

# The Enzymatic Mechanisms for Deoxythymidine Synthesis in Human Leukocytes

## IV. COMPARISONS BETWEEN NORMAL AND LEUKEMIC LEUKOCYTES

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**ABSTRACT** (1) Synthesis of deoxythymidine by either direct transfer of deoxyribosyl to thymine (pyrimidine deoxyribosyltransferase) or by a coupled deoxynucleoside phosphorylase mechanism is approximately twofold greater with normal leukocyte extracts (55 to 88% granulocytes) than with extracts prepared from leukocytes obtained from patients with chronic myelogenous leukemia. Activities in lymphocytes (normal or leukemic) are one-fifth the activity of normal granulocytes.

(2) The lower activity in chronic myelogenous leukemia remains at 50% of normal even when patients are in hematologic remission with a normal per cent mature granulocytes in the peripheral blood.

(3) The leukemic enzyme could not be distinguished from the normal by pH optima, thermal stability, or kinetic properties. The  $K_m$ 's for the deoxyribosyl acceptor and deoxyribosyl donors were identical for both enzymes. Both are subject to substrate inhibition by thymine and to inhibition by purine bases with similar  $K_i$ 's. In addition, the transferase component of both the leukemic and the normal cell enzyme is activated by phosphate and arsenate. It appears, therefore, that there is no qualitative difference between the enzyme obtained from leukocytes of patients with chronic myelogenous leukemia and the enzyme obtained from normal leukocytes, suggesting that the difference in total cell activity is due to an actual decrease in amount of enzyme in chronic myelogenous leukemia or to a mixed cell population, one with a normal quantity of enzyme and the other with little or no active enzyme.

(4) In both the normal cell and the leukemic cell extracts, transferase and phosphorylase activities could

not be separated. The ratio of the two activities remained constant over a 140- and a 230-fold purification in normal and leukemic cell extracts, respectively. These and other observations indicate that transferase and phosphorylase activities are associated with the same protein.

(5) The metabolism of pyrimidine and purine deoxynucleosides is similar for normal and leukemic cells. Catabolism of all deoxynucleosides tested was by direct phosphorolysis, except for deoxyadenosine which required initial deamination to deoxyinosine before phosphorolysis. In contrast to the greater rates of pyrimidine deoxynucleoside synthesis and cleavage with normal leukocyte extracts, the rates of purine deoxynucleoside synthesis and cleavage were approximately twofold greater with extracts prepared from cells of patients with chronic myelogenous leukemia. There was no significant difference in the rate of phosphorolytic cleavage of pyrimidine nucleosides (uridine) between the CML and normal leukocyte extracts.

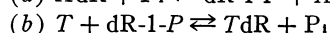
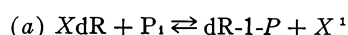
## INTRODUCTION

It has been known for some time that pyrimidine products of DNA degradation can be reutilized for the synthesis of new nucleic acid molecules. These studies have generally been concerned with reutilization of deoxynucleosides and deoxynucleotides as, for example, the several observations on the reutilization of deoxythymidine (1-3). Much less information is available on the reutilization of free pyrimidine bases (thymine, uracil, and cytosine), particularly in mammalian cells. One mechanism by which this could occur would be through the synthesis of pyrimidine deoxynucleosides from a pyrimidine base and a deoxyribosyl donor.

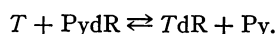
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The enzyme *trans-N*-deoxyribosylase, which catalyzes deoxynucleoside synthesis, has been well characterized in bacterial systems (4-7). The absence of this enzyme in human tissues led Beck to hypothesize that human tissues may lack a mechanism by which the deoxyribosyl moiety can be transferred from one base (purine or pyrimidine) to another (8). However, we have recently demonstrated that crude extracts of human leukocytes actively catalyze transfer of deoxyribosyl to thymine and to uracil, a reaction catalyzed by deoxythymidine phosphorylase (9). There is evidence in human spleen (10) and in normal human leukocytes (11, 12) indicating that the one enzyme protein, deoxythymidine phosphorylase, catalyzes synthesis of deoxythymidine or deoxyuridine by two distinct enzymatic mechanisms:

(1) coupled deoxynucleoside phosphorylase



(2) pyrimidine deoxyribosyltransferase



Reaction (1) was initially characterized by Friedkin and Roberts (13).

The capacity of leukocyte extracts from patients with CML to support deoxythymidine synthesis by either the coupled or transferase mechanism is reduced to approximately one-half of normal. The decreased activity in CML leukocytes was found even though leukocyte differential counts were comparable to those in the normal controls and in donors who were in clinical and hematologic remission (14). These observations suggested the possibility of a defect in the enzyme in CML and prompted an investigation of the comparative kinetic properties of the CML and normal leukocyte enzymes. The purpose of the present communication is to report the data obtained from these comparisons and the results of the relative activities of enzymes involved in the metabolism of pyrimidine and purine deoxynucleosides in normal and leukemic leukocytes.

## METHODS

### Source of leukocytes

Leukocytes were obtained from the peripheral blood of normal volunteers and from patients with CML. The cells from the normal donors consisted primarily of mature granulocytes (55 to 88% granulocytes). The CML patients had prominent leukocytosis (peripheral leukocyte counts over

<sup>1</sup> Abbreviations used either in the text or in the figures: XdR, any purine or pyrimidine deoxynucleoside; PydR, pyrimidine deoxynucleoside; X, any purine or pyrimidine base; Py a pyrimidine base; TdR, deoxythymidine; UdR, deoxyuridine; AdR, deoxyadenosine; IdR, deoxyinosine; T, thymine; U, uracil; Hx, hypoxanthine; dR-1-P or dR-1-PO<sub>4</sub>, deoxyribose-1-P; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemia.

50,000/mm<sup>3</sup>) with a population of cells of variable maturity. All had moderate splenomegaly. None of these patients was in a blast phase and none was receiving antileukemic therapy. Bone marrow preparations of the CML patients were all positive for the Philadelphia chromosome. Studies of various enzymatic activities of CML patients in complete hematologic remission were also performed and those results have been reported in a preliminary communication (14).

### Isolation of leukocytes

Leukocytes were obtained and extracts prepared by methods previously described (9). All experiments were performed with cellular extracts. Lymphocytes were isolated by the nylon wool column technique (15).

