VOL. 5, NO. 11, NOVEMBER 1966

An Isotopic Assay for Dihydrofolate Reductase*

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ABSTRACT: An isotopic method with tritiated folate, generally labeled, has been developed for the assay of dihydrofolate reductase. The tetrahydrofolate that formed in the enzymatic reaction was oxidized with potassium nitrite in order to release tritiated water. The residual substrate was adsorbed onto charcoal, and an aliquot of the supernatant fluid was counted by liquid scintillation.

An assay for dihydrofolate reductase with generally labeled tritiated folate is presented which is based on the release of tritiated water from tetrahydrofolate. Folate labeled by gaseous exchange with tritium contained isotope in both the pteridine and *p*-aminobenzoylglutamate moieties (Johns *et al.*, 1961). The tritium in the pteridine ring becomes labile when folate is reduced to tetrahydrofolate. Barring an isotope effect, oxidation of the reduced pteridine ring releases one-half of the radioactivity at position 7. The cleavage of the C_9–N_{10} bond releases a pteridine product which can be oxidized (Webb, 1955). The residual substrate is removed by adsorption onto charcoal, and an aliquot of the tritiated water formed by the oxidation of the pteridine ring of tetrahydrofolate is measured by liquid scintillation counting. The assay has been standardized against the Bratton–Marshall reaction for tetrahydrofolate (Werkheiser *et al.*, 1962).

Methods

Rat liver was selected as a source of dihydrofolate reductase for the present study because of the high level of enzyme activity in this organ and because of the earlier studies on the reduction of folate by this source of enzyme (Werkheiser, 1961; Roberts and Hall, 1965). Many of the conditions of other methods for dihydrofolate reductase from rat liver were found to apply. The 20% homogenate of rat liver in 0.01 M Tris, pH 7.0, was centrifuged at 30,000*g* for 25 min. The supernatant fluid was stored at −20° and diluted as required for assay.

Reagent. A 35-μl aliquot of stock buffer solution composed of 0.7 M 3,3-dimethylglutarate, 0.07 M citrate, and 0.07 M $MgCl_2$, adjusted to pH 6.1 with NaOH, was combined with 4 μl of NADPH[1] (10 mg/ml) in 0.05 M sodium carbonate buffer, pH 10, 7 μl of generally labeled folate (0.6 mg/ml), sp act. 35–200 μc/μmole, and 5 μl of water or methotrexate (100 μg/ml). This reagent was prepared fresh each day. The folate was stored in small aliquots which were lyophilized and stored dry at −10°. This lyophilization also reduced the blank by about one-half. The NADPH was stored between 0 and 4° and prepared fresh each month (Lowry *et al.*, 1961). Methotrexate, 4 × 10^{-6} M, was added to the complete system and incubated for a control.

Procedure. A 20-μl aliquot of the supernatant fluid of rat liver homogenate (one volume of 20% homogenate plus three volumes of 0.01 M Tris, pH 7.0) was incubated for 40 min at 37° with 5 μl of reagent. At the end of the incubation, 50 μl of trichloroacetic acid–KNO_2 (nine volumes of 15% plus one volume of 0.63%) was added to precipitate the protein and to oxidize the tetrahydrofolate. After 10 min at room temperature, 80 μl of charcoal (100 mg/ml), grade NF, Merck, Rahway, N. J., was added and the suspension was centrifuged for 10 min at 200*g*. A 77-μl aliquot was removed for liquid scintillation counting in 10 ml of BBOT solution. Lang–Levy pipets were used to deliver the solutions (Lowry *et al.*, 1954).

The trichloracetic acid–KNO_2 solution was prepared fresh just prior to use with KNO_2 which was prepared fresh each Monday. The charcoal was kept in suspension with a small magnetic stirrer and pipetted with a modified Lang–Levy pipet on which the orifice and constriction were slightly enlarged to avoid clogging.

