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## Thymidine Catabolism by Normal and Leukemic Human Leukocytes \*

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The pyrimidine deoxyribonucleoside, thymidine, is converted enzymatically to the free pyrimidine, thymine, in a variety of tissues, including liver, spleen, and bone marrow (1-4). Little information is available concerning human leukocyte catabolism of thymidine, although it has been used extensively in isotopic form to study leukocyte DNA synthesis (5-8).

While investigating the influence of various leukocyte homogenates on the uptake of tritiated thymidine ( $H^3TdR$ ) by isolated leukocytes from patients with chronic myelocytic leukemia (CML), we obtained data that suggested the presence of thymidine phosphorylase activity. This report presents the evidence for activity of this enzyme in human leukocytes and the values obtained for this enzyme activity in different types of leukocytes.

### Methods

**Leukocyte isolation.** Whole blood was drawn from patients with various hematologic disorders and from normal volunteers. Leukocytes were isolated by a modification of the method of Fallon and associates (9). Streptokinase-streptodornase was not used. The isolated cells were washed once with 10 ml of 5% glucose, resuspended in the same volume, and counted in an electronic cell counter.<sup>1</sup> The cells were then centrifuged for 15 minutes at  $2,100 \times g$  and resuspended in the appropriate solution at the desired concentration. Cells to be assayed for thymidine phosphorylase were resuspended in isotonic saline for counting. Aspirated bone marrow specimens were treated in a similar fashion.

Lymph<sup>2</sup> was obtained by cannulation of the thoracic duct of a patient with a malignancy who was being studied for circulating tumor cells. It was collected in heparin (1,000 U per 50 ml of lymph) on ice and cen-

trifuged at  $2,100 \times g$  for 15 minutes. The sample contained only 3 erythrocytes per 100 leukocytes and no tumor cells. Further separation of erythrocytes and leukocytes was not attempted, and the sample was homogenized directly for enzyme assay.

**Leukocyte preparations.** Homogenates for incubation with intact CML cells were made in a homogenizer<sup>3</sup> at 5,000 rpm for 15 minutes in the cold, with  $10^8$  to  $10^9$  leukocytes per milliliter of 0.1 M phosphate buffer, pH 7.2, and 0.25 g of glass beads (average diameter, 0.2 mm) per  $10^8$  cells. The mixture was centrifuged for 20 minutes at  $37,000 \times g$  and  $4^\circ C$ , and the supernatant fluid was refrigerated overnight and used the following day.

Supernatant solutions from leukocytes were prepared by incubating normal or CML cells,  $10^9$  per ml, in phosphate buffer for 2 hours at  $37^\circ C$ , and centrifuging at  $37,000 \times g$ . Homogenates of the residual cells were made as above in a concentration of  $10^9$  leukocytes per milliliter.

Homogenates for thymidine phosphorylase assay were prepared as above, with  $1.25 \times 10^8$  cells per milliliter of 0.15 M Tris-HCl buffer, pH 7.1, and 0.5 g of glass beads. Assays were performed on the day of preparation.

**CML cell uptake of  $H^3TdR$ .** Two  $\times 10^7$  freshly isolated CML cells in 0.2 ml of 5% glucose were added to 0.3 ml of autologous heparinized plasma (500 U of heparin per 25 ml blood), 1.2 ml of Robinson's salt mixture (10), pH 7.4, supplemented with 100 mg glucose per 100 ml and 209 mg sodium bicarbonate per 100 ml, and 0.2 ml of the leukocyte homogenate being tested or 0.1 M phosphate buffer as a control. After incubation for 1 hour at  $37^\circ C$  in a screw-top vial,  $1 \mu c$  of  $H^3TdR$  (SA, 0.36 c per mmole)<sup>4</sup> in 0.1 ml of isotonic saline was added and the incubation continued for an hour. In some experiments, hereafter referred to as "preincubation experiments," the  $H^3TdR$  was incubated first with the leukocyte homogenate and Robinson's solution for an hour. The CML cells and plasma were then added and the incubation continued for another hour. All incubations were performed in duplicate. The incubations were terminated by placing the vials in ice, after which the contents were transferred quantitatively with 2 ml of isotonic saline to test tubes that were centrifuged at  $1,400 \times g$  for 10 minutes at  $4^\circ C$ . The cells were washed three times with saline, which was sufficient to remove

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<sup>1</sup> Coulter Electronics, Inc., Chicago, Ill.

<sup>2</sup> Kindly provided by Dr. George Irvin, Surgery Branch, National Cancer Institute, Bethesda, Md.

<sup>3</sup> MSE homogenizer, Measuring and Scientific Equipment, Ltd., London, England.

<sup>4</sup> Schwarz BioResearch, Inc., Orangeburg, N. Y.

extracellular radioactivity. They were then dissolved in 0.5 ml of 5% sodium hydroxide at 60° C for 15 minutes, and 0.1 ml of the resulting solution was added to 18 ml of a scintillation mixture, 70% toluene, 30% methanol containing 3 g of 2,5-diphenyloxazole (PPO) and 0.050 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene (dimethyl POPOP) per L. After 48 hours at -5° C, the samples were counted in duplicate in a liquid scintillation spectrometer.<sup>5</sup> Quenching was found to be negligible with the use of internal standards.

