Effect of 5-Diazouracil on the Synthetic and Catabolic Utilization of Thymine in Rat Liver[1] (36193)

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Recent studies in this laboratory examined the regulation of the opposing pathways of thymidine metabolism (1, 2). These investigations revealed that the utilization of thymidine for DNA synthesis (synthetic pathway) and the degradation of thymidine to CO_2 (catabolic pathway) in rat liver exhibited an opposing pattern in differentiation, in regeneration and in hepatomas. Under these circumstances of development and altered growth conditions, it is assumed that long-term control mechanisms are in play involving regulation of gene expression (1–3). These results also suggested to us that there might exist control mechanisms that can achieve rapid alterations in the balance of opposing pathways of nucleic acid metabolism. We have explored the action of several drugs on nucleic acid metabolism and the present report deals with the effects of 5-diazouracil on the metabolic fate of thymine. This compound is of interest because it irreversibly inhibited dihydrouracil dehydrogenase (4,5-dihydrouracil:NADP oxidoreductase, EC 1.3.1.2) (4) which is the rate-limiting enzyme of the catabolic pathway of pyrimidines (5, 6). This observation was of relevance for our studies, since we perceived in it a possible tool for exploring rapidly-acting control mechanisms in nucleic acid metabolism. The present paper reports that administration of 5-diazouracil markedly decreases the catabolism of thymine but increases the synthetic utilization of thymine 4-fold and the acid-soluble pool 7-fold in 2 hr.

Materials and Methods. Animals and chemicals. Three-week-old male Wistar rats were kept in separate cages and Purina laboratory chow and water were available *ad lib.* Thymine-2-^{14}C was purchased from New England Nuclear Corporation, Boston, MA, 5-diazouracil and cycloheximide from Sigma Chemical Company, St. Louis, MO, and actinomycin D from Merck Sharp & Dohme, West Point, PA.

Experimental procedures. Thymine-2-^{14}C (sp act 2 mCi/mmole), 5-diazouracil (5.0 mg/kg), actinomycin D (50 μg/kg), cycloheximide (876 mg/kg), or distilled water were given by ip injections.

In vivo *effect of diazouracil on incorporation of thymine into DNA.* Two hr after thymine injection the rats were stunned, decapitated, and exsanguinated. Livers were removed and 100-mg slices were placed in separate 25-ml Erlenmeyer flasks containing 6 ml of 10% trichloroacetic acid (TCA). The flasks were shaken for 30 min at 37° in a Dubnoff metabolic shaker, then the tissues were homogenized, transferred to 10-ml volumetric flasks and brought to volume with distilled water. Aliquots of 0.6 ml were pipetted into separate Millipore filter apparatuses which contained glass fiber discs (934AH glass fiber filter, 2.4 cm, Reeve Angel, Clifton, NJ) and 5 ml of 10% ice-cold TCA. Suction was applied and filter discs were washed successively with cold 10-ml portions of 10% TCA, alcohol:ether (1:1), and ether. The filters were air-dried for 1 min by suction, then placed in scintillation vials to which were added 10 ml of scintillator fluid (0.03% dimethyl POPOP and 0.5% PPO in toluene from New England Nuclear). The vials were then counted on a Packard Tri Carb liquid scintillation spectrometer (model 314 EX). These techniques are described in detail elsewhere (2).

In vivo *effect of diazouracil on the degradation of thymine to CO_2.* The *in vivo* effect of 5-diazouracil on thymine degradation was measured by assaying CO_2 production from

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TABLE I. Effect of 5-Diazouracil Injection on Incorporation of Thymine into DNA in Liver.[a]

Incorporation of thymine into DNA (dpm/g/hr)					
Controls	Hr after 5-diazouracil injection	Days of 5-diazouracil treatment			
0	2	1	2	3	4
275 ± 20 (100)	1202 ± 176 (437)[a]	1593 ± 101 (579)[b]	1766 ± 240 (642)[b]	1362 ± 74 (495)[b]	1597 ± 166 (581)[b]

[a] Daily ip injections of 5-diazouracil (5.0 mg/kg) were given. Rats were killed and livers removed 2 hr after injection with thymine-2-^{14}C. Controls were injected with distilled water. Means ± SE of 3 or more rats are given with percentages in brackets.