Quantitative adsorption of the degradative products of tetrahydrofolate onto charcoal was not observed. When the aliquot for scintillation counting was lyophilized or evaporated at 95° to dryness, a nonvolatile product was observed with some batches of tritiated folic acid. Identification of the nonvolatile product remains incomplete because of the change to other batches of folate of lower specific activity. This product moved with R_F 0.85 in 0.1 M potassium phosphate buffer, pH 7.0. The presence of a nonvolatile product increases the sensitivity of the assay over the theoretical yield from the oxidation of the pteridine ring and does

* Laboratories of Pharmacology, Children's Cancer Research Foundation, and Department of Pathology, Harvard Medical School, Boston, Massachusetts. *Received August 1, 1966.* This investigation was supported by Contract No. PH-43-62-169 from the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health.

[1] Abbreviations used: NADPH, reduced nicotinamide–adenine dinucleotide phosphate; BBOT solution, 2,5-bis-2-(5-*t*-butylbenzoxazolyl)thiophene dissolved in organic solvents according to instructions of Packard Instrument Co., Inc., LaGrange, Ill.

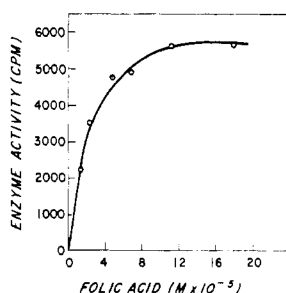

FIGURE 1: Concentration curve of folic acid.

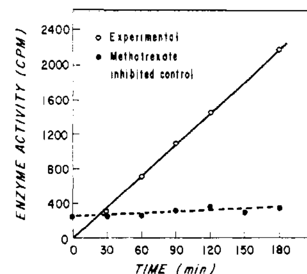

FIGURE 3: Linearity of enzyme reaction with time.

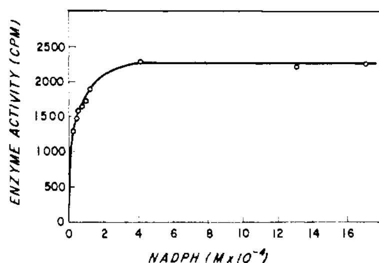

FIGURE 2: Concentration curve of NADPH.

TABLE I: Degradation of Tetrahydrofolate.[a]

Condition	Cpm
Water	1205
3% H_2O_2	1267
Satd aqueous I_2	1445
0.63% KNO_2	1508

[a] The enzymatic reaction was terminated by placing the tubes in ice water; 2.5 μl of water, H_2O_2, iodine solution, or KNO_2 was added and after 10 min the solutions were diluted with an equal volume of 15% trichloroacetic acid and extracted with charcoal for counting. A methotrexate-inhibited control was used for each condition, and the difference for the experimental condition minus the blank is reported.

not require any modification of the procedure for standardizing the method.

Standardization of Method. The isotopic method was standardized by relating it to the Bratton–Marshall reaction for *p*-aminobenzoylglutamate (Werkheiser *et al.*, 1962). The incubation system was enlarged tenfold for this study. The reaction was terminated with the addition of 250 μl of 15% trichloroacetic acid and centrifuged for 10 min at 200*g*. KNO_2 (5 μl of 0.63%) was added to 65 μl of the supernatant fluid and assayed as above.

To 265 μl of the trichloroacetic acid supernatant fluid was added at 3-min intervals 22 μl of 0.63% KNO_2, 22 μl of 2.5% ammonium sulfamate, and 22 μl of 0.5% napthylethylene diamine·2HCL. The solution was centrifuged for 10 min at 200*g* and after 20 min the absorbancy at λ 560 mμ was measured. An extinction coefficient of 55,600 was used to standardize the color reaction (Zakrzewski, 1960). The isotopic assay was then standardized in counts per minute per millimicromole of *p*-aminobenzoylglutamate formed from the product, tetrahydrofolate.