### Enzyme assays

(1) *Deoxythymidine phosphorylase*. This enzyme catalyzes the reversible synthesis and phosphorolytic cleavage of deoxythymidine and measurements were made in both directions. Deoxythymidine synthesis was measured by following formation of deoxythymidine-<sup>14</sup>C from the reaction of thymine-<sup>14</sup>C with deoxyribose-1-P. Details of the procedure have been previously described (9). Cleavage of deoxythymidine was measured by following thymine production from deoxythymidine spectrophotometrically (16) or by following conversion of deoxythymidine-<sup>14</sup>C to thymine-<sup>14</sup>C. Assay conditions were described previously (9).

(2) *Pyrimidine deoxyribosyltransferase* activity was measured by following deoxythymidine-<sup>14</sup>C formation from the reaction of thymine-<sup>14</sup>C and deoxyuridine in the presence of high (0.1 M) concentrations of arsenate. Arsenate is used to prevent formation of deoxyribose-1-P so that determination of newly synthesized deoxythymidine is a measure of deoxythymidine formed only by direct transfer of deoxyribosyl to thymine and not through a deoxyribose-1-P intermediate which may result from phosphorolysis of deoxyuridine. Details of the assay have been reported (9).

(3) *Uridine phosphorylase* was assayed by following uracil production from uridine spectrophotometrically at 290 mμ. The reaction mixture in a final volume of 0.5 ml included: 10 mM uridine; 0.1 M phosphate buffer, pH 7.2; 0.05 M Tris-HCl buffer, pH 7.2; and from 0.5 to 0.7 mg protein obtained from crude leukocyte extracts. Incubations were at 37°C for 20 min. Labeled substrates and products were separated by descending paper chromatography (9).

(4) *Deoxyadenosine deaminase* was assayed by determining the rate of deoxyinosine-8-<sup>14</sup>C formation from deoxyadenosine-8-<sup>14</sup>C. Reaction mixtures contained 10 mM deoxyadenosine-2-<sup>14</sup>C (46.5 mc/mmole). The buffers and conditions were otherwise as described for uridine phosphorylase.

(5) *Purine nucleoside phosphorylase* was assayed by measuring hypoxanthine-8-<sup>14</sup>C production from deoxyinosine-8-<sup>14</sup>C (4.2 mc/mmole), but otherwise experimental conditions were identical to the assay for uridine phosphorylase. Hypoxanthine was not catabolized significantly during the period of assay, indicating that leukocytes contain minimal xanthine oxidase activity. Its determination could, therefore, be used for measurement of deoxyinosine cleavage. Separation of purine bases from purine deoxynucleosides was by descending paper chromatography (17).

### Purification of deoxythymidine phosphorylase

The details of the purification of the enzyme from normal leukocytes has been previously described (17). The pro-

cedure used for purification of the CML leukocyte enzyme is described below.

All procedures were carried out at 4°C. Approximately  $5 \times 10^{10}$  CML leukocytes were homogenized 10 min in a Virtis homogenizer and centrifuged 30 min at 40,000 *g*. The supernatant (fraction I) was treated with 1 M acetic acid to bring the pH to 5.2 and centrifuged 30 min at 40,000 *g*. The supernatant (fraction II) was treated with solid ammonium sulfate to 55% saturation and allowed to stand 45 min, and then centrifuged at 40,000 *g* for 30 min. The precipitate (fraction III) was taken up in 5 ml of 0.01 M sodium phosphate buffer, pH 7.3, containing 4 mM dithiothreitol (buffer A) and applied to a  $2.5 \times 15$ -cm G-25 Sephadex column and eluted with buffer A. The samples from the protein peak were combined and applied to a  $1 \times 10$ -cm DEAE cellulose column and eluted with buffer A, containing 0.75 or 1.0 M NaCl. Elution of the enzyme was accomplished in a small volume (1–2 ml) that was then dialyzed overnight against buffer A. The dialyzed material was applied to a  $1 \times 60$ -cm DEAE cellulose column and a convex salt gradient was initiated by permitting buffer A containing 1 M NaCl to pass into a 250 ml mixing chamber containing only buffer A. The samples containing the enzyme were combined and dialyzed for 10–15 hr against 0.1 M Tris-HCl buffer, pH 7.2. This fraction (fraction IV) was a 230-fold purification over fraction I. 1 ml contained approximately 0.00025 mg of protein and from 1 to 2 units of enzyme. (A unit of enzyme was defined as that amount

of protein that cleaved 1  $\mu$ mole of deoxythymidine in 1 hr.) Except when specifically indicated, fraction I was the source of enzyme. The principal objective in purification was to determine if the CML leukocyte enzyme like the normal leukocyte enzyme (11) contained both phosphorylase and transferase activities.

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (18) with bovine serum albumin as the standard. Phosphate was determined by the method of Chen, Toribara, and Warner (19).

## RESULTS

A comparison of purine nucleoside phosphorylase, deoxythymidine phosphorylase (measured in the direction of deoxythymidine cleavage), and pyrimidine deoxyribosyltransferase activities of various leukocyte extracts is illustrated in Fig. 1. Normal leukocytes contain approximately two-fold greater activity of both deoxythymidine phosphorylase and pyrimidine deoxyribosyltransferase than CML leukocytes, and approximately fivefold greater activity than normal lymphocytes or lymphocytes from patients with CLL. In contrast, CML leukocyte extracts contain approximately 1.5- to 2-fold more purine nucleoside phosphorylase than normal leukocytes (see also Fig. 9). As with the pyrimidine enzyme activities, pu-

