THYMINE 7-HYDROXYLASE ACTIVITY IN NORMAL AND LEUKEMIC LEUKOCYTES

E. K. Schandl
Life Sciences Center
Nova University
Fort Lauderdale, Florida 33314

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Summary. Thymine 7-hydroxylase activity was detected in normal and leukemic leukocytes of Fischer 344 rats as witnessed by the conversion of thymine-2-^{14}C to 5-hydroxymethyluracil. All the enzyme activity was found inside the cells, but occasionally low levels of activity were recovered from the plasma. The catalysis of the reaction was stimulated by ascorbic acid, α-ketoglutaric acid and Fe^{2+}. Leukemic cells produced up to eight times as much product as normal leukocytes.

Introduction. Thymine can be catabolyzed, for example, by an oxidative pathway whereby the first step is its conversion to 5-hydroxymethyluracil (HMU). Several mammalian systems (1, 2) as well as the mold Neurospora crassa (3) possess thymine-7-hydroxylase, the catalytic agent of this conversion. Highly active cell-free extracts were prepared from Neurospora (4), but only low levels of activity were detected in beef liver, rat liver and lamb kidney (2). The cofactor requirements for all systems were reported to be ascorbate, α-ketoglutarate, Fe^{2+} (4) and GSH (2, 5). Up to this time there is no report available concerning thymine 7-hydroxylase activity in malignant systems. This presentation describes the conversion of thymine to HMU in leukemic and normal leukocytes.

Materials and Methods.

Cells. Leukemic white blood cells, originally drawn from a germfree Fischer 344 rat, bearing a spontaneous mononuclear leukemia, were serially passaged in conventional Fischer 344 rats. Normal white blood cells were obtained from Fischer 344 rats. All animals used were

about three months old. Leukemic blood was collected at the terminal stage of the disease.

Buffers and Chemicals.

The complete incubation mixture was composed of 1 mM ascorbate, 0.5mM α-ketoglutarate (α-KG) and 0.5 mM Fe^{2+} in 50 mM Tris, adjusted to pH 8.0 with concentrated HCl. Phosphate buffered saline (PBS), contained, per liter, 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 (anhydrous) and 0.2 g KH_2PO_4 (anhydrous), pH 7.00. Ascorbate, GSH and NADPH were purchased from Sigma Chemical Co., non-radioactive thymine, HMU and α-KG from Calbiochem. Thymine-2-C^{14} was obtained from New England Nuclear and the specific activity was adjusted to 3 mC/mmole with cold thymine. In each experiment, 0.144 μC of T was used per ml medium

Procedures. Blood was collected in a heparinized syringe by cardiocentesis. Red cells were sedimented by mixing each 1 ml of blood with 0.4 ml of 6% dextran in PBS (6). 1 x 10^8 normal or leukemic white blood cells used in each separate experiment were separated from the plasma by centrifugation at 500 x g, then suspended in 2 ml of the appropriate incubation mixture and poured into tubes containing the radioactive substrate. All procedures were carried out at 2°. Incubations were at 37° for 60 min. in a shaking water bath, terminated by the addition of 4 volumes of absolute ethanol. The mixtures were shaken vigorously and allowed to stand for at least 30 minutes. The precipitable materials were pelleted by the aid of a clinical centrifuge. 0.2 ml aliquots were applied on Whatman No. 1 chromatography paper on top of already applied T and HMU chromatography markers. Two dimensional chromatography was carried out in an ethyl acetate, formic acid and water solution (70:20:10) as described (7). The UV absorbing spots were cut out and placed in vials with 5 ml of counting fluid, 4 g Omnifluor (New England Nuclear) per liter scintillation grade toluene (Fischer Scientific Co.)

Table 1

Thymine 7-Hydroxylase Activity: Normal vs. Leukemic Leukocytes

Cells	5-Hydroxymethyluracil Formed (mμmoles)
Normal[a]	0.4
Leukemic[b]	3.4

Cells were incubated for 60 min in complete incubation mixtures: 1 mM ascorbate, 0.5 mM ∝-KG, 0.5 mM Fe^{2+} in 50 mM Tris, pH 8.0.

[a]The average of four experiments, 3 in triplicate and one in duplicate, is given. Standard deviations = 0.1; the range of the average values of the duplicate and triplicate experiments was 0.5-0.3=0.2.

[b]The average of six experiments, all in duplicate, is given. Standard deviation = 1.6; the range of the average values of the duplicate experiments was 5.7-1.7=4.0.

and were counted for 10 min per sample in a Packard 2425 liquid scintillation counter (efficiency: 90% for ^{14}C). Non-enzymic and/or t=0 min enzymic controls accompanied each experiment. All results are interpreted in terms of net radiation, i.e., above background and control. All experiments were carried out in duplicate or triplicate, and they agreed within 10% of the mean value.

Results and Discussion

The conversion of thymine to 5-hydroxymethyluracil occurred in normal and leukemic leukocytes (Table 1). However, it can be seen that leukemic cells possessed a greater catalytic activity, on the average eight-fold higher than the non-leukemic leukocytes. The values obtained for normal cells varied very little from one experiment to another indicating that a low level of activity is inherent to leukocytes. Variations

Table 2

Thymine 7-Hydroxylase Activity in Leukemic White Blood Cells vs. that in the Plasma

Experiment	% Activity
Cells + Plasma[a]	100
Cells[b]	100
Plasma[c]	14
Cells + Plasma +NaCl[d]	64

Cells were prepared and the experiments carried out as described in Procedures.

[a]The plasma obtained from $1x10^8$ cells per each of the duplicate experiments was added back after sedimentation of the cells. The total volume was brought to 2 ml with the complete incubation mixture.

[b]An equal volume of PBS to that of plasma used in above was added to the cells and the total volume was adjusted to 2 ml with the complete incubation mixture.

[c]The same amount of plasma as used in the above experiments was brought to 2 ml with the complete incubation mixture.

[d]Same as the first experiment above, except 0.15 M NaCl was added to the complete incubation mixture. Incubations were for 60 min.

were observed in the malignant cells, but in every case there was at least three-fold higher activity in malignant than in normal cells.

Leukemic leukocytes were used in the following experiments described because of the higher thymine 7-hydroxylase activity present in these cells. Table 2 shows that the enzymic activity was intracellular. Some experiments, however, indicated that a portion of the enzyme was present in the plasma, probably due to leakage from

Table 3

Effect of Cofactors on Thymine 7-Hydroxylase Activity

Complete Incubation Mixture		5-Hydroxymethyluracil Formed (mμmoles)
Omissions	Additions	
None	None	2.6
Ascorbate, α-KG Fe^{2+}	PBS[a]	1.4
Fe^{2+}	None	1.9
Ascorbate	None	1.7
α-KG	None	1.8
None	GSH	1.0
None	NADPH	0.9

In each of the duplicate experiments, $1x10^8$ cells were incubated in 2 ml of incubation mixture for 60 min.

[a]Cells were suspended in 2 ml of PBS.

the cells during the manipulations. The addition of NaCl for isotonic conditions resulted in a lower enzymic activity.

Apparently the inclusion of ascorbate, α-KG and Fe^{2+} in the incubation mixture yielded the highest formation of HMU. The omission of any of these substances resulted in a decreased activity. Cells were incubated in PBS to simulate cellular conditions. The results of such incubations witnessed of about 50% lesser activity than those in complete incubation medium containing ascorbate, α-KG and Fe^{2+}. GSH and NAPH were inhibitory when included in the complete incubation media.

The increased role of thymine 7-hydroxylase in leukemic leukocytes in comparison with normal leukocytes remains to be investigated.

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