A Direct Assay for Dihydrofolate Reductase in Human Leukocytes

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The 4-amino folic acid antagonists are effective in the treatment of choriocarcinoma in women, acute lymphocytic leukemia and, when given by perfusion, of metastatic squamous cell carcinoma. These antagonists inhibit the enzymatic conversion of folic acid (F) to tetrahydrofolic acid (FH4) and there is good evidence that their biological effects result from this inhibition. The relationship of this "target" enzyme to tumor response and tumor resistance to the antagonists is being studied in a number of laboratories. This paper describes a simplified, direct method of assay of dihydrofolic reductase activity in human leukocytes. The advantages of this method, over methods previously described, are that (1) crude lysates of white cells can be used, (2) endogenous regeneration of NADPH does not interfere, (3) as few as 0.6 x 10⁸ cells are adequate for this assay, and (4) a constant source of supply of highly purified formate activating enzyme is not necessary.

Dihydrofolic reductase catalyzes reaction (1) as follows:

Reaction (1)

\[ 7,8\text{-dihydrofolic acid} + \text{NADPH} + \text{H}^+ \rightleftharpoons 5,6,7,8\text{-tetrahydrofolic acid} + \text{NADP}^+ \]

Tetrahydrofolic acid serves as a "one carbon" carrier for various reactions including the biosynthesis of thymidylic acid as in reaction (2):

Reaction (2)

\[ \text{deoxyuridylic acid} + 5\text{-}10\text{-methylene} \text{tetrahydrofolic acid Mg}^{++} \rightleftharpoons \text{deoxythymidylic acid} + 7,8\text{-dihydrofolic acid} \]

Dihydrofolic acid (FH₂) is regenerated by reaction (2) and can be again converted to tetrahydrofolic acid by reaction (1). Thus reactions (1) and (2) are cyclic and interdependent. Dihydrofolic reductase is usually measured spectrophotometrically by observing the decrease in optical absorbancy at 340 mμ due to NADPH oxidation. In some crude tissue preparations, particularly those from human leukocytes, the simultaneous regeneration of NADPH by other enzymes invalidates this 340 mμ measurement. Bertino et al. have assayed dihydrofolic reductase by coupling reaction (1) with reaction (3) mediated by added formate activating enzyme:

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* NADPH = reduced nicotinamide-adenine dinucleotide phosphate;
  NADP = oxidized nicotinamide-adenine dinucleotide phosphate.
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Reaction (3)

\[ 5,6,7,8\text{-tetrahydrofolic acid} + \text{formate} + \text{ATP Mg}^{++}, \text{K}^{+}, \text{NH}_{4}^{+} \]

\[ \rightarrow \text{N}^{10}\text{-formyl tetrahydrofolic acid} + \text{ADP} \]

The resulting N\(^{10}\)-formyl tetrahydrofolic acid is then converted by acidification to N\(^{5}\), N\(^{10}\)-methenyltetrahydrofolic acid, which is measured at its absorption maximum of 355 nm. This technic effectively avoids errors due to regeneration of NADPH but requires the availability of highly purified formate-activating enzyme and depends upon the quantitative production of the N\(^{5}\), N\(^{10}\)-methenyltetrahydrofolic acid from the product of reaction (1). The present method avoids the need for these steps.

Direct assay of dihydrofolic reductase (reaction 1) dependent upon the disappearance of NADPH at 340 nm has also been carried out by Bertino et al. However, this could only be done with enzyme which had been purified about 20-fold from acetone-dried leukocytes, since the purification procedure destroys the NADP reductase, which would otherwise interfere with this particular assay system.

The method described in the present paper is based on the change in absorbancy at 282 nm coincident to the conversion of dihydrofolic acid to tetrahydrofolic acid. It involves incubation of dihydrofolic acid with a simple preparation of the enzyme from leukocytes.

**PROCEDURE**

Leukocytes are separated from whole blood by differential dextran sedimentation and osmotic shock. The white cells are then suspended in 0.05 M potassium phosphate buffer (pH 7.5) with 0.01 M 2-mercaptoethanol, to give a final concentration of \(3 \times 10^8\) cells per ml. The cells are fragmented for 4-5 minutes in a Virtis Homogenizer at 30,000 rpm with sand glass. The homogenate is centrifuged for 45 minutes at 17,300 x g. The supernatant is assayed for dihydrofolic reductase. All procedures are carried out at 2-4 °C.