[b] Statistically significant difference from control value ($p < .05$).

thymine-2-^{14}C (sp act 55 mCi/mmole) by the method of Ferdinandus *et al.* (2) The *in vitro* effect of 5-diazouracil on the degradation of thymine to CO_2 was assayed in the same way except that 5-diazouracil was added to the incubation medium and not injected into the animal.

Acid-soluble pool. Two hr after injection with labeled thymine the livers were removed and 100-mg slices were placed in separate flasks containing 5 ml of ice-cold Krebs–Ringer phosphate buffer (pH = 7.4), then washed three times with 5-ml portions of this buffer. The tissues were transferred to ice-cold homogenizing tubes which contained 1 ml of 0.5 *N* perchloric acid. The tissues were homogenized and centrifuged, and the supernatants were neutralized with 7 *N* KOH. The precipitates were removed by centrifugation and 100 μl of the acid-soluble material were placed in scintillation vials. The samples were air-dried, filled with scintillator fluid and counted.

Results. The effect of 5-diazouracil injection on incorporation of thymine into DNA in liver is shown in Table I. Two hr after 5-diazouracil injection there was a more than 4-fold increase in DNA synthesis. Subsequent daily injections of 5-diazouracil did not produce a statistically significant increase in DNA synthesis above the high value observed at 2 hr after the first injection.

In view of these results the 2 hr period after drug injection was selected for more detailed analysis. The question of interest at this point was whether the stimulation of the synthetic utilization of thymine entailed a concurrent decrease in the catabolic pathway of this pyrimidine. In order to examine this mechanism, groups of rats were injected with labeled thymine. The experimental animals were given ip injections of 5-diazouracil and the controls were injected with distilled water; the rats were killed 2 hr after the injection. The activity of the synthetic pathway was ascertained by determining the incorporation of the injected labeled precursor into DNA from samples of the liver. The activity of the catabolic pathway was determined by preparing slices from livers of control and 5-diazouracil-treated rats, incubating them with thymine-2-^{14}C and assaying the production of $^{14}CO_2$. A representative series of experiments is given in Table II. The results show that administration of 5-diazouracil caused an increase in 2 hr in the synthetic utilization of thymine to 492% of the controls and inhibited the activity of the catabolic pathway of thymine to 13% of that observed in control rat liver.

Addition of labeled thymine to liver slices permitted the *in vitro* measurement of the dose response inhibition by 5-diazouracil of the degradative pathway of thymine to CO_2. As Fig. 1 shows, under these circumstances a 50% inhibition was observed at a 5-diazouracil concentration of 0.02 m*M*. The *in vitro* incubation of 5-diazouracil in liver slices caused a dose-dependent progressive decrease in the degradation of thymine to CO_2 (Fig. 1), whereas this technique did not lead to conditions in the slice system which exhibit the marked stimulation of the synthetic pathway revealed only under *in vivo* circumstances (Tables I and II).

TABLE II. Effect of 5-Diazouracil Injection on Thymine Incorporation into DNA and Thymine Degradation to CO_2.[a]

Experimental Groups	Catabolic pathway Thymine to CO_2 (dpm/g/hr)	Synthetic pathway Thymine to DNA (dpm/g/hr)
Controls	356000 ± 32000 (100)	275 ± 20 (100)
5-Diazouracil	47000 ± 257 (13)[b]	1353 ± 122 (492)[b]

[a] The activity of the synthetic pathway was determined by injecting thymine-2-^{14}C ip and measuring its incorporation into DNA. The activity of the catabolic pathway was determined by measuring $^{14}CO_2$ production from thymine-2-^{14}C in liver slices from rats previously injected ip with 5-diazouracil. Rats were killed 2 hr after 5-diazouracil injection. Means ± SE of 3 or more rats are given with percentages in brackets.

