 10
Control for 9618A	5			2.0 ± 0.1	112 ± 6
Control for 9618B	4			1.8 ± 0.1	82 ± 3
Control for 7777	3			2.2 ± 0.2	116 ± 35
Control for 9618A2	3			2.6 ± 0.1	87 ± 2
Normal liver (Wistar)	4			2.2 ± 0.3	93 ± 13
Sham-operated (Wistar)	4			2.3 ± 0.3 (100)	108 ± 18 (100)
24-hr regenerating liver (Wistar)	4			1.7 ± 0.2 (74)	87 ± 9 (81)
Hepatomas					
44	3	6	9	2.5 ± 0.3 (89)[a]	111 ± 15 (94)
47-C	4	5	8	1.5 ± 0.1 (52)[a]	87 ± 7 (64)[a]
7787	2	15	7	2.3 ± 0.8 (94)	102 ± 38 (80)
9618A	6	6	5.8	1.1 ± 0.1 (55)[a]	55 ± 5 (49)[a]
9618B	4	5	4.5	1.2 ± 0.1 (69)[a]	59 ± 6 (73)[a]
7777	4	66	1.3	0.9 ± 0.2 (41)[a]	45 ± 16 (39)[a]
3924A	14	277	1.0	0.5 ± 0.05 (29)[a]	36 ± 5 (38)[a]
3683F	4	280	0.5	0.3 ± 0.04 (9)[a]	12 ± 1 (10)[a]
9618A2	4	19	0.4	0.3 ± 0.04 (12)[a]	13 ± 1 (15)[a]

[a] Values statistically significantly different from the respective controls.

per mg DNA basis, the activities were 0.28 μmole/mg DNA/hr for newborn and 0.71 μmole/mg DNA/hr for adult rats, corresponding to a 2.7-fold increase from newborn to adult. The rise of the dihydrouracil dehydrogenase activity in differentiating liver is in parallel with the behavior of the activities of thymidine phosphorylase and uridine phosphorylase (12), β-ureidopropionase and dihydrouracil hydrase (4, 5). The behavior of these *enzymes* involved in the catabolism of thymidine or uridine to CO_2 agrees well with the pattern observed for the activity of the *overall catabolic pathway* which degrades thymidine or uridine to CO_2 (3).

Correlation of the Activity of Dihydrouracil Dehydrogenase with Growth Rate of Hepatomas

Investigation was carried out to establish the relationship of this enzyme activity to hepatoma growth rate. Table 3 gives the activities of dihydrouracil dehydrogenase in hepatomas of different growth rates in comparison with the activities observed in the liver of control animals of the same strain, sex, and age, killed concurrently with the tumor-bearing rats. In parallel with the increase in hepatoma growth rate, there was a decrease in the activity of dihydrouracil dehydrogenase. In the slow-growing tumors the enzyme activity was near normal values, but in the more rapidly growing tumors it was markedly decreased and in the most rapidly growing hepatomas in the series (3683F, 9618A2) it was 15% or less of that in livers of corresponding control normal rats.

The activity of this enzyme in normal liver is high as compared to those involved in the synthetic utilization of thymidine into DNA. For instance, whereas dihydrouracil dehydrogenase activity in normal rat liver is 20.7 μmoles/g protein/hr (Table 1), the activities of thymidine kinase,

thymidylate kinase, and DNA polymerase were 2.4, 0.420, and 0.056 μmoles/g protein/hr, respectively (8). It appears that the activity of the degradative pathway is high in normal liver and predominates over the synthetic pathway. The decrease in the catabolic pathway activity and the decrease in activity of this rate-limiting catabolic enzyme are significantly correlated with the increased growth rate of the tumor tissue. The increase in activity of the synthetic enzymes also occurs concomitant with the increase in hepatoma growth rate.

