DIHYDROFOLIC REDUCTASE IN HUMAN LEUKEMIC LEUKOCYTES*

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Received October 12, 1960

Dihydrofolic reductase from avian and mammalian liver catalyzes the pyridine nucleotide-dependent reduction of dihydrofolate shown in reaction (I). The

$$7,8\text{-dihydrofolate} + \text{TPNH} + H^+ \rightleftharpoons 5,6,7,8\text{-tetrahydrofolate} + \text{TPN}^+ \quad (I)$$

enzyme is inhibited by extremely low concentrations (ca. 10^{-8} M) of the folic acid antagonists, aminopterin and amethopterin (Futterman and Silverman, 1957; Futterman, 1957; Osborn and Huennekens, 1958; Osborn, et al., 1958; Zakrzewski and Nichol, 1958; Peters and Greenberg, 1958; 1959; Zakrzewski, 1960). Because these anti-folic compounds produce a remission of symptoms in certain acute leukemias, it seemed desirable to examine normal and leukemic white cells for the presence of dihydrofolic reductase and its sensitivity to the inhibitors.

Leukocytes were isolated from human blood‡ by a rapid procedure involving sedimentation of the red cells in the presence of dextran, removal of platelets by centrifugation, and selective lysis of the residual red cells by a brief exposure to a hypotonic medium. Lysates were prepared by disrupting the white cells in a hi-speed Virtis homogenizer or by extraction with phosphate buffer of acetone-dried preparations of the cells. Details of this procedure will be

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* Paper I in the series "Studies on normal and leukemic white cells". A preliminary account of this work was presented at the American Federation for Clinical Research, Atlantic City, May, 1960. This investigation was supported in part by an Institutional Research Grant from the American Cancer Society to the University of Washington, and by a grant from the American Cancer Society (P-203).

** Postdoctoral fellow of the National Heart Institute, U.S. Public Health Service.

‡ We are indebted to Drs. D.N. Coleman, Q.B. DeMarsh, D.M. Donohue, C.A. Finch, J.R. Hartmann and A.R. Stevens for providing us with blood from leukemia patients.

described elsewhere (Bertino, et al., to be published).

The endogenous regeneration of TPNH in lysates precludes the use of the customary spectrophotometric assay (TPNH disappearance) for dihydrofolic reductase (reaction (1)). However, when reaction (1) was coupled with reaction (2), the

$$\text{formate} + \text{ATP} + \text{tetrahydrofolate} \xrightleftharpoons{Mg^{++},\ K^{+} \text{ or } NH_4^{+}} N^{10}\text{-formyl tetrahydrofolate} + \text{ADP} + P_i \qquad (2)$$

appearance of N^{10}-formyl tetrahydrofolate from dihydrofolate was observed in leukemic lysates (Table 1). Reaction (2) is catalyzed by endogenous formate-

Table 1

Component Study for the Coupled System
Dihydrofolic Reductase-Formate Activating Enzyme

Component Omitted	N^{10}-formyl tetrahydrofolate
	mμmoles
None	40.9
TPNH	11.8
Dihydrofolate	0
Formate	6.3
ATP	8.7
Mg^{++} and NH_4^{+}	3.6

The complete system contained in a total volume of 1.0 ml., 100 μmoles of Tris buffer, pH 8.5, 20 μmoles of 2-mercaptoethanol, 0.30 μmole of dihydrofolate, 0.4 μmole of TPNH, 50 μmoles of sodium formate, 10 μmoles of $MgCl_2$, 50 μmoles of NH_4Cl, and 1.5 mg. of protein extracted from acetone-dried cells from an acute myelogenous leukemic patient. After incubation at 37° for 1 hour, the mixture was deproteinized with 0.5 ml. of 1.5 N. perchloric acid; denatured protein was removed by centrifugation. Acidification converts the product, N^{10}-formyl tetrahydrofolate, to N^{5}, N^{10}-methenyl tetrahydrofolate which was measured at 355 mμ ($\epsilon = 22 \times 10^6$ cm^2/mole). Determinations were made against a water blank and corrected for a control tube in which the lysate protein had been omitted.

activating enzyme. Dialysis of the lysate against 2×10^{-3} M phosphate buffer, pH 6.0, for 12 hours largely destroys the formate-activating enzyme but not the dihydrofolic reductase; activity of the coupled system can be restored by the addition of a formate-activating enzyme, such as the highly purified preparation

from Micrococcus aerogenes (kindly supplied by Dr. H. R. Whiteley).

Dihydrofolic reductase has been purified approximately 20-fold from extracts of acetone-dried leukemic leukocytes by treatment with protamine sulfate, fractionation with solid ammonium sulfate (55-90% saturation) and adsorption and elution from calcium phosphate gel. With the purified enzyme, the dihydrofolate-dependent disappearance of TPNH can be demonstrated readily. TPN was shown to be a product by its reactivity in the glucose-6-P dehydrogenase system. Tetrahydrofolate was demonstrated as the other product by its ability to be coupled with the formate-activating enzyme, as described above, or by a direct spectrophotometric experiment (Fig. 1). Dihydrofolate (Curve A, λ_{max} at 282 mμ)