The enzyme is assayed spectrophotometrically at 282 nm (\(A_{\text{max}}\) for dihydrofolic acid). The experimental 1 ml. silica cuvette contained 0.9 ml. of a solution of 0.05 M potassium phosphate buffer pH 7.5 containing 0.01 M 2-mercaptoethanol, 0.01 ml. of potassium phosphate buffer containing 0.06 \(\mu\)moles of NADPH, 0.01 ml. of water containing 0.03 \(\mu\)moles of dihydrofolic acid and 0.1 ml. of leukocyte enzyme preparation. The blank cuvette contained all of the above with the exception of dihydrofolic acid. A decrease in absorbancy at 282 nm over a 30-minute period is measured at 28 °C.

The activity of the enzyme is then expressed as millimicromoles of dihydrofolic acid reduced per milligram of protein per hour at 28 °C. This is calculated by the use of a factor expressing the difference in the molar extinction coefficients (\(A_{\text{m}}\)) at 282 nm of dihydrofolic and tetrahydrofolic acids. This factor has been determined by two different methods. The first method involved the experimental determination of the change in optical absorbancy occurring at 282 nm when 0.01 ml. of a highly purified chicken liver enzyme (containing no NADP reductase) was employed to reduce FH\(_2\) completely by reaction (1). The reaction was measured by use of the published\(^8\) molar extinction coefficients for the optical absorbancy changes occurring at 340 nm. The observed changes in optical absorbancy in this experiment were −0.269 at 340 nm and −0.080 at 282 nm. The change at 340 nm is due to combination of losses of 6220 optical absorbancy units per mole for NADPH → NADP and 5050 units per mole for FH\(_2\) → FH\(_{1}\), which add up to 11,270 units per mole for the overall reaction. On this basis the observed loss of 0.269 units of optical absorbancy at 340 nm represents the reduction of 0.0238 \(\mu\)moles of FH\(_2\). This per-
mits calculation of the change in molar extinction at 282 m\(\mu\) for this reaction and yields a value of 3361. This change at 282 m\(\mu\) is primarily attributable to the reduction of FH\(_2\) because the differences in optical absorbancy between NADPH and NADP at 282 m\(\mu\) is negligible. Identical values for change in absorbancy at 340 and 282 m\(\mu\) were obtained in an experiment with another source of enzyme. Enzyme from livers of mice bearing a methotrexate-resistant variant of leukemia L1210 (rich in dihydrofolic reductase) was used. This enzyme preparation did not have NADP reductase. Experimental conditions were the same as for the chicken liver experiment.

FH\(_2\) used in these experiments were prepared by a procedure described by Friedkin, Crawford and Misra.\(^1\) The actual amount of FH\(_2\) was calculated from the absorbancy measurement using a molar extinction value of 26,100 at 282 m\(\mu\).\(^2\) FH\(_2\) present before the reaction started was: 

\[
\frac{0.720}{26.1} = 0.027 \text{ \(\mu\)moles} \quad (\text{refer to figure 4}).
\]

After completion of the reaction, the amount of FH\(_4\) produced was calculated using a molar extinction value of 25,000 for FH\(_4\) at 297 m\(\mu\)\(^2\) and was: 

\[
\frac{0.695}{25.0} = 0.027 \text{ \(\mu\)moles}.
\]

Thus, there was a 100% conversion of FH\(_2\) to FH\(_4\). The reaction was complete in about 5 minutes. In this experiment the experimental cuvette contained 1 ml of potassium phosphate buffer, 0.01 ml of potassium phosphate buffer containing 0.06 \(\mu\)moles of NADPH, and 0.01 ml of water containing 0.03 \(\mu\)moles of FH\(_2\). The blank cuvette contained all the above except FH\(_2\). Curve A was recorded. One-hundredth ml of the enzyme from livers of mice bearing a methotrexate-resistant variant of leukemia L1210 was then added to each cuvette. Curves B and C of figure 4 were recorded at 3/4- and 5-minute intervals respectively. Further metabolism or breakdown of FH\(_4\) did not occur for at least the next 1-hour period. This was confirmed by repeated spectral recordings of the product of the reaction, FH\(_4\), indicating its stability in the phosphate buffer with mercaptoethanol.

The second approach to determine \(\Delta A_m\) for FH\(_2\) \(\rightarrow\) FH\(_4\) involved calculations from established molar extinction coefficients for pure samples of the two compounds\(^1\(^1\),\(^2\) adjusted to 282 m\(\mu\) from reliable ultraviolet absorption spectral data.