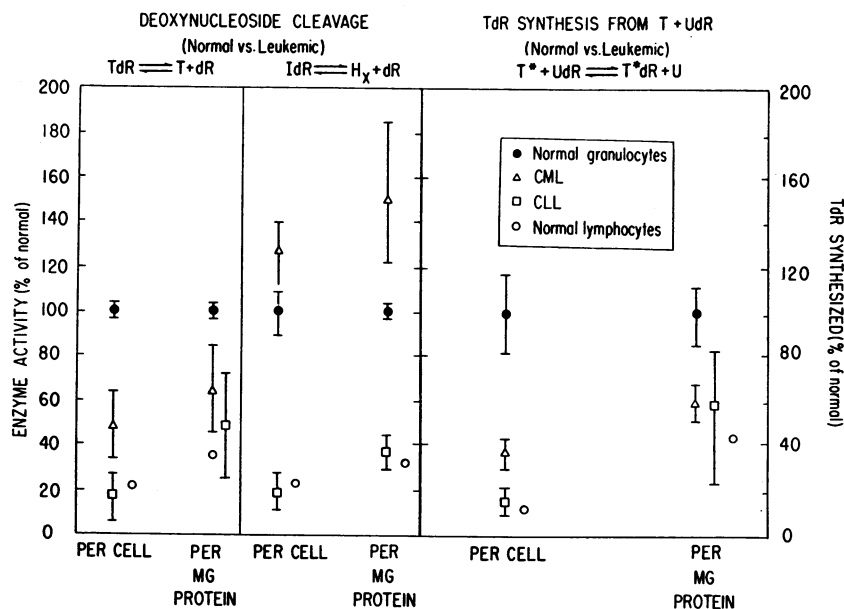


FIGURE 1 Comparison of levels of deoxythymidine phosphorylase, purine nucleoside phosphorylase, and pyrimidine deoxyribosyltransferase activities in normal and leukemic leukocyte extracts. Results are expressed as per cent of the normal granulocyte activity both on a per cell ( $10^8$  leukocytes) or per milligram of protein bases. Deoxythymidine phosphorylase activity is determined by following cleavage of deoxythymidine (left panel); purine nucleoside phosphorylase by measurement of deoxyinosine cleavage (center panel); and the transferase levels by measuring deoxythymidine synthesis from thymine and deoxyuridine. Details of assay are described in Methods. Points and accompanying lines are the mean values and standard deviations.

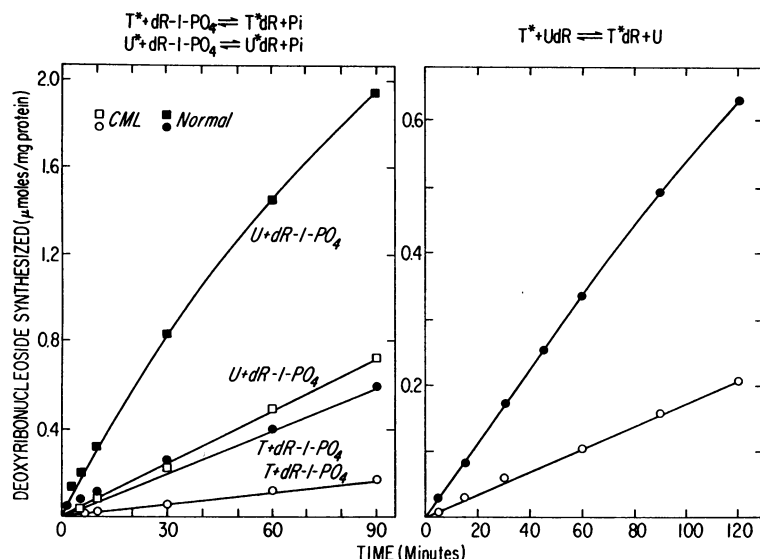


FIGURE 2 *Left panel*: Time course of deoxythymidine and deoxythymidine synthesis with deoxyribose-1-P as the deoxyribosyl donor catalyzed by deoxythymidine phosphorylase. The rates with both the normal and CML leukocyte enzyme are illustrated. The reaction mixture in a final volume of 0.5 ml contained 5  $\mu$ moles of thymine- $^{14}$ C or uracil- $^{14}$ C; 5  $\mu$ moles of deoxyribose-1-P; 25  $\mu$ moles of Tris-HCl buffer, pH 7.2; and 25  $\mu$ moles of succinate buffer, pH 7.2.

*Right panel*: Time course of deoxythymidine synthesis with deoxyuridine as the deoxyribosyl donor. The normal and CML leukocyte enzymes are compared. The reaction mixture contained 5  $\mu$ moles of thymine- $^{14}$ C; 5  $\mu$ moles of deoxyuridine; 25  $\mu$ moles of Tris-HCl buffer, pH 7.2; 25  $\mu$ moles of succinate buffer, pH 7.2; and 50  $\mu$ moles of sodium arsenate. Fraction I was the enzyme source in both experiments.

rine enzyme activity was least in lymphocytes, and there was no significant difference in activities between normal and leukemic lymphocytes. The results presented in Fig. 1 are the mean values and standard deviations of the activities in leukocytes from 40 normal volunteers (predominantly granulocytes), 25 patients with CML, 7 patients with CLL, and 5 normal volunteers (lymphocytes only). The lower activity of deoxythymidine phosphorylase in CML leukocyte extracts does not appear to be the consequence of an inhibitor present in these cells. Experiments in which equal amounts of leukocyte extracts from CML patients and leukocyte extracts from normal volunteers were mixed gave values that approximated the calculated mean activity of each assayed alone.

#### Pyrimidine deoxyribosyltransferase in CML patients in remission

Since persistently low levels of deoxythymidine phosphorylase and pyrimidine deoxyribosyltransferase activities were found in leukocytes obtained from patients with CML (approximately 50% of the activity of normal granulocytes), an investigation was carried out to determine if the low values were simply the result of cellular immaturity or were, in fact, associated with the leukemic process. Pyrimidine deoxyribosyltransferase was assayed in nine patients with CML in remission who were not receiving chemotherapy. These patients had approximately the same number of mature granulocytes in their peripheral blood as the normal controls. Paired assays revealed that the enzymatic activity remained at approximately 50% of normal despite apparent clinical and hematologic remission. A report of these findings has been published (14).

#### Properties of the enzyme in CML leukocytes

**pH optima.** With both the normal and CML-enzyme the pH optimum for deoxythymidine synthesis from thymine and dR-1-P (phosphorylase) is from 5.9 to 6.0. The optimum is 7.2 for deoxythymidine synthesis from thymine and deoxyuridine (transferase).

**Heat denaturation.** The rate of enzyme denaturation was determined by heating the enzyme preparation at 60°C for various periods, cooling, and then assaying for deoxythymidine synthesis. The  $t_{1/2}$  for inactivation of both transferase and phosphorylase with either CML or normal enzyme is approximately 10 min.

**Time curves.** Equilibration of deoxythymidine or deoxyuridine synthesis with deoxyribose-1-P as the deoxyribosyl donor for normal and CML cell extracts was not observed over a 90 min period (Fig. 2, left panel). Similarly, over a 120 min assay period, equilibration was not obtained with deoxyuridine as the deoxyribosyl donor (Fig. 2, right panel). Maximum rates of deoxynucleoside synthesis are not obtained in these experiments (Fig. 2), both because of the inhibitory concentrations (10 mM) of pyrimidine bases used in these experiments and the suboptimal concentrations of deoxyuridine (right panel). Substrate inhibition by the pyrimidine bases is discussed below.