In one experiment, the residual media from incubation were treated with 0.2 ml of 70% perchloric acid in the cold to precipitate protein and centrifuged at  $2,100 \times g$  for 10 minutes. The supernatant fluid was neutralized with 0.38 ml of 6 N potassium hydroxide and recentrifuged. Two-tenths ml of the resulting supernatant fluid was applied to Whatman 3 MM filter paper for descending chromatography.

*Distribution of intracellular radioactivity.* The method of Williams and Schilling (11) was used to separate the acid soluble fraction, protein, RNA, and DNA from cells incubated as described above with the following modifications: 1) Centrifugation was at  $2,100 \times g$  at 4° C for 10 minutes. 2) RNA was hydrolyzed by suspending the precipitated nucleic acid in 0.5 ml of 0.3 N sodium hydroxide and incubating 1 hour at 37° C. 3) DNA was precipitated by chilling and adding 0.5 ml of cold 0.5 N hydrochloric acid. 4) The protein residue and DNA were dissolved in 0.5 ml 0.5 N sodium hydroxide by heating at 80° C for 15 minutes. 5) Radioactivity was determined as above.

*Incubation of H<sup>3</sup>TdR with cell-free leukocyte preparations.* Mixtures were prepared with 1.2 ml of Robinson's solution, supplemented with glucose and sodium bicarbonate as above, 150  $\mu$ g of thymidine, 1  $\mu$ c of H<sup>3</sup>TdR, and 0.2 ml of either the supernatant solution from incubation of leukocytes in phosphate buffer or the homogenate prepared from the original cells. The mixtures were shaken gently at 37° C for 1 hour and the reaction stopped by boiling for 5 minutes. The leukocyte preparations were added at the time of boiling in the control tubes. After centrifugation at  $2,100 \times g$  for 10 minutes, 0.1 ml of each sample was chromatographed in several systems on Whatman 3 MM filter paper.

*Paper chromatography.* The solvents employed for descending chromatography were butanol, water, and ammonia (60:30:10) (12), ethyl acetate, water, and formic acid (60:35:5) (13), and isobutyric acid, water, and ammonia (66:33:1) (14).

Standards of thymine, thymidine, 5'-thymidine triphosphate (TTP), dihydrothymine (DHT),<sup>6</sup> and  $\beta$ -ureidoisobutyric acid (BUIB) were chromatographed with the samples. BUIB was prepared from DHT and dilute alkali, and both compounds were identified by spraying the dried chromatogram with dilute alkali followed by *p*-dimethylaminobenzaldehyde (15). The other com-

pounds were located by inspection with an ultraviolet lamp. The chromatograms were cut into small sections, placed in scintillation vials, and counted in the usual manner. Recovery of radioactivity when H<sup>3</sup>TdR was chromatographed was 85 to 90%.

*Thymidine phosphorylase assay.* Assay of thymidine phosphorylase activity was performed by arsenolysis (3), with 32  $\mu$ moles of thymidine per milliliter of 0.1 M arsenate and 0.42 M succinate buffer, pH 5.9, as a substrate solution. The assay mixture consisted of 0.1 ml of the enzyme preparation in Tris buffer added to 0.1 ml of the substrate solution. The final pH was 5.9. Fifteen minutes at 37° C was the optimal time for routine assay. At the end of the incubation, 0.5 ml of cold 3% perchloric acid was added, and the mixture was allowed to stand at least 1 hour in the cold. After centrifugation at  $2,100 \times g$  for 15 minutes, 0.5 ml of the supernatant fluid was added to 2 ml of 0.3 N sodium hydroxide. The differences in OD<sub>300</sub> between the experimental and control tubes, to which the perchloric acid had been added before the enzyme, were measured in a spectrophotometer (16). All assays were performed in duplicate, and correction was made for any increase in absorption in the absence of thymidine. Under these conditions, the molar extinction coefficient for thymine is  $3.62 \times 10^3$ . One unit of enzyme was defined as that quantity which catalyzed the formation of 1  $\mu$ mole of thymine per hour. Results were expressed as units per 10<sup>8</sup> leukocytes and as units per milligram of protein. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (17), using bovine serum albumin<sup>7</sup> as a standard and correcting for absorption by Tris.

*Statistics.* The significance of the differences of means of enzyme activity and of the inhibition of H<sup>3</sup>TdR uptake was calculated by the *t* test (18).

## Results

*Inhibition of thymidine uptake.* When CML cells were incubated with various leukocyte homogenates for 1 hour, H<sup>3</sup>TdR added, and the mixture incubated an additional hour, inhibition of isotope uptake in varying degrees was observed (Table I). Since the homogenates were prepared at different concentrations of cells per unit volume of buffer, comparison of the effect was made by calculating the percentage of inhibition per 10<sup>7</sup> cells used to prepare the homogenate. The normal leukocyte homogenates inhibited H<sup>3</sup>TdR uptake by 7.2 to 16.3%, CML homogenates by 4.1 to 6.1%, and chronic lymphocytic leukemia (CLL) homogenates by 0 to 2.5%. The difference of the mean inhibitory effects between normal and CML is significant ( $p < 0.05$ ), as is

<sup>5</sup> Packard Instrument Co., La Grange, Ill.

<sup>6</sup> All obtained from California Corp. for Biochemical Research, Los Angeles, Calif.

<sup>7</sup> Armour Pharmaceutical Co., Kankakee, Ill.