Experimental Section

The isotopic assay depends upon the use of conditions which allow the tetrahydrofolate to be quantitatively degraded (Roberts and Hall, 1965). For this reason, 2-mercaptoethanol was omitted from the procedure. Tetrahydrofolate undergoes degradation under these conditions, but the addition of KNO_2, H_2O_2, or iodine increases the release of radioactivity (Table I).

The concentration curve with folate is presented in Figure 1. This curve was corrected for an increase in the methotrexate-inhibited control which occurs with the increase in the concentration of folate.

The response to increase in the concentration of NADPH is shown in Figure 2. The utilization of an endogenous NADPH-regenerating system, based on the oxidation of citrate, lowered the concentration of added NADPH which was required for long incubations (Werkheiser, 1961).

The enzyme reaction was linear for 3 hr and was accompanied by a slow increase in the methotrexate-inhibited control (Figure 3). This increase in the control occurs as the result of a degradation of the substrate under the conditions of the assay or as the result of side reactions with the folate (Roberts, 1961; Johns *et al.*, 1964).

The method is proportional to enzyme concentration (Figure 4) when the concentration of the homogenate was equivalent to 8% (grams per volume), or less. Methotrexate stoichiometrically inhibited dihydrofolate reductase in this assay when graded amounts of drug were added (Figure 5). The assay has been adapted to the measurement of the methotrexate content of boiled extracts of white blood cells as was reported by Werkheiser *et al.* (1962) with the Bratton–Marshall assay.

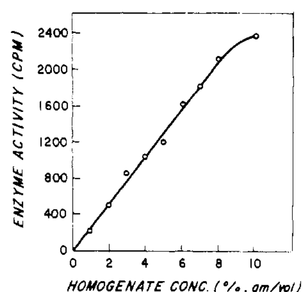

FIGURE 4: Linearity with enzyme concentration.

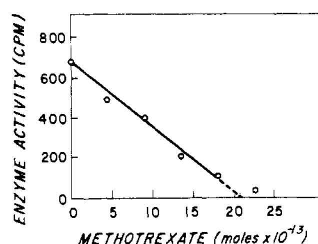

FIGURE 5: Methotrexate titration of enzyme activity. The concentration of the enzyme was reduced, and the time of incubation increased to 2 hr.

The concentration of dihydrofolate reductase was reduced, and the time of incubation increased to improve the sensitivity of the method when the assay was adapted to the measurement of methotrexate. The stoichiometric inhibition of dihydrofolate reductase indicates that dissociation of the drug–enzyme complex will not interfere with the *in vitro* measurement of the *in vivo* level of enzyme activity.

The enzyme reaction was measured at pH 6.1. This is a pH where the NADPH-regenerating system was functional and dihydrofolate reductase activity remained high. Maximal binding of methotrexate to dihydrofolate reductase was observed (Bertino *et al.*, 1964) in this pH range.

Discussion

This isotopic assay for dihydrofolate reductase is very simple and extremely sensitive. The stoichiometric inhibition by methotrexate under the conditions of the assay avoids the problems encountered by Roberts and Hall where dissociation of the methotrexate–enzyme complex may have occurred with dihydrofolate as substrate (Roberts and Hall, 1966b). The simplicity of the absorption of the substrate onto charcoal suggests an advantage of the present assay when compared with the electrophoretic method with isotopic folate (Rothenberg, 1965).

Dihydrofolate was reduced about one hundred times more rapidly than folate, but the availability of tritiated folate of high specific activity negates this advantage. The conditions of the present assay are not applicable to the use of dihydrofolate.

With variation of the period of incubation and of the concentration of the homogenate the present method has been applicable to the measurement of the dihydrofolate reductase activity of normal and leukemic human leukocytes (Roberts, 1966; Roberts and Hall, 1966a), cell cultures of human leukemic leukocytes, and rodent lymphocytic leukemic cells and murine ascites tumor cells.[2]

Acknowledgment

The author gratefully acknowledges the technical assistance of Mr. John Finesilver and Miss Ellen Loehr in the execution of this study.

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[2] D. Roberts, unpublished observation