**Substrate specificity.** With deoxyribose-1-P as the deoxyribosyl donor, the rate of deoxynucleoside synthesis is approximately two- to three-fold greater with uracil as the deoxyribosyl acceptor than with thymine (Fig. 2). Uracil is also the favored deoxyribosyl acceptor with deoxyuridine as the deoxyribosyl donor (9). In neither case did cytosine serve as a substrate. These relationships were found with both the normal and the CML cell extracts. The  $K_m$  for deoxyribose-1-P

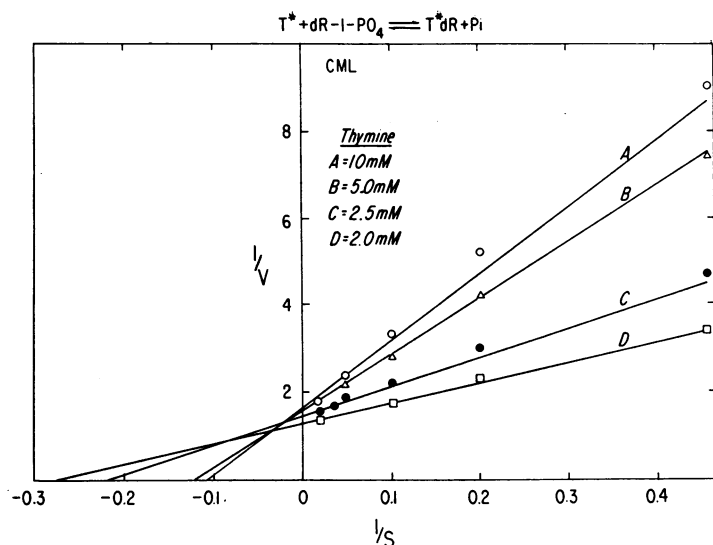


FIGURE 3 Lineweaver-Burk plot of the velocity of deoxythymidine synthesis catalyzed by CML leukocyte deoxythymidine phosphorylase as a function of the concentration of deoxyribose-1-P (S). The thymine concentrations are indicated in the figure. The enzyme source in these experiments was fraction IV.

is from 2 to 4 mM for the enzyme obtained from normal leukocytes (9, 17), and similar values were obtained with the CML leukocyte enzyme (see curve D of Fig. 3). The  $K_m$  for deoxyuridine is approximately 15 mM for the normal leukocyte enzyme (9). A similar  $K_m$  for deoxyuridine was obtained with the CML enzyme. This can be calculated from the data shown in Fig. 4 at non-inhibitory concentrations of thymine (2 mM).

#### Substrate inhibition of deoxythymidine synthesis by pyrimidine bases

The determination of a  $K_m$  for the pyrimidine base is complicated by substrate inhibition. Using normal leu-

kocyte extracts, Gallo, Perry, and Breitman demonstrated that both uracil and thymine inhibit deoxythymidine synthesis markedly with deoxyribose-1-P as the deoxyribosyl donor (9). The inhibition by thymine is greater than the inhibition by uracil. The same degree of inhibition was observed with the CML enzyme (Figs. 3 and 5) and, in addition, as with the normal cell enzyme, Lineweaver-Burk plots reveal that the inhibition involves a change in both the apparent  $K_m$  for deoxyribose-1-P as well as  $V_{max}$  (Fig. 3).

Deoxythymidine synthesis from thymine and deoxyuridine is also inhibited by thymine but not by uracil. The kinetics of inhibition with the normal leukocyte

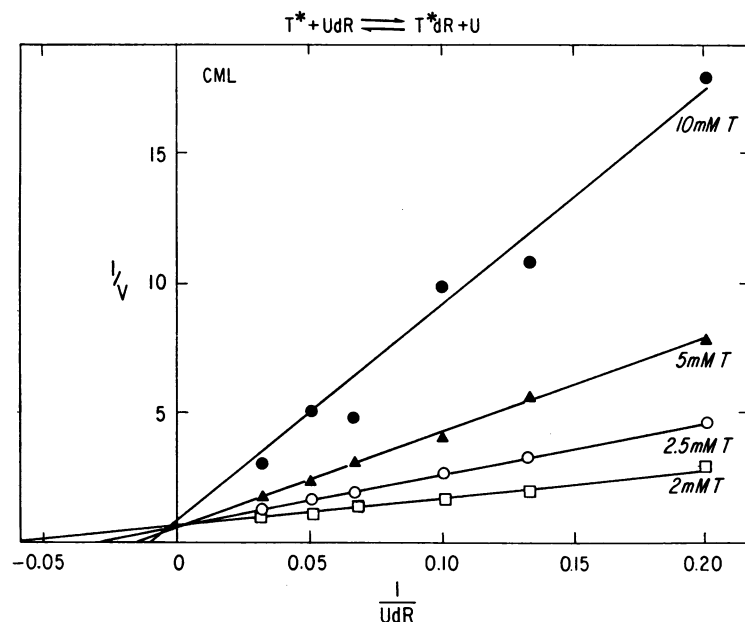


FIGURE 4 Lineweaver-Burk plot of the velocity of deoxythymidine synthesis catalyzed by CML leukocyte pyrimidine deoxyribosyltransferase as a function of the concentration of deoxyuridine. The thymine concentrations are indicated in the figure. Fraction I was the source of enzyme.