[b] Statistically significant difference from respective control value ($p < .05$).

Both results, the fact that diazouracil can cause a rise in the synthetic pathway in 2 hr, and that the stimulation cannot be demonstrated *in vitro*, posed a question regarding the mechanism of the increase observed *in vivo*.

Among the possible mechanisms there were two that seemed of interest to examine. To ascertain whether stimulation of the incorporation of thymine into DNA involved an increase in the synthesis of enzymes in the synthetic pathway of DNA or alterations in the acid-soluble pool, a series of experiments were carried out.

The effects of actinomycin D and cycloheximide on the action of 5-diazouracil were examined in order to explore whether the rapid stimulation of the synthetic utilization of thymine entails new RNA and possibly new enzyme protein synthesis. Pretreatment of the rats with actinomycin D (50 μg/kg injected ip) or cycloheximide (876 mg/kg injected ip) 1 hr before injection with 5-diazouracil did not prevent the more than 4-fold increase of thymine incorporation into DNA. Further experiments demonstrated that actinomycin D or cycloheximide (same doses as above) injected concurrently with 5-diazouracil also failed to affect the rapid rise of DNA syn-

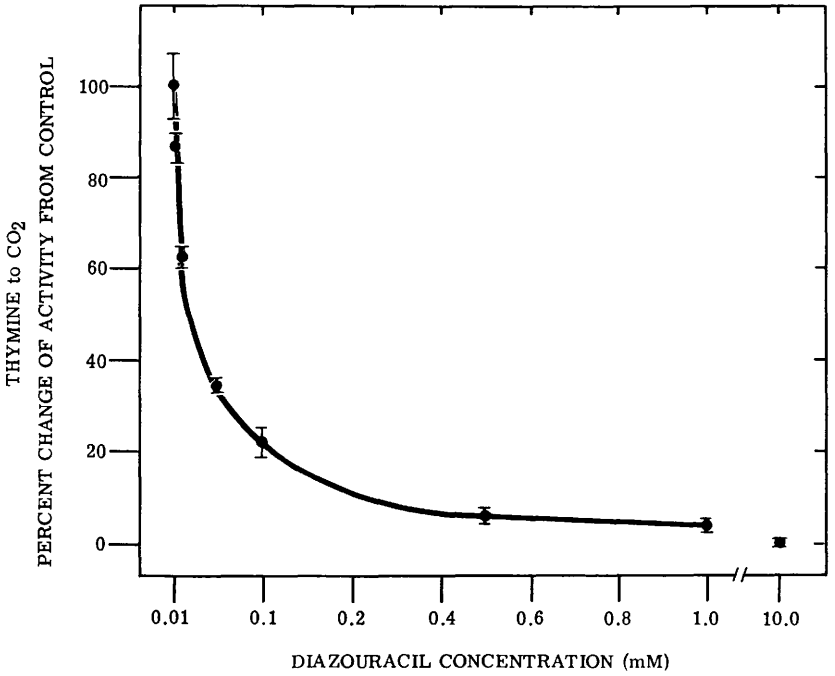

FIG. 1. *In vitro* dose-response effect of diazouracil on the degradation of thymine-2-^{14}C to CO_2 in a liver slice system.

TABLE III. Effect of 5-Diazouracil on the Acid-Soluble Pool.[a]

Experimental Groups	dpm/g
Control	490 ± 72 (100)
5-Diazouracil	3611 ± 306 (737)[b]

[a] Animals were killed 2 hr after 5-diazouracil and thymine-2-^{14}C injections. Means ± SE of 4 rats in each group are given with percentages in brackets.

[b] Statistically significant difference from control ($p < .05$).

thesis. The rapid increase of thymine incorporation into DNA produced by 5-diazouracil therefore did not appear to depend on new RNA or enzyme biosynthesis.