TABLE I  
Inhibition of CML cell H<sup>3</sup>TdR uptake by leukocyte homogenates\*

Type	Patient	Concentration of homogenate†	Inhibition of H <sup>3</sup> TdR uptake‡	Inhibition per 10 <sup>7</sup> cells of homogenate	Mean	Preincubation inhibition of H <sup>3</sup> TdR uptake§
			%	%	%	%
Normal	1	1.16 × 10 <sup>8</sup>	38	16.3	12.3	95
	2	2.7 × 10 <sup>8</sup>	73	13.5		99
	3	3.0 × 10 <sup>8</sup>	43	7.2		
CML	1	1.0 × 10 <sup>8</sup>	10.8	5.4	5.0	98
	2	2.7 × 10 <sup>8</sup>	33	6.1		98
	3	6.0 × 10 <sup>8</sup>	49	4.1		98
	4	1.0 × 10 <sup>9</sup>	89	4.5		98
CLL	1	1.0 × 10 <sup>8</sup>	1	0.5	1.0	98
	2	2.7 × 10 <sup>8</sup>	0	0		88
	3	6.0 × 10 <sup>8</sup>	30	2.5		

\* CML = chronic myelocytic leukemia; H<sup>3</sup>TdR = tritiated thymidine; CLL = chronic lymphocytic leukemia.  
 † Cells per ml of buffer.  
 ‡ CML cells and 0.2 ml homogenate incubated together; H<sup>3</sup>TdR added 1 hour later.  
 § H<sup>3</sup>TdR and 0.2 ml homogenate incubated together; CML cells added 1 hour later.

that between normal and CLL (p < 0.02), and CML and CLL (p < 0.01).

Some of the homogenates were also tested in preincubation experiments. Under these conditions, there was nearly complete inhibition (88 to 98%) of isotope uptake (Table I), and differences in inhibitory capacity between the different types of homogenates were not evident.

These results suggested differing capacities of

different types of leukocyte homogenates to inhibit H<sup>3</sup>TdR uptake by intact CML cells when competing for isotope simultaneously with them, in contrast to the virtually complete inhibitory capacity shown by all three types of homogenates under preincubation conditions.

In one experiment in which the three types of homogenates, prepared at identical concentrations, were tested simultaneously, duplicate incubation

TABLE II  
Effect of leukocyte homogenates on uptake of H<sup>3</sup>TdR by CML cells, intracellular isotope distribution, and residual radioactivity in the medium\*

Type of homogenate†	Cell radioactivity		Distribution of cell radioactivity				Residual radioactivity in medium‡		DHT or BUIB
	Inhibition		DNA	RNA	Acid-soluble	Protein	Thymine	Thymidine	
Regular incubation§	cpm	%	%	%	%	%	%	%	%
Control	981		90.2	2.2	6.5	1.1	45	45	10
Normal	265	73	95.3	0.5	1.9	2.3	71	4	25
CML	662	33	94.1	1.3	3.8	0.8	85	4	11
CLL	1,065	0	91.2	6.9	1.3	0.6	77	2	21
Preincubation									
Control	1,317						52	40	8
Normal	10	99					76	2	22
CML	22	98					71	2	27
CLL	26	98					70	2	28

\* H<sup>3</sup>TdR = tritiated thymidine; CML = chronic myelocytic leukemia; CLL = chronic lymphocytic leukemia; DHT = dihydrothymine; BUIB = β-ureidoisobutyric acid.  
 † 2.7 × 10<sup>8</sup> cells per ml of buffer.  
 ‡ Ethyl acetate:water:formic acid chromatography system (13).  
 § CML cells and 0.2 ml homogenate incubated together; H<sup>3</sup>TdR added 1 hour later.  
 || H<sup>3</sup>TdR and 0.2 ml homogenate incubated together; CML cells added 1 hour later.

mixtures were analyzed for distribution of intracellular radioactivity and for identification of radioactive compounds in the residual medium (Table II). DNA contained 90 to 95% of the intracellular radioactivity in all samples under the first incubation conditions. In the preincubation experiment the total intracellular radioactivity was very small, and the accuracy of the cell fractionation method was not adequate to permit conclusions concerning its distribution. Chromatography of the residual media in the ethyl acetate: water: formic acid system demonstrated radioactive peaks corresponding to the positions of thymine and DHT or BUIB, as well as thymidine. Little thymidine remained in the media to which homogenates had been added. In the butanol: water: ammonia system, radioactive peaks corresponded to the positions of thymidine, thymine, and BUIB. The deproteinization process used (treatment with perchloric acid followed by potassium hydroxide) would have converted any DHT to BUIB. No radioactivity corresponding to the position of the thymine nucleotides (TMP, TDP, or TTP) was found when the media were chromatographed in the isobutyric acid: water: ammonia system. These results suggested that the inhibitory effect of the homogenates was produced by the conversion of thymidine to thymine and, perhaps, to additional products of thymine catabolism. The CML cells in the absence of homogenates also seemed to carry out these reactions, but to a lesser degree. There was no inhibition of the conversion of thymidine to DNA intracellularly, since no increase in acid-soluble radioactivity was found.

A CML homogenate from Donor 4 (Table I), prepared at a concentration of  $10^9$  cells per ml, inhibited the uptake of  $1 \mu\text{c}$  of tritiated deoxyuridine (SA, 1.3 c per mmole)<sup>4</sup> by 72%, or 3.6% per  $10^7$  cells in the same experiment in which its inhibition of  $\text{H}^3\text{TdR}$  uptake (89%) was determined.