- Molar extinction for FH\(_2\) at 282 m\(\mu\) = 26,100
- Molar extinction for FH\(_4\) at 282 m\(\mu\) = 22,180
- \(\Delta A_m\) for the change in molar extinction = 3,920

A value of 3.360 was used as the change in molar extinction at 282 m\(\mu\) when FH\(_2\) is reduced to FH\(_4\). This value was chosen over the value obtained from method 2 because it is directly derived from enzyme assays.

RESULTS

The enzyme activity is proportional to concentrations both for mouse liver enzyme (fig. 1) and for human leukocytes (fig. 2) over the ranges studied.

In the case of mouse liver enzyme, a 6-minute incubation period was used because of higher activity of the enzyme, whereas in the case of human leukocytes a 30-minute incubation was necessary to detect a measurable amount of change in absorbancy.

Figure 3 shows the spectrum of the reaction mixture taken against the blank at zero time. Curve A represents the spectrum of dihydrofolate with a \(\lambda_{\text{max}}\) at 282 m\(\mu\). Curve B taken after 30 minutes when the reaction is complete represents the spectrum of tetrahydrofolate with a \(\lambda_{\text{max}}\) at 297 m\(\mu\). The source of dihydrofolate reductase was from the white cells of a patient with chronic myelogenous leukemia (CML).
Fig. 1.—Effect of enzyme concentration on dihydrofolate reductase activity. Enzyme obtained from livers of mice bearing a MTX-resistant variant of leukemia L1210 (enzyme diluted 2x). Activity expressed as total change in absorbancy at 282 mµ over a 6-minute period.

Figure 4 shows the spectrum of the reaction mixture when dihydrofolate reductase was obtained from the liver of a mouse bearing methotrexate-resistant leukemia L1210.

In the region of 330-370 mµ, curve C of figure 4 was below the base line, whereas curve B of figure 3 was well above the base line. This is because the spectrum was taken against a blank containing NADPH and enzyme. In figure 4, NADPH present in the blank cuvette is not converted to NADP, but in the experimental cuvette most of NADPH is oxidized to NADP with no endogenous regeneration of NADPH. In the case of white cells (fig. 3), this does not occur because of the regeneration of NADPH. Marked flattening of the line presumably represents the mechanical lower cut-off of the spectrophotometer. Under the experimental condition described above, the enzyme is quite stable when kept frozen and the results of the assay are reproducible as shown in table 1.

White cell preparations obtained from patient J.A. on two separate occasions within an interval of 1 week gave the same values of enzyme activity.
Fig. 2.—Activity of dihydrofolate reductase of leukocytes from a patient with chronic myelogenous leukemia. Activity expressed as a total change in absorbancy at 282 μM over a 30-minute period.

The values are subject to variation depending upon the differential count of the white cells at the time the blood is withdrawn from the patient. Since mature granulocytes have very little or no dihydrofolate reductase activity, a higher percentage of immature cells in the peripheral blood would give a higher enzyme level.

Using this assay procedure, dihydrofolate reductase was determined on various types of human leukocytes (table 2). Activity is expressed in millimicromoles of dihydrofolate reduced per milligram of protein per hour at 28°C. Protein content was determined by the method of Lowry et al.14

**DISCUSSION**

A major area of importance of this enzyme system relates to cancer chemotherapy. The folic acid antagonists inhibit this “target” enzyme with
Fig. 3.—Reduction of dihydrofolate to tetrahydrofolate. The experimental 1 ml. cuvette (1 cm. lightpath) contained 0.9 ml. of 0.05 M potassium phosphate buffer with 0.01 M 2-mercaptoethanol, 0.01 ml. of potassium phosphate buffer containing 0.06 μmoles of NADPH, 0.01 ml. of water containing 0.03 μmoles of dihydrofolic acid, and 0.1 ml. of enzyme preparation from chronic myelogenous leukemia cells. The reference cell had the identical mixture except for omission of dihydrofolate. Spectra were taken with the Cary Recording Spectrophotometer Model 15. Curve A was recorded immediately after the addition of dihydrofolate; curve B was recorded after 30 minutes.

resultant major therapeutic benefit for some patients with certain types of neoplastic disease. The identification of potential responders and an explanation for lack of response may be possible from quantitative studies of folic acid reductase in clinical material. The development of resistance to folic acid antagonists in some rodent tumor systems has been associated with a marked increase in folic acid reductase activity. The development of refractoriness to these agents in man also occurs but has not been satisfactorily explained at a biochemical level.