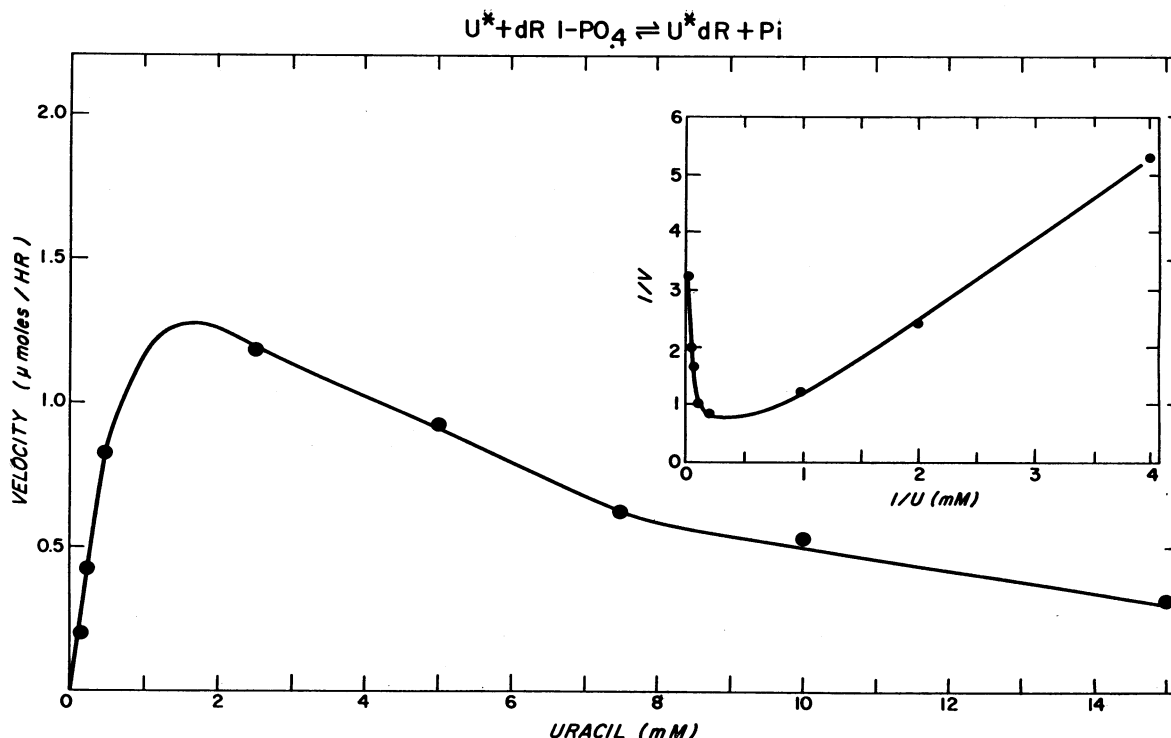


FIGURE 5 Substrate inhibition of CML leukocyte deoxythymidine (deoxyuridine) phosphorylase by uracil. The reaction mixture contained 5  $\mu$ moles of deoxyribose-1-P and the indicated concentrations of uracil. Fraction I was the source of enzyme.

enzyme revealed only a change in  $K_m$  for deoxyuridine with no change in  $V_{max}$ , indicating that excess thymine interferes with the binding of deoxyuridine (9). Similar kinetics were found when the CML enzyme was used (Fig. 4).

Fig. 6 illustrates that the magnitude of substrate inhibition by thymine of deoxythymidine synthesis from thymine and deoxyuridine (pyrimidine deoxyribosyltransferase) is identical with both the normal enzyme and the CML leukocyte enzyme. As anticipated from the competitive kinetics shown in Fig. 4, the curves illustrated in Fig. 6 intercept at a common point that represents the  $K_i$  for thymine (approximately 1.5 mM for both enzymes).

Thymine also inhibits the phosphorolytic cleavage of deoxythymidine, in which case it is an example of product inhibition. This has been demonstrated in bacterial systems (20) as well as in mammalian tissues (16, 17, 21). The possible regulatory significance of thymine in controlling the intracellular pool of deoxythymidine by two mechanisms, substrate inhibition and product inhibition, has been the subject of previous speculation by Gallo and associates (9, 17). It is of interest in this regard that unlike the human leukocyte enzyme, *E. coli* deoxythymidine phosphorylase is subject only to product

inhibition, since there is no substrate inhibition of deoxythymidine synthesis by thymine with the *E. coli* enzyme (R. Gallo and T. R. Breitman, unpublished observations.)

#### Activation of pyrimidine deoxyribosyltransferase by phosphate and arsenate

Previous studies with the normal leukocyte enzyme revealed anion activation of the transferase mechanism for deoxythymidine synthesis by phosphate and arsenate (9). The activation by phosphate was not due to formation of deoxyribose-1-P from deoxyuridine, since concentrations of phosphate that were optimal for activation of deoxythymidine synthesis from thymine and deoxyuridine markedly inhibited deoxythymidine synthesis from thymine and deoxyribose-1-P. Similar characteristics of the enzyme were also found with the CML enzyme (Fig. 7).

#### Purine base and purine deoxynucleoside inhibition of deoxythymidine synthesis

The normal leukocyte enzyme has been shown to be subject to inhibition by purines (17, 22). A comparison of the effect of various concentrations of different purines on the CML and normal enzymes is presented in Fig. 8.

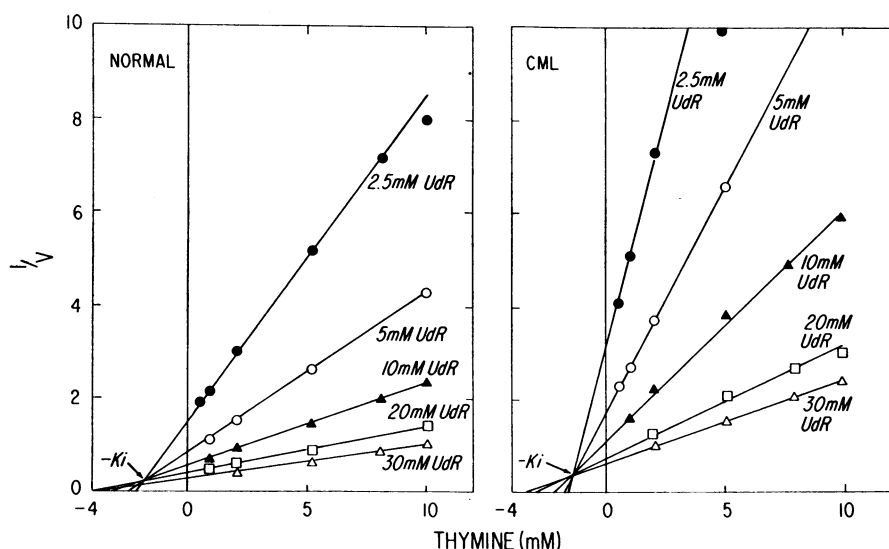


FIGURE 6 Deoxythymidine synthesis from thymine and deoxyuridine. Comparison of pyrimidine base (thymine) substrate inhibition of the normal leukocyte and CML leukocyte pyrimidine deoxyribosyltransferase. The reciprocal of the velocity of deoxythymidine synthesis is plotted as a function of the concentration of thymine with several concentrations of deoxyuridine. The  $K_i$ 's for thymine inhibition of both enzymes are indicated. The enzyme source in these experiments was fraction IV.

Inhibition is observed with the 6-oxypurines and 6-mercaptapurine and the magnitude of inhibition is the same for both enzymes. Kinetic studies of inhibition revealed a slight change in  $K_m$  for deoxyribose-1-*P* and a change in  $V_{max}$  (17).<sup>2</sup> This "mixed type" inhibition is similar to the inhibition by thymine, but unlike thymine, purines did not inhibit the enzyme in the direction of deoxythymidine cleavage. The kinetics were identical for the normal cell and CML cell enzyme.