No attempt was made to purify the inhibitory factor extensively, but when prepared from normal leukocytes, it was found in the precipitate after 40% saturation with ammonium sulfate. It was not dialyzable, and activity was lost after heating to  $60^\circ\text{C}$  for 30 minutes.

Several experiments suggested that less thymidine uptake by intact cells occurred with in-

TABLE III  
Effect of phosphate concentration on  $\text{H}^3\text{TdR}$  uptake by CML cells\*

	Experi- ment	Phosphate concentration	Uptake	Inhibi- tion
		$M \times 10^{-3}$	<i>cpm</i>	%
Cells alone	1	1.95	1,577	
		11.95	981	
	2	1.95	1,567	
		11.95	895	
	3	0.	1,521	
		10.0	850	
With homogenate†	1	0.95		8
		5.6		77
	2	0.95		8
		5.6		77
	3	17.5		81
		17.5		81

\* CML = chronic myelocytic leukemia.  
†  $10^9$  cells per ml.

creased phosphate concentration (Table III), either in the presence or absence of CML homogenates. Since the former were separate experiments, a single experiment was designed to study the effect of increasing phosphate concentration on the inhibitory activity of a CML homogenate ( $10^9$  cells per milliliter) prepared in Tris buffer. Isosmotic quantities of Tris were substituted for phosphate in the Robinson's solution, and phosphate concentration was varied by the addition of different concentrations of phosphate buffer. The results showed that the inhibition of  $\text{H}^3\text{TdR}$  uptake increased with increased concentration of phosphate (Table IV). The data closely resemble those of Sköld (19) for deoxyuridine phosphorylase. When the data are plotted according to the method of Lineweaver and Burk (20), a straight line results (Figure 1), suggesting that phosphate has a substrate role in inhibiting  $\text{H}^3\text{TdR}$  uptake by intact cells under these conditions.

TABLE IV  
Effect of phosphate concentration in inhibition of  $\text{H}^3\text{TdR}$  uptake by CML cells\*

Phosphate concentration	Inhibition
$M \times 10^{-3}$	%
0.16	25
0.5	43
2.0	58
8.0	63

\* Chronic myelocytic leukemia (CML) homogenate,  $10^9$  cells per ml 0.15 M Tris.

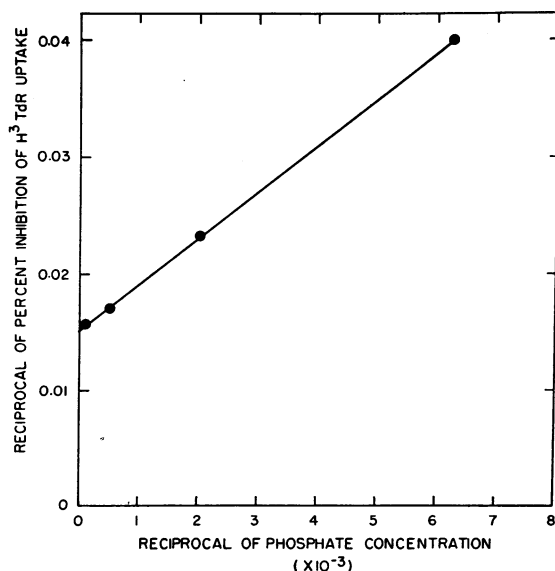


FIG. 1. RELATIONSHIP BETWEEN INHIBITION OF TRITIATED THYMIDINE (H<sup>3</sup>TdR) UPTAKE AND PHOSPHATE CONCENTRATION. The reciprocal of the percentage of inhibition of isotope uptake by chronic myelocytic leukemia (CML) cells effected by a CML homogenate in Tris buffer is plotted against the reciprocal of the concentration of phosphate in the incubation mixture.

*Thymine formation by leukocyte preparations.* When the supernatant solution from incubation of CML or normal leukocytes in phosphate buffer or the homogenate from the residual cells was incubated with H<sup>3</sup>TdR and thymidine, a radioactive peak corresponding to the position of thymine was found when the incubation mixture was chromatographed (Table V). No such peak was found when the leukocyte preparations were heated to 60° C before incubation. No evidence for DHT, BUIB, or thymine nucleotide formation was found in this cell-free system when other chromatographic systems were used. Although the preparations from the normal leukocytes seemed to have more converting activity than the CML preparations, no definite conclusion is possible since only one experiment of this type was performed.

*Thymidine phosphorylase activity of peripheral leukocyte and bone marrow homogenates.* A modification of the arsenolysis assay method of Friedkin and Roberts (3) was used to take advantage of the spontaneous decomposition of the reaction product, deoxyribose-1-arsenate. Under the conditions used, the increase in OD<sub>300</sub> in

TABLE V  
Formation of thymine from thymidine by cell-free leukocyte preparations

Preparation	Thymine spot visible with ultraviolet	Radioactivity*	
		Thymidine	Thymine
Supernatant solution†			
Normal	+	66	34
CML	0	78	22
Heated supernatant solution‡			
Normal	0	100	0
CML	0	100	0
Homogenate of residual cells§			
Normal	+	24	76
CML	+	50	50

\* Descending paper chromatography in ethyl acetate: water: formic acid (13).

† Supernatant solution after incubation of 10<sup>9</sup> cells in 0.1 M phosphate buffer, pH 7.2, for 2 hours. CML = chronic myelocytic leukemia.

