A New Assay of Thymidylate Synthetase Activity Based on the Release of Tritium from Deoxyuridylate-5-³H*

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SUMMARY

The tritium atom of deoxyuridylate-5-³H is obligatorily displaced in the thymidylate synthetase reaction and equilibrates with water. Accordingly, a new and simple assay for the enzyme has been devised which is based on the measurement of the tritium released. This method shows essentially the same substrate requirements and pH optimum as the spectrophotometric assay. It has the advantage of much greater sensitivity and lower blanks and is applicable to crude extracts without modification. The ratio of tritium released to dihydrofolate formed is less than 1.0 and is considered to be the result of an isotope effect.

The assays of thymidylate synthetase activity, which have been evaluated recently by Friedkin (2), can be divided into two types: (a) methods based on the use of a labeled substrate and the subsequent isolation of the radioactive thymidylate or a derivative by chromatography and (b) the spectrophotometric method of Wahba and Friedkin (3) which depends on the change of absorbance at 338 mp resulting from the oxidation of 5,10-methenyltetrahydrofolate to 7,8-dihydrofolate.

The spectrophotometric method, while rapid and reliable, is limited in sensitivity and, with crude preparations of enzyme, frequently exhibits significant increases in absorbance even in the absence of the substrate, deoxyuridine 5'-phosphate. Although the radioisotope-chromatographic methods are capable of greater sensitivity, they are time consuming and tend to suffer from interfering side reactions and high blank values (4, 5).

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1 For example, crude extracts of Escherichia coli R2 brought about the degradation of dTMP to thymidine, and thence to thymine, at a rate of 1.7 mmoles per min per mg of protein at 25°, pH 7.4, and in the presence of 0.01 M magnesium acetate. With a thymine-dependent organism, E. coli B3, the value was 1.0 m mole

(2), displacing tritium which then equilibrates with the ions of water. The procedure has the advantage of great sensitivity and simplicity, is relatively rapid, and shows low blanks even in crude extracts.

MATERIALS AND METHODS

Biological Materials—Escherichia coli R2, a derivative of strain B (6), was grown aerobically in 40-liter batches in a Biogen apparatus (American Sterilizer Company, Erie, Pennsylvania) per min per mg. The nature of the blank values and the sensitivity of radioisotope column methods has been investigated and discussed by Lomax (4).
in a glycerol-Casamino acids-salts medium adapted from that of Fraser and Jerrel (7) as described previously (5, 8). At concentrations between 6 x 10^6 and 3 x 10^8 per ml, the cells were harvested in a cooled Sharplies centrifuge and stored as a paste at -20°.

The preparations of lysates of T2 bacteriophage and of infected E. coli R2 have been described elsewhere (5, 8).

Enzymes—Thymidylate synthetase from E. coli was prepared essentially by the method of Friedkin et al. (9). The procedure was modified by substituting Sephadex G-100 for Sephadex G-75 in the preparation of Fraction IV, and resulted in a greater purification (27 to 60 times the specific activity of the crude extract). However, in most of the experiments performed with E. coli enzyme which are reported here, Fraction III was used. Details are given in the figure legends. Thymidylate synthetase induced by infection of E. coli with T2r+ phage was separated from the host enzyme by chromatography of crude extracts on DEAE-cellulose columns, as described previously (8). Wheat phosphotransferase was prepared and assayed as described by Barner and Cohen (10). A water extract of lyophilized Crotalus adamanteus venom was used as the source of 5'-nucleotidase.

Deoxyuridylate-5'-H—Wheat phosphotransferase was used to phosphorylate deoxyuridine-5'-H according to the following reaction (see Reference 10).