#### Evidence that human leukocyte pyrimidine deoxyribosyltransferase and deoxythymidine phosphorylase activities are associated with the same protein

Zimmerman has reported that the transferase and phosphorylase activities were not separated after a 3000- and a 30-fold purification of the human spleen and *E. coli* enzymes, respectively (10). In addition, these purifications did not alter the ratios of the activities, which suggested to him that both activities were a function of the same protein. We have confirmed these find-

<sup>2</sup> Inhibition by purines is not due to depletion of substrate (deoxyribose-1-*P*) by reaction with purines to form a purine deoxynucleoside, since the concentration of deoxyribose-1-*P* in these experiments is in excess (10 mM). Furthermore, the purified enzyme is completely free of purine nucleoside phosphorylase, the enzyme which catalyzes a reaction of purines with deoxyribose-1-*P*, yet inhibition by purines is still observed (17).

ings with the enzyme activities from normal leukocytes. Over a 140-fold purification transferase and phosphorylase activities were constant. Furthermore, both enzyme activities had identical rates of heat denaturation (11). As part of the investigation of the properties of the CML enzyme, purification was carried out along with comparisons of transferase and phosphorylase activities. The yield of purified enzyme (fraction IV) by this method varied from 5 to 10% of fraction I and in the purification presented in Table I it was 6%. As shown in Table I, over a 250-fold purification the two activities could not be separated and the ratio of transferase to phosphorylase remained constant. It is concluded therefore that like the normal leukocyte and spleen protein, one CML protein probably has both transferase and phosphorylase activity.

#### Comparison of pyrimidine nucleoside phosphorylytic cleavage (uridine phosphorylase) in normal and CML leukocyte extracts

Uridine is the most commonly used precursor to measure RNA synthesis and pool sizes of uridylate. Variation in the rate of catabolism by phosphorolysis between normal cells and leukemic cells could lead to erroneous conclusions concerning differences observed in rates of RNA synthesis or pool sizes when this compound is used as a precursor. Furthermore, as shown in this communication, we have observed significantly lower

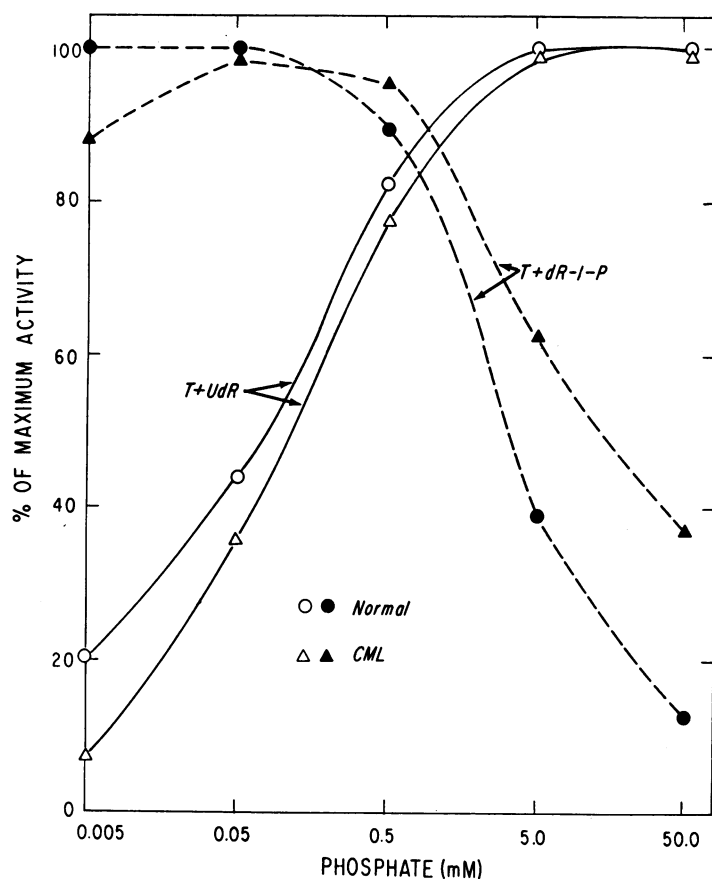


FIGURE 7 Phosphate activation of deoxythymidine synthesis from thymine plus deoxyuridine and inhibition of deoxythymidine synthesis from thymine plus deoxyribose-1-P, comparison of the normal and CML leukocyte enzymes. The leukocyte extracts were dialyzed until phosphate free, and then phosphate was added to the concentrations indicated in the figure. Note the absence of deoxythymidine synthesis from thymine and deoxyuridine (deoxyribosyltransferase) in the absence of phosphate. That the marked increase in deoxythymidine synthesis with addition of phosphate is due to an activation of the enzyme rather than a consequence of deoxyribose-1-P production from deoxyuridine is indicated by the marked phosphate inhibition of deoxythymidine synthesis from thymine and deoxyribose-1-P at concentrations that activate the transferase. Fraction I was the source of enzyme.

levels of enzymes involved in the phosphorolysis of pyrimidine deoxynucleosides (deoxythymidine phosphorylase) in CML leukocyte extracts. The possibility was considered that CML leukocytes may have in general lower levels of enzymes involved in the metabolism of pyrimidine pentosides. The data in Table II demonstrate that this is not the case. The rate of phosphorolysis of uridine is not significantly different in CML leukocyte extracts from patients in relapse or in remission from the rate observed with normal leukocyte extracts.

#### Comparison of pyrimidine deoxynucleoside and purine deoxynucleoside metabolism in normal and leukemic leukocytes

As previously discussed, pyrimidine deoxynucleoside (deoxythymidine or deoxyuridine) synthesis and cleavage are greater in normal cell extracts than in extracts from CML cells. The mechanism for cleavage appears to be entirely phosphorolytic in both cell types and the mechanisms for synthesis also appear identical in normal and leukemic leukocytes. As shown in the center panel of Fig. 1 and the right panel of Fig. 9, cleavage of purine deoxynucleosides (deoxyinosine) is 1.5- to 2-fold greater in CML leukocyte extracts than in normal

leukocyte extracts. Fig. 9 (right panel) also illustrates that cleavage of deoxyinosine, like that of pyrimidine deoxynucleosides, is by a phosphorolytic (or arsenolytic) mechanism since the rate in the presence of 0.1 M arsenate is approximately three-fold greater than in the absence of arsenate. The low rate of cleavage in the arsenate might be attributed to a hydrolytic mechanism. However, this is not the case. Rather, it is due to the endogenous phosphate (0.25–0.32 mM) of the leukocyte homogenate, since after prolonged dialysis of the homogenate all enzymatic activity was lost but could be restored by the addition of phosphate.