‡ 60° C for 30 minutes.

§ 10<sup>9</sup> cells per ml phosphate buffer.

alkali after 15 minutes' incubation was proportional to the concentration of the homogenate being tested (Table VI, Figure 2). With longer incubation there was less proportionality, pre-

TABLE VI  
Relation of thymidine phosphorylase activity to homogenate concentration

Homogenate source and concentration	Time of incubation in minutes			
	5	15	30	45
CML,* 1.25 × 10 <sup>8</sup> cells per ml	OD <sub>300</sub>	OD <sub>300</sub>	OD <sub>300</sub>	OD <sub>300</sub>
Undiluted	0.052	0.125	0.238	0.312
Diluted 1:4	0.017	0.036	0.100	0.123
Diluted 1:8	0.000	0.017	0.052	0.055
CML, 5 × 10 <sup>8</sup> cells per ml				
Undiluted	0.091	0.177	0.371	0.372
Diluted 1:2	0.047	0.110	0.196	0.256
Diluted 1:4	0.010	0.061	0.118	0.145
CML, 1.25 × 10 <sup>8</sup> cells per ml				
Undiluted		0.095	0.162	
Diluted 1:2		0.045	0.103	
Normal, 1.25 × 10 <sup>8</sup> cells per ml				
Undiluted		0.115	0.162	
Diluted 1:2		0.066	0.101	

\* CML = chronic myelocytic leukemia.

TABLE VII  
*Thymidine phosphorylase activity of leukocyte homogenates*

Patient	Diagnosis	Leukocyte count	Activity	Protein	Remarks
		$10^3/mm^3$	$U/10^8$ leukocytes	$mg/10^8$ leukocytes	
R. E.	Normal	10.5	5.07	5.40	
T. J.	Normal	4.2	4.79	3.12	
E. Y.	Normal	5.6	3.55	4.0	
H. M.	Normal	5.9	4.05	3.08	
R. C.	Normal	8.2	4.67	3.80	
L. S.	Normal	4.2	3.80	3.40	
G. R.	CLL*	372.0	1.55	1.40	
G. N.	CLL	130.0	1.08	1.18	
A. R.	CLL	26.0	1.48	1.38	
D. R.	CLL	24.0	1.02	1.09	
J. L.	CLL	125.0	0.52	0.76	
L. H.	CLL	231.0	0.49	0.46	Receiving prednisone, 5 mg per day
N. B.	CML	243.0	2.94	3.08	
W. P.	CML	185.0	2.94	2.88	Receiving cyclophosphamide
M. B.	CML	82.0	3.00	2.24	
O. D.	CML	41.8	2.32	3.00	
R. H.	CML	63.8	2.72	3.20	
C. W.	CML	162.0	2.50	5.40	
J. B.	CML	42.6	1.76	2.20	
B. F.	CML	39.0	1.95	2.28	Therapy with phenylalanine mustard without effect
M. W.	CML-remission	4.2	5.04	4.10	Phenylalanine mustard-induced remission
R. S.	CML-remission	9.5	6.37	4.28	6-Mercaptopurine (6-MP)-induced remission
E. K.	CML (blastic crisis)	80.0	0.43	2.46	Blasts, 75%, 10% progranulocytes; receiving 6-MP, prednisone without effect
L. S.	ALL	119.0	0.12	2.24	Untreated; 74% blasts, 15% prolymphocytes
W. P.	ALL	56.0	0.86	3.60	Receiving cyclophosphamide without effect; 99% blasts
J. S.	ALL	84.6	1.63	1.68	Prednisone, 6-MP, vincristine. Count rapidly falling from 800,000; 95% blasts
A. F.	AML	53.5	0.19	3.24	Receiving 6-MP, prednisone without effect; 97% blasts
V. T.	AML	162.0	1.39	3.88	Untreated; 75% blasts, 23% promyelocytes
E. S.	AML	8.0	1.35	2.96	Untreated; 89% blasts
J. B.	Leukemoid reaction; Hodgkin's disease	37.0	3.80	2.28	
W. J.	Psoriasis		Bone marrows 2.07	1.94	Normal peripheral blood and bone marrow
J. B.	CML-relapse		2.26	2.96	
C. T.	Chondrosarcoma	3.7	Thoracic duct lymph 0.68	6.15	Lymphocytes, 100%; 3 erythrocytes per 100 leukocytes; no tumor cells

\* CLL = chronic lymphocytic leukemia; CML = chronic myelocytic leukemia; ALL = acute lymphocytic leukemia; AML = acute myelocytic leukemia.

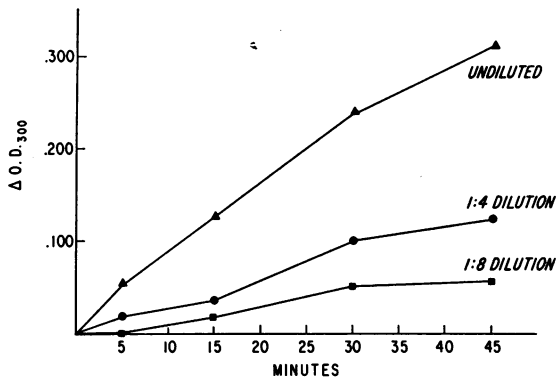


FIG. 2. THYMIDINE PHOSPHORYLASE ACTIVITY OF DIFFERENT CONCENTRATIONS OF A CHRONIC MYELOCYTIC LEUKEMIA LEUKOCYTE HOMOGENATE.

sumably due to product inhibition of the reaction by thymine (3).