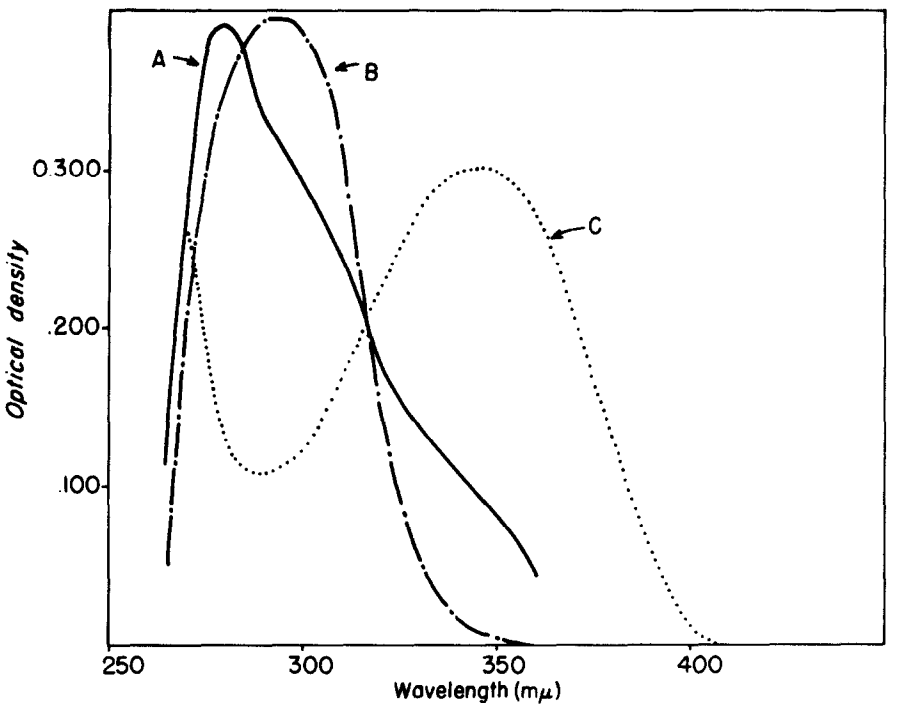

Figure 1. Tetrahydrofolate formation in the dihydrofolic reductase reaction. The experimental silica cuvette contained 0.018 μmole of dihydrofolate, 10 μmoles of 2-mercaptoethanol, 10 μmoles of $MgCl_2$, 100 μmoles of glucose-6-P, 20 μg. of purified glucose-6-P dehydrogenase (Sigma), 50 μmoles of Tris buffer, pH 8.5, 0.8 mg. of purified reductase from acute leukemic cells, and water to make 1.0 ml. The blank cuvette was identical except for the omission of dihydrofolate. Spectra were determined with the Beckman Recording Spectrophotometer, Model DK-1. Curve A was taken and 0.012 μmole of TPNH was added to each cuvette; after 20 min., Curve B was taken. To each cuvette was then added: 25 μmoles of formate, 25 μmoles of NH_4Cl, 0.1 μmole of ATP, and 10 μg. of purified formate-activating enzyme from M. aerogenes. After incubation for 30 min. the mixtures were deproteinized with 0.1 ml. of 2.5 N. perchloric acid, the denatured protein was removed by centrifugation, and the final spectrum was recorded (Curve C). Curves B and C were corrected to a 1.0 ml. volume.

was converted first to tetrahydrofolate (Curve B, λ_{max} at 298 mμ) via reaction (1), and then to N^{10}-formyl tetrahydrofolate by reaction (2) followed by transformation of the latter compound by acidification to the N^5, N^{10}-methenyl derivative (Curve C, λ_{max} at 350 mμ). The pH optimum for reaction (1) is 8.3 in Tris or phosphate buffer: K_m values† for TPNH and dihydrofolate in reaction (1) are 1.2×10^{-6} M and ca. 10^{-6} M, respectively. Dihydrofolic reductase from leukemic white cells, like its counterpart in other tissues, is inhibited by minute levels of the folic acid antagonists (Table II).

Table II

Inhibition of Dihydrofolic Reductase
by Anti-folic Compounds

Inhibitor	Inhibition
	percent
Amethopterin	57
Aminopterin	64
Dichloroamethopterin	85

The following components were added to a silica cuvette: 0.08 μmole of TPNH, 10 μmoles of 2-mercaptoethanol, inhibitors at a final concentration of 1.0×10^{-8} M, 100 μmoles of Tris buffer, pH 8.5, 0.8 mg. of purified reductase from acute leukemic cells, and water to make 10 ml. The optical density was determined at 340 mμ against a blank containing only buffer, and 0.04 μmole of dihydrofolate was added to start the reaction. The change in optical density was measured for a 10 min. period and corrected for controls without TPNH or dihydrofolate.

In the presence of excess formate-activating enzyme, the coupled reaction is linearly dependent upon the amount of dihydrofolic reductase, and it is thus possible to measure the level of the latter enzyme in white cell lysates. Dihydrofolic reductase has been found in all cases of acute leukemia (12 patients) investigated to date. In terms of dihydrofolate converted, the average level is

† With dihydrofolic reductase from sheep liver, Peters and Greenberg (1958) found that the K_m values for dihydrofolate are 6.2×10^{-6} M at pH 5.0 (DPNH as the cofactor), and 1.0×10^{-5} M at pH 7.0 (TPNH as the cofactor).

about 0.05 μmole/ml./hr./mg. of lysate protein[††]. The enzyme occurs at approximately the same level in patients with chronic myelogenous leukemia. Dihydrofolic reductase activity, measured by the above assay methods, has not been detected in normal or chronic lymphatic leukemia cells.

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†† For comparison, the levels of formate-activating enzyme and glucose-6-P dehydrogenase in lysates from acute leukemia are 0.7 and 4.0 μmoles/ml./hr./mg. protein, respectively.

The authors are indebted to Dr. M. I. Freeman and Mrs. A. Alenty for their assistance in this problem.

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