All deoxynucleosides studied (deoxyguanosine, deoxyinosine, deoxythymidine, and deoxyuridine), except for deoxyadenosine, were cleaved by direct phosphorolysis to produce their respective pyrimidine bases and deoxyribose-1-P. Adenine was not isolated from the reaction mixture with deoxyadenosine as substrate, but instead deoxyinosine and hypoxanthine quickly appeared. The results indicate that deoxyadenosine is initially deaminated to deoxyinosine before phosphorolysis. The data presented in the left panel of Fig. 9 illustrate that, as with purine nucleoside phosphorylase activity, deoxyadenosine deaminase activity is approximately threefold



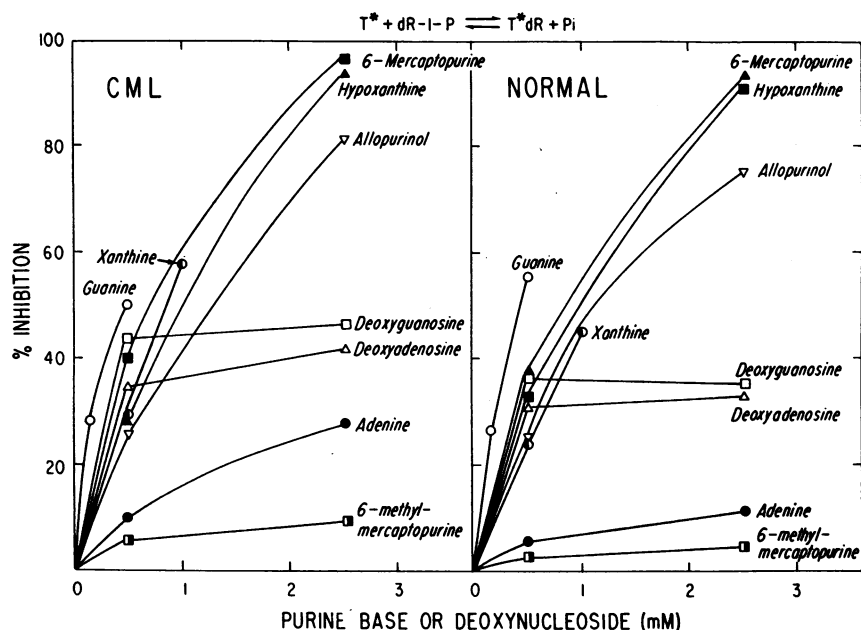


FIGURE 8 Purine inhibition of deoxythymidine synthesis from thymine and deoxyribose-1-P; comparison of the normal and CML leukocyte enzyme. The reaction mixture in a final volume of 0.5 ml contained 2.5  $\mu$ moles of thymine- $^{14}C$ , 5  $\mu$ moles of deoxyribose-1-P, buffers as described in the legend to Fig. 2, and the indicated concentrations of purines. Results were expressed as per cent inhibition compared with the control with no purine added. Fraction I was the source of enzyme.

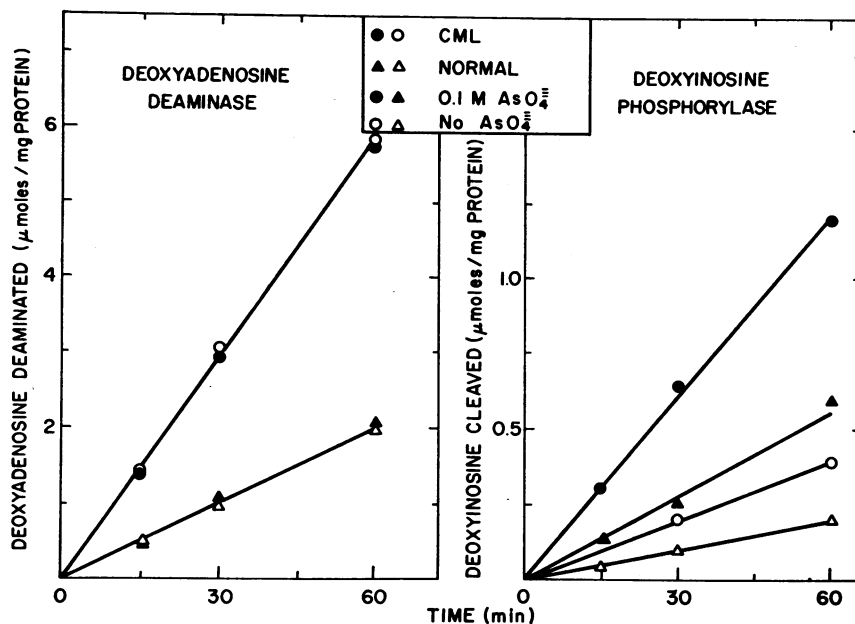


FIGURE 9 Comparison of the levels of deoxyadenosine deaminase and purine nucleoside phosphorylase activities in normal and CML leukocyte extracts. Details of assay are described in Methods.

TABLE I  
*Purification of CML Leukocyte Deoxythymidine Phosphorylase and Pyrimidine Deoxyribosyltransferase*

Fraction	Specific activity		Purification		Ratio of Phosphorylase to transferase
	Deoxythymidine phosphorylase	Pyrimidine deoxyribosyltransferase	Deoxythymidine phosphorylase	Pyrimidine deoxyribosyltransferase	
I	1.4	1.6			0.87
II	1.8	2.1	1.3	1.3	0.84
III	5.2	5.6	3.7	3.5	0.93
IV	322	398	230	249	0.81

greater in CML leukocyte extracts than in normal leukocyte extracts.

As reported by Gallo and Breitman, pyrimidine deoxynucleosides support deoxythymidine synthesis catalyzed by leukocyte enzyme preparations by either of two mechanisms: direct transfer of deoxyribosyl to thymine or by initial phosphorolytic cleavage of the pyrimidine deoxynucleoside to deoxyribose-1-*P*. The latter compound reacts with thymine to form deoxythymidine. Purine deoxynucleosides do not act as deoxyribosyl donors by the

direct transfer mechanism but function only through a deoxyribose-1-*P* intermediate (11). These findings with normal leukocytes were confirmed with CML leukocytes. No direct transfer of deoxyribosyl from deoxyinosine, deoxyadenosine, or deoxyguanosine to either hypoxanthine, xanthine, adenine, guanine, allopurinol, or 6-mercaptopurine could be demonstrated.