Results of the assays of the leukocyte and bone marrow homogenates from normal individuals and various patients are shown in Table VII.

When expressed as activity per  $10^8$  leukocytes, the means of all the leukemic homogenates were significantly lower than that of the normal ( $p < 0.001$ ) (Table VIII, Figure 3). The CML homogenates were higher than those from CLL and the acute leukemias (AL) ( $p < 0.001$ ). CLL and AL homogenates did not differ significantly when compared in this fashion ( $p < 0.70$ ).

When the data were expressed as enzyme activity per milligram of homogenate protein, i.e., specific activity, the normal homogenates were still significantly higher than CML ( $p < 0.01$ ) and AL ( $p < 0.001$ ). The difference between normal and CLL is only suggestive ( $p < 0.10$ ),

TABLE VIII

Thymidine phosphorylase: mean activities of various types of leukocyte homogenates

Group	No. of patients	Activity*	Protein*	SA*
		U/ $10^8$ leukocytes	mg/ $10^8$ leukocytes	
Normal	6	4.32 ± 0.25	3.80 ± 0.35	1.17 ± 0.10
CML (relapse)†	8	2.52 ± 0.15	3.16 ± 0.35	0.83 ± 0.06
CLL	6	1.02 ± 0.18	1.05 ± 0.15	0.97 ± 0.05
AL	7	0.85 ± 0.23	2.87 ± 0.29	0.33 ± 0.12

\* Mean ± SE.

† CML = chronic myelocytic leukemia; CLL = chronic lymphocytic leukemia; AL = acute leukemia.

but there was no significant difference between CML and CLL ( $p < 0.20$ ). The difference between CLL and AL was significant ( $p < 0.001$ ), as was the difference between CML and AL ( $p < 0.01$ ). The explanation for these results when the data are compared in different ways is that the CLL cells are smaller than the other leukocytes. As a result, CLL homogenates contain less protein than other types of homogenates prepared from the same number of cells and have a higher specific activity.

The enzyme activity of the one patient (J. S.) with acute leukemia who was approaching a remission was the highest in the AL group, with

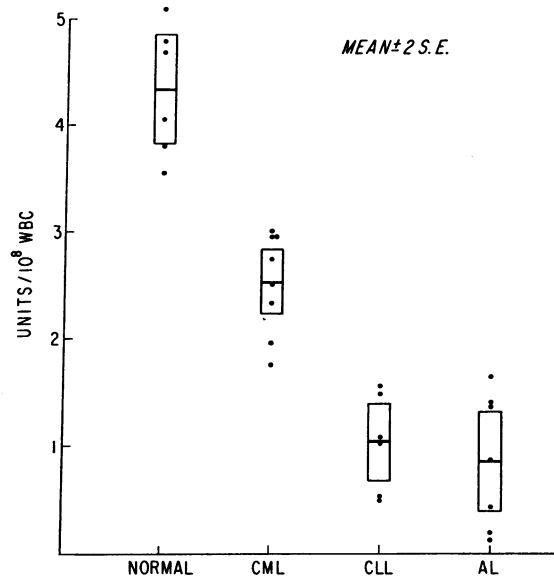


FIG. 3. THYMIDINE PHOSPHORYLASE ACTIVITY OF DIFFERENT TYPES OF LEUKOCYTE HOMOGENATES. CML = chronic myelocytic leukemia; CLL = chronic lymphocytic leukemia; AL = acute leukemia.

a nearly normal specific activity, even though he had a leukocyte count of  $84,000$  per  $\text{mm}^3$  with 95% blasts. He had been on antileukemic therapy for 3 days with a rapidly falling leukocyte count. The activity of the cells from a patient with CML in blastic crisis (E. K.) was within the acute leukemia range and is included in the calculations for the latter group.

The leukemic patients who were being treated but were still in relapse (B. F., L. H., W. P., and A. F.) did not have significantly different values for enzyme activity from untreated patients with



the same type of leukemia. Two CML patients in remission and a patient with a leukemoid reaction had enzyme activities in or slightly above the normal range. The normal and CML bone marrow values were lower than the normal peripheral leukocyte values. The thoracic duct lymphocytes had very low levels of enzyme activity. Erythrocytes had no detectable activity and did not inhibit active leukocyte preparations.

*Correlation of thymidine phosphorylase activity and inhibition of  $H^3TdR$  uptake.* Homogenates prepared identically in Tris buffer ( $1.25 \times 10^8$  cells per ml) from normal, CML, CLL, and acute myelocytic leukemia (AML) leukocytes were assayed simultaneously for thymidine phosphorylase activity and for their ability to inhibit  $H^3TdR$  uptake by CML cells. Two incubation conditions were studied. There was good correlation between enzyme activity and inhibition of isotope uptake when the cells and homogenate were incu-

bated together (Figure 4). The normal homogenate was most active, followed by the CML, CLL, and AML homogenates in order. This relationship was also found in the preincubation experiments, except that the activity of the normal and CML homogenates appeared to be equal, since both caused virtual complete inhibition of  $H^3TdR$  uptake. The AML homogenate, which had negligible enzyme activity, increased the uptake of the isotope by the CML cells.