This paper presents an assay system which involves a single reaction step. It is simple and direct. The method permits the measurement of very low levels of dihydrofolate reductase, such as seen in normal leukocytes. It is
Fig. 4.—The source of the enzyme was from the liver of mice bearing a methotrexate-resistant variant of leukemia L1210. Enzyme was diluted two-fold with phosphate buffer. Because of higher activity of the enzyme, curve A was recorded with NADPH and FH₂ against a blank containing NADPH alone. 0.01 ml. of enzyme was then added to each cuvette; after 2½ minutes, curve B was recorded; after 5 minutes, curve C was recorded. (See text for details.)

hoped that this method will facilitate studies on the changes which occur in dihydrofolate reductase levels in leukocytes of patients undergoing therapy with folic acid antagonists. The values of dihydrofolate reductase activity obtained in various forms of human leukocytes by using this assay procedure are in good agreement with the values obtained by coupled assay system of Bertino et al.¹⁵

SUMMARY

A new direct spectrophotometric assay for dihydrofolate reductase has been developed. The reaction is observed at 282 mμ and is based on the disappearance of dihydrofolate. Based on this assay, the values for normal leukocytes are 0–5 μmoles of dihydrofolate reduced to tetrahydrofolate, per mg. of protein per hour at 28 C. The respective values for chronic myelogenous leukemic granulocytes are 25–67, chronic lymphocytic leu-
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Table 1.—Stability of Enzyme and Reproducibility of Enzyme Assay

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Day Assayed</th>
<th>Amount of Enzyme Used</th>
<th>Enzyme Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient J. A. (CML)†</td>
<td>day of preparation</td>
<td>0.1 ml.</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>11 days later</td>
<td>0.1 ml.</td>
<td>0.040</td>
</tr>
<tr>
<td></td>
<td>13 days later</td>
<td>0.1 ml.</td>
<td>0.040</td>
</tr>
<tr>
<td>Patient A. S. (ALL)‡</td>
<td>day of preparation</td>
<td>0.4 ml.</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>day of preparation</td>
<td>0.2 ml.</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>7 days later</td>
<td>0.2 ml.</td>
<td>0.018</td>
</tr>
<tr>
<td>Patient R. S. (CML)</td>
<td>day of preparation</td>
<td>0.3 ml.</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td>25 days later</td>
<td>0.3 ml.</td>
<td>0.050</td>
</tr>
</tbody>
</table>

It can be seen that, over a period of days, there is virtually no change in enzyme activity. *Enzyme activity expressed as a total change in absorbancy over a 30-minute period.
†CML = chronic myelogenous leukemia; ALL = acute lymphocytic leukemia.

Table 2

<table>
<thead>
<tr>
<th>No. of Subjects</th>
<th>No. of Assays</th>
<th>Enzyme Median</th>
<th>Activity Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal leukocytes</td>
<td>3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>CML* leukocytes</td>
<td>5</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>CLL* leukocytes</td>
<td>5</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>ALL* leukocytes</td>
<td>3</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Leukemoid reaction</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

*CML = chronic myelogenous leukemia; CLL = chronic lymphocytic leukemia; ALL = acute lymphocytic leukemia.
‡High values of dihydrofolate reductase activity found in chronic lymphocytic leukemic leukocytes were obtained from subjects who had received corticosteroids within 2 weeks of enzyme assay.

kemia 0–18, acute lymphocytic leukemia 7–19, and for leukocytes of leukemoid reaction 4 to 5.

SUMMARIO IN INTERLINGUA

Esseva disveloppate un nove directe essayo spectrophotometric pro reductase de dihydrofolato. Le reaction es observate a 282 mα e ha su base in le disparition de dihydrofolato. Secundo iste essayo, le valor pro leucocytos normal, exprimite per mg de proteina per hora a 28 C, es 0 a 5 mμmol de dihydrofolato reducete a tetrahydrofolato. Le correspondente valores pro granulocytos de chronic leucemia myelogene es 25 a 67, pro illos de chronic leucemia lymphocytic 0 a 18, pro illos de acute leucemia lymphocytic 7 a 19, e pro leucocytos de reaction leucemoid 4 a 5.

REFERENCES


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