### DISCUSSION

Studies of cellular metabolism, enzyme mechanisms, and even biochemical approaches to the study of neoplasia can be conveniently studied with human leukocytes. One significant disadvantage, however, is the usual presence of a mixed population of cells. This problem can be circumvented with normal leukocytes by the separation of granulocytes from lymphocytes by passage of the cells through a nylon column (15). Enzyme activity of the total cells and of the isolated lymphocytes can be measured. The difference between the two is primarily due to the mature granulocyte. These experiments have been performed with deoxythymidine phosphorylase and with pyrimidine deoxyribosyltransferase, and more than 80% of both activities appears to be associated with the granulocyte. Evidence has been presented in this communication and by Gallo and associates elsewhere (11) that transferase and phosphorylase activities are associated with the same protein. Studies with leukemic leukocytes revealed that CLL cells (more than 95% lymphocytes) also contained low levels of both enzyme activities. However, these values were not lower than normal lymphocytes. Transferase and phosphorylase activities of leukocytes obtained from CML patients in relapse with peripheral leukocytosis and containing the typical spectrum of cells at various stages of maturation were approximately one-half of the activities found in the mature normal granulocyte (Fig. 1). Patients with CML in relapse usually have leukocytosis. Therefore adequate numbers of cells are readily obtained and for that reason comparisons are often made between enzyme activities of leukocytes from this type of CML patient with leukocytes from normal individuals. However, this results in

TABLE II  
*Uridine Phosphorylase Activity in Leukocyte Extracts Obtained From Patients with CML in Relapse, CML in Remission, and From Normal Controls*

Source of leukocyte extracts	Enzyme activity	Mature granulocytes in final leukocyte preparation
	$\mu\text{moles/mg protein/hr}$	% of total leukocytes
Normal		
1	0.20	81
2	0.24	84
3	0.28	80
4	0.18	71
5	0.24	77
6	0.21	65
7	0.16	82
8	0.29	76
Mean	0.23	
CML in relapse		
1	0.30	10
2	0.23	5
3	0.19	34
4	0.25	23
Mean	0.24	18
CML in remission		
1	0.32	66
2	0.26	60
3	0.20	83
4	0.28	86
5	0.22	71
Mean	0.26	60

a comparison of leukocytes at various stages of differentiation (CML) with mature cells (normal) so that the differences may only be a consequence of differences in the level of maturation rather than a result of the leukemic process. For this reason, a study was made of the level of pyrimidine deoxyribosyltransferase of leukocyte extracts of 14 CML patients in complete hematologic remission (14). The granulocytes in the peripheral blood of these patients were morphologically mature, and the mean per cent granulocytes was quite comparable to the normal controls. In 16 of 17 paired assays of the leukocyte enzyme activity of these 14 CML patients, the results were below the normal (mean, 50% of normal) and were not significantly different from the enzyme levels of the mixed population of cells from the CML patients in relapse. In nine instances the CML patients in remission were not receiving chemotherapy so that a drug effect could reasonably be ruled out.

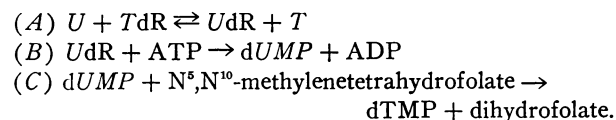
This demonstration of an enzyme deficiency in leukemia is unique with the exception of the reports of reduced leukocyte alkaline phosphatase in CML. This has been reported to remain low in some patients in remission despite the presence of mature granulocytes in the peripheral blood (23-25). Although it is tempting to relate the reduced enzyme activities to the Philadelphia chromosome, correlation between the two was not observed, since in the one CML patient in remission in which the enzyme activity did return to normal, the cells remained positive for the Philadelphia chromosome.

There are several possible explanations for the lower enzyme activity for the synthesis of deoxythymidine in CML leukocyte extracts as compared to normal leukocytes: (1) the presence of an inhibitor in the CML cells; (2) an alteration at the catalytic site of the enzyme; and (3) an actual decrease in the number of enzyme molecules.

The first consideration, the presence of an inhibitor in the CML cell extracts, has largely been excluded by the mixing experiments with normal leukocytes. As to the second possibility, no qualitative functional difference between the normal leukocyte enzyme and the CML enzyme was identified in the study reported in this communication. It appears, therefore, that the lower activity of the CML enzyme which persists even though patients are in complete hematologic remission, is the result of a decrease in the number of enzyme molecules rather than a consequence of an altered catalytic function. Alternatively, the reduction to approximately 50% of normal could be explained by two populations of cells, one without enzyme activity and the other with a normal enzyme level. At present there appears to be no way to test this hypothesis.

Although the metabolic importance of the enzymatic synthesis of pyrimidine deoxynucleosides has not been clearly established, these reactions allow for redistribu-

tion of deoxyribosyl from one deoxynucleoside DNA precursor to another, as for example, it may permit the interconversion of the salvage and de novo pathways for thymine deoxynucleotide synthesis as illustrated by the following reaction sequence:



In reaction (A) the deoxyribosyl moiety of a compound in the salvage pathway, deoxythymidine (TdR), is redistributed to another pyrimidine deoxynucleoside, deoxyuridine (UdR), catalyzed by pyrimidine deoxyribosyltransferase. In the presence of ATP, deoxyuridine can be directly phosphorylated to deoxyuridylate (dUMP), a key intermediate in the de novo pathway. This reaction (B) is catalyzed by deoxythymidine kinase. Deoxyuridylate is then directly converted to deoxythymidylate (dTMP) as shown in reaction (C), and is catalyzed by the important enzyme of the de novo pathway, deoxythymidylate synthetase. Furthermore, as demonstrated by Gallo and associates (9), the leukocyte enzyme is subject to the influence of modifiers, e.g., activation by anions, substrate inhibition by thymine, product inhibition by thymine (17), and inhibition by purines (17). The inhibition by purines is complex and is critically dependent upon the thymine concentration, higher concentrations of thymine augmenting inhibition by purines (17). From this data and other observations, Gallo has presented a hypothesis for the role of this enzyme in contributing to the essential DNA precursor, dTMP, under conditions in which there has been a stimulus to DNA synthesis. It was emphasized that the control of this enzyme intracellularly may be particularly dependent on the thymine concentration (17).

In the comparative studies on the metabolism of some purine compounds, the levels of purine nucleoside phosphorylase and deoxyadenosine deaminase were measured and found to be significantly higher in CML leukocyte extracts than in normal leukocytes. It should be emphasized that the assays of purine nucleoside phosphorylase and deoxyinosine phosphorylase were performed only with leukocyte extracts obtained from CML patients in relapse. The metabolic significance of the finding that CML leukocytes contain lower levels of enzymes catalyzing the metabolism of pyrimidine deoxynucleosides but higher levels of enzymes catalyzing reactions in the metabolism of purine nucleosides and deoxynucleosides is not apparent from these studies and awaits further investigation.

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