*Effect of hydroxyurea and phytohemagglutinin.* Hydroxyurea,<sup>8</sup> which has clinical activity in CML (21, 22), caused 30% inhibition of  $H^3TdR$  uptake by CML cells at a concentration of 1.5 mg per L and 80% at 15 and 150 mg per L. The last two concentrations correspond to the plasma levels after doses of the drug ranging from 15 to 100 mg per kg in man (23, 24). At a concentration of 76 and 7.6 mg per L, hydroxyurea had no effect on thymidine phosphorylase activity and was additive with it in inhibiting  $H^3TdR$  uptake by CML cells.

Phytohemagglutinin has growth-stimulating effects on leukocyte cultures (25) and has been reported to inhibit leukocyte alkaline phosphatase when thymidylic acid (TMP) is used as a substrate (26). At a concentration corresponding to 0.4 ml of phytohemagglutinin<sup>9</sup> per 10 ml of culture medium there was no effect on thymidine phosphorylase activity, and one-half this concentration had no effect on  $H^3TdR$  uptake by CML cells.

### Discussion

These experiments have demonstrated inhibition of  $H^3TdR$  uptake into CML cells by a non-dialyzable, heat-labile leukocyte factor that was more effective as phosphate concentration was increased and when it was preincubated with  $H^3TdR$  before addition of the cells. When the cells alone were incubated with  $H^3TdR$ , the residual radioactivity of the medium suggested the formation of thymine, DHT, and possibly BUIB. The inhibitory factor increased the amount of these compounds found in the medium while decreasing the amount of residual thymidine. Similar compounds have recently been found in stud-

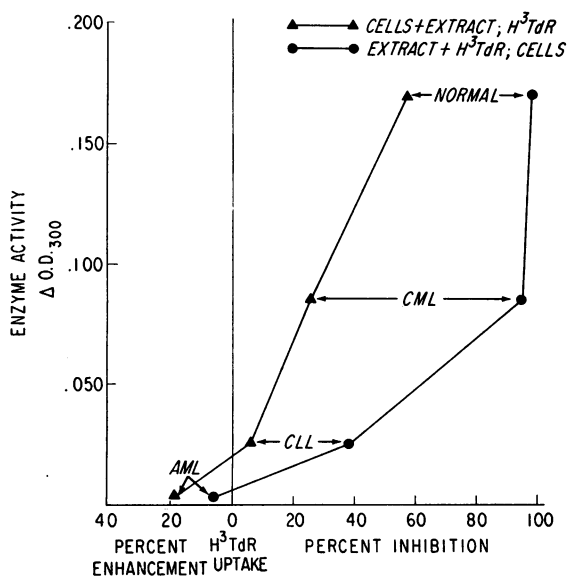


FIG. 4. CORRELATION OF THYMIDINE PHOSPHORYLASE ACTIVITY OF LEUKOCYTE HOMOGENATES WITH INHIBITION OF TRITIATED THYMIDINE UPTAKE BY CHRONIC MYELOCYTIC LEUKEMIA CELLS.  $H^3TdR$  = tritiated thymidine; CML = chronic myelocytic leukemia; CLL = chronic lymphocytic leukemia; AML = acute myelocytic leukemia.

▲—▲: Cells and homogenate incubated for 1 hour;  $H^3TdR$  added and incubated an additional hour. ●—●: Homogenate incubated with  $H^3TdR$  for 1 hour; cells and plasma added and incubated an additional hour. Homogenates prepared in Tris buffer,  $1.25 \times 10^8$  cells per ml.

<sup>8</sup> E. R. Squibb and Sons, New York, N. Y.

<sup>9</sup> Difco Laboratories, Detroit, Mich.

ies of H<sup>3</sup>TdR uptake by plant tissues (27). Normal and CML leukocytes were capable of elaborating this factor when incubated in phosphate buffer.

Partial purification of the inhibitory factor showed that it was precipitable at 40% saturation with ammonium sulfate, as is human spleen (4) and horse liver (3) thymidine phosphorylase. It also inhibited CML cell uptake of tritiated deoxyuridine, which is a substrate for thymidine phosphorylase in other species (3). Good correlation was found between thymidine phosphorylase activity and the ability to inhibit H<sup>3</sup>TdR uptake into CML cells. It may be concluded that this inhibitory effect is due to thymidine phosphorylase.

Craddock (28) reported that normal intact human granulocytes or homogenates had a greater inhibitory effect than leukemic cells on labeling of dog thoracic duct lymphocytes with H<sup>3</sup>TdR. Dog lymphocytes and erythrocytes had little effect. Because of the studies reported above, his results may be attributed, at least in part, to different amounts of thymidine phosphorylase in the cells tested.

An enzyme catalyzing the conversion of thymidine to thymine was first described by Deutsch and Laser (1) in 1929, who found activity in beef bone marrow and rabbit leukocytes. Klein (2) in 1935 confirmed these findings and found similar activity in beef kidney, spleen, lung, lymph nodes, and other tissues. The enzyme was activated by arsenate and phosphate. Friedkin and Roberts (3) purified thymidine phosphorylase from horse liver and described its kinetics and substrate specificity. They also found activity in human bone marrow. Zimmerman (4) has recently surveyed enzyme activity in many human tissues, including spleen and various tumors. There have been no reports of thymidine phosphorylase activity in human peripheral leukocytes. A purine nucleoside phosphorylase is present in guinea pig leukocytes (29).