Table 3 also shows that dihydrouracil dehydrogenase activity in the regenerating liver underwent only a small decrease of about 26% as compared to activities in the liver of sham-operated control rats. This is in good agreement with previous work (4). Since there is no similar extent of decrease in the regenerating liver which proliferates at a rate comparable to that of the rapidly growing hepatomas, the decrease in the dihydrouracil dehydrogenase activity in the liver tumors appears to be specific to neoplasms. A comparison with the rapidly growing, newborn rat liver also indicates a difference; in the liver of newborn and young rats, high activity of dihydrouracil dehydrogenase is present.

Since this is thought to be the rate-limiting enzyme in the degradation of thymidine and uridine, a comparison is given of the behavior of dihydrouracil dehydrogenase activity and the activity of the degradative pathway as measured by studying the catabolism of thymidine to CO_2 in slices from hepatomas of different growth rates (Chart 3). This chart shows that there is a correlation between the growth rate of the hepatomas and the decrease in dihydrouracil dehydrogenase activity and in the overall CO_2-producing pathway. The mathematical aspects of the correlation were calculated by Spearman's method, which indicated that all correlations were statistically significant ($p < 0.05$).

These results are in line with the rate-limiting role proposed for dihydrouracil dehydrogenase in the catabolic pathway of uracil. The Molecular Correlation Concept (16) groups the behavior of biochemical parameters according to their relation to tumor growth rate. Accordingly, the metabolic parameters that correlate with growth rate belong to Class 1 and the behavior of dihydrouracil dehydrogenase places the enzyme in this class. The close linking of dihydrouracil dehydrogenase activity with tumor proliferation rate provides further evidence in support of the Molecular Correlation Concept.

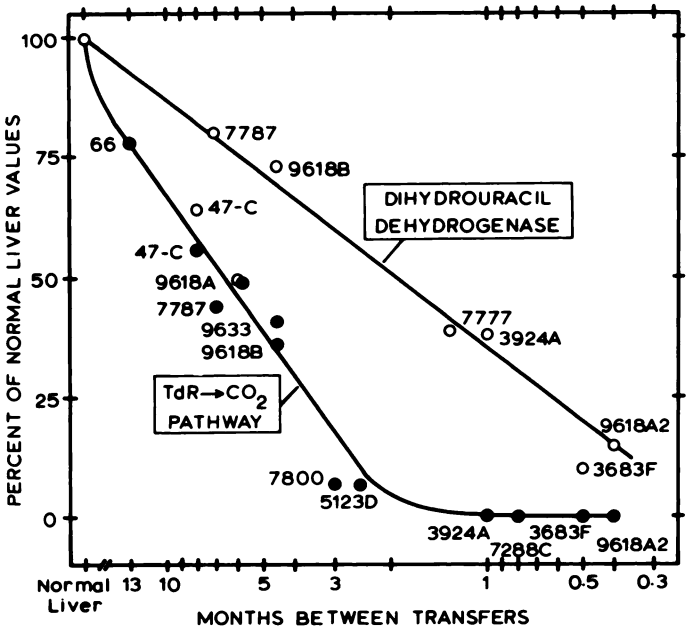

Chart 3. Correlation with hepatoma growth rate of the activity of dihydrouracil dehydrogenase and of the activity of the overall degradative pathway of thymidine to CO_2. Dihydrouracil dehydrogenase activities were assayed in supernatant fluid, and values were taken from Table 3. The degradation of thymidine to CO_2 was determined in slices and values were taken from the work of Ferdinandus *et al.* (3). The growth rates are as in Table 3. Spearman's rank correlation coefficients, $r' = 6\,\Sigma d^2/[n(n^2 - 1)]$, were calculated for the relationships shown in the chart. For the 8 tumor series, dihydrouracil dehydrogenase activity *versus* growth rate gave $r' = 0.88$. In the same series the dihydrouracil dehydrogenase activity *versus* thymidine (TdR) to CO_2 pathway activity gave $r' = 0.79$. In both cases, the correlation was significant ($p < 0.05$).

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