The possibility that the rapid rise in DNA synthesis caused by 5-diazouracil was due to increased availability of biosynthetic precursors from thymine was investigated by examining the acid-soluble pool. Table III shows that injection of 5-diazouracil caused in 2 hr a 7-fold increase in the acid-soluble pool.

Discussion. These results reveal that it is possible to produce rapidly a metabolic imbalance in the relationship of the synthetic and catabolic pathways of pyrimidine utilization by injection of 5-diazouracil. Previous work (1, 2) showed that in the liver of newborn rats the incorporation of thymidine to DNA was very high and it gradually decreased during differentiation to very low levels in the adult. In contrast, the catabolic utilization of thymidine was high in the liver of the newborn rat, and it further increased during differentiation, being approximately twice as high in the adult as in the newborn. Treatment with actinomycin blocked the rise in the activity of the catabolic pathway but did not affect the decrease in the anabolic pathway. Similar opposing behavior was observed after partial hepatectomy which resulted in marked changes in the behavior of thymidine metabolism. After operation the activity of the synthetic pathway increased, while that of the catabolic pathway decreased. Such events in differentiation develop over a number of days and weeks, and in regeneration they evolve in about 18 hr after operation. These coordinated alterations were interpreted in terms of repression and derepression of the genic expression for the respective pathways (1–3).

On the other hand the rapid action of diazouracil which also leads to marked alterations in the ratio of the synthetic and catabolic pathways becomes apparent in 2 hr. The profound changes produced by this drug are not blocked by inhibitors of RNA and protein synthesis (actinomycin and cycloheximide). The extensive decrease in the degradation of thymine to CO_2 achieved in *in vivo* by 5-diazouracil and also demonstrated in a dose-dependent fashion *in vitro* slice studies may well be attributed to the inhibitory action of this compound on dihydrouracil dehydrogenase (4). This interpretation is also supported by the close correlation between the behavior of the overall catabolic pathway of thymidine and that of dihydrouracil dehydrogenase (6). It is of considerable interest that inhibition of the catabolic pathway goes hand-in-hand with stimulation of the synthetic pathway. This may be accounted for by an increased availability of thymine or thymidine which is reflected in the observed 7-fold rise of the acid-soluble pool (Table III). The antagonistic behavior of the opposing pathways of thymidine utilization manifested in differentiation, in regeneration and in hepatomas has a counterpart in the rapid metabolic imbalance caused by this compound. Thus, the evidence suggests that the synthetic and catabolic utilization of pyrimidines is a closely coordinated process which is subject to regulation both at the level of gene expression and at the level of enzyme activity. These considerations also indicate that 5-diazouracil is a valuable experimental tool for analyzing the control of thymidine metabolism and experiments are in progress utilizing this compound in the study of the regulation of nucleic acid metabolism in differentiation, in regeneration and in liver tumors.

Summary. Injection of 5-diazouracil increased in 2 hr the incorporation of thymine-2-^{14}C into DNA of rat liver over 4-fold and it elevated the level of the acid-soluble pool 7-fold. Continued treatment with 5-diazouracil over a 4-day period did not increase the in-

corporation of labeled thymine above the level achieved at 2 hr after administration. Treatment with 5-diazouracil decreased the degradation of thymine to CO_2 to 13% of the values observed in control rats. Addition of 5-diazouracil to liver slices caused a dose-dependent progressive decrease in the degradation of thymine to CO_2 yielding a 50% inhibition at 0.02 m*M*. Actinomycin D or cycloheximide, whether injected 1 hr before or simultaneously with 5-diazouracil, did not inhibit the stimulation of the incorporation of thymine into DNA produced by the diazouracil treatment. Thus, 5-diazouracil, which irreversibly inhibits hepatic dihydrouracil dehydrogenase, can rapidly cause a marked imbalance in pyrimidine metabolism leading to a rise in the synthetic utilization and a decrease in the catabolism of thymine.

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