Many investigators have demonstrated differences in enzyme levels, particularly those concerned with DNA metabolism, between rapidly growing and stable tissues. Thymidine kinase and thymidylate kinase activities increase in regenerating rat liver with a simultaneous decrease in the

activities of enzymes concerned with pyrimidine catabolism (30, 31). Potter, Pitot, and Ono (32) have shown generally decreased pyrimidine catabolic activity in rat hepatomas. Wheeler, Alexander, and co-workers (33-35) have obtained similar results in studies of purine metabolism in animal tumors. Smith, Baker, and Sullivan (36) have shown increased levels of enzymes regulating de novo pyrimidine synthesis in CML leukocytes, but to our knowledge, enzymes involved in pyrimidine catabolism in leukocytes have not been studied. A simplified version of the current concept of thymidine catabolism is shown in Figure 5.

The physiologic role of thymidine phosphorylase has not been defined. The equilibrium of the thymidine-thymine reaction is favorable to nucleoside synthesis in cell-free systems (37). Major pathways of pyrimidine deoxyribonucleotide synthesis, however, are thought to bypass the nucleoside level. Friedkin and Kalckar (37) have speculated that, *in vivo*, the nucleoside phosphorylases "might serve as catalysts in regulatory pathways dealing mainly with the breakdown of nucleic acids." It is possible that thymidine phosphorylase may function as an enzyme in DNA synthesis. Sköld (19) has reported increased levels of deoxyuridine phosphorylase, probably the same enzyme as thymidine phosphorylase, in regenerating rat liver. De Verdier and Potter (38), however, have found decreased levels of a similar enzyme in regenerating rat liver and very

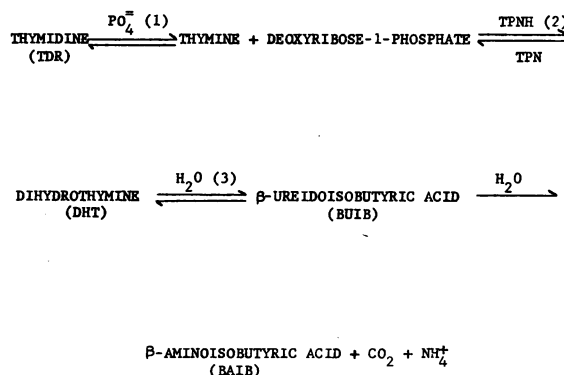


FIG. 5. THYMIDINE CATABOLISM [Modified from Fink and associates (13)]. (1) Thymidine phosphorylase; (2) hydroypyrimidine dehydrogenase; (3) hydroypyrimidine hydrase.

low levels in Dunning hepatoma. Furthermore, thymine is poorly utilized by most tissues (39) and by CML cells (5, 11), contrary to what would be expected if thymidine phosphorylase were an important enzyme in DNA synthesis.

Although thymidine is probably not a major substrate for DNA formation, it is readily utilized by those cells that have adequate thymidine kinase. Physiologically, thymidine may represent a step in the degradative pathway of thymidylate as part of the cellular control of DNA synthesis.

Whether low levels of thymidine phosphorylase are characteristic of leukemic cells per se or are merely reflections of leukocyte immaturity remains an open question. The low value of the one normal marrow assayed suggests that immature leukocytes have low activity that increases as the cells mature. Consistent with this is the observation of relatively high activity in a patient (J. S.) with AL approaching remission and the high activities in two patients (M. W. and R. S.) with CML in remission.

Since the value obtained for the enzyme activity of thoracic duct lymphocytes was low, the enzyme in normal peripheral leukocytes would seem to be located primarily in the granulocytes.

The data in this report suggest the further conversion of thymine to additional products, probably DHT and, perhaps, BUIB by intact CML cells. The failure to find evidence for formation of these compounds in the cell-free system may be due to the absence or inactivation of the necessary enzymes in the homogenates used or to the lack of TPNH. This pyridine nucleotide has been shown to be a required cofactor in the conversion of thymine to DHT in other tissues (40).

Certain considerations have been pointed out in the interpretation of experiments using H<sup>3</sup>TdR as a DNA label. These include radiation effects (41), reutilization (42), and low thymidine kinase activity (43). To these should be added the fact that different cell populations contain different levels of thymidine phosphorylase activity, which may profoundly affect their uptake of H<sup>3</sup>TdR. In addition, one cell population may influence the uptake of H<sup>3</sup>TdR by another through elaboration of the enzyme into the immediate environment.

### Summary

1) Leukocyte homogenates inhibit the uptake of tritiated thymidine by chronic myelocytic leukemia cells. The degree of inhibition correlates with the level of thymidine phosphorylase activity.

2) Thymidine phosphorylase activity is also found in the medium after intact leukocytes are incubated in phosphate buffer.

3) Normal leukocytes contain significantly higher levels of thymidine phosphorylase activity than leukemic leukocytes. Leukocytes from patients with leukemia in remission had higher enzyme levels than those from patients in relapse.

4) In addition to demonstrating thymidine phosphorylase activity converting thymidine to thymine, chronic myelocytic leukemia cells *in vitro* can reduce thymine to a compound corresponding chromatographically to dihydrothymine, or  $\beta$ -ureidoisobutyric acid, or both.

5) Low thymidine phosphorylase activity may be a function of cellular immaturity and capacity for DNA synthesis, rather than a characteristic of leukemia per se.

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