Deoxyuridine-5'-H + AMP → dUMP-5'-H + adenosine

The reaction mixture contained 38.4 mm AMP, 19.2 mm potassium acetate buffer (pH 5.2), 1.92 mm unlabeled deoxyuridine, 50 μC of deoxyuridine-5'-H, and enzyme in a final volume of 0.52 ml and was incubated for 34 hours at 32°. The entire reaction mixture was streaked on Whatman No. 3MM paper and developed by descending chromatography for 15 hours with isopropanol-water, 66:1:33 (v/v). Four bands were found, corresponding to dUMP, AMP, deoxyuridine, and adenosine. The dUMP and deoxyuridine bands were eluted with 1% ammonium hydroxide and taken to dryness repeatedly, and the residue was dissolved in 1 ml of water. The concentration of dUMP was determined from the molar extinction coefficient of 10.2 x 10^6 liter mole⁻¹ cm⁻¹ at 262 μm (11). The yield of dUMP-5'-H was usually 20 to 40%, based on deoxyuridine. Alternatively, dUMP-5'-H was isolated from phosphotransferase reaction mixtures by chromatography on columns, 0.6 x 8 cm, of Dowex 1-X8 (formate) resin, 200 to 400 mesh. After the unadsorbed nucleosides were removed by a water wash, the nucleotides were eluted by means of a convex gradient of formic acid ranging from 0 to 4 M. AMP and dUMP-5'-H were eluted at 0.90 M and 2.35 M formic acid, respectively. The specific activity was essentially constant throughout the dUMP region. Both isolation procedures yielded material with the characteristic ultraviolet absorption properties of a deoxyuridine derivative. The synthesized dUMP-5'-H cochromatographed with carrier dUMP in ethanol-1.0 M ammonium acetate, pH 7.5, 7.3 (v/v), on Whatman No. 1 paper. The identity of dUMP-5'-H also was established through conversion to deoxyuridine-5'-H with snake venom 5'-nucleotidase, and the resulting compound cochromatographed with deoxyuridine in the ethanol-ammonium acetate and isobutyrate-ammonium hydroxide solvents.

Spectrophotometric Assay for Thymidylate Synthetase—The optical method of Wabba and Friedkin (3), which measures the increase in absorbance at 338 μm upon conversion of 5,10-methylenetetrahydrofolate to dihydrofolate, was used as the standard assay in these studies. Measurements were made in a Beckman DU spectrophotometer combined with an automatic cuvette changer and a recorder system having an expanded scale (Gilford Instruments Laboratories, Oberlin, Ohio). Full scale normally was 0.1 A. The assay mixture contained: 42 mM Tris-Cl (pH 7.4), 26 mM MgCl₂, 15.8 mM formaldehyde, 106 mM 2-mercaptoethanol, 1.06 mM EDTA, 0.30 mM dl-l-tetrahydrofolate, and the enzyme in a final volume of 1 ml. The reaction was started by the addition of 0.050 μmole of dUMP, a control reaction containing no dUMP was run simultaneously for each concentration of enzyme. One unit of enzyme activity is defined as that amount of enzyme which catalyzes the formation of 1 μmole of dihydrofolate per min at 25°, calculated on the basis of a change in absorbance of 6.6 x 10⁻⁶ liter mole⁻¹ cm⁻¹ (3). Thymidylate Synthetase Assay by Release of Tritium from Deoxyuridylate-5'-H—Thymidylate synthetase was measured by collecting the water of the reaction mixture by sublimation and assaying it for the tritium displaced from dUMP-5'-H. The following procedure was used. The concentrations of the components of the reaction mixture were those used in the spectrophotometric assay except for the substitution of dUMP-5'-H for dUMP. The final volume was 0.5 ml. Variations are given in the figure legends. Incubations were performed in the side arms of Thunberg tubes in a 25° bath. Alternatively, an aliquot of a reaction mixture could be transferred to a side arm after incubation. The reaction was stopped by freezing the mixture as a thin shell in a Dry Ice-ethanol bath. The side arm then was connected to the Thunberg tube and evacuated on a vacuum pump to less than 4.58 mm of mercury, the pressure at the triple point of water. Under these conditions, the water of the reaction mixture sublimed and condensed in the lower portion of the tube, which was held in a Dry Ice-ethanol bath. When sublimation was complete (about 1 hour), a 0.2-ml aliquot of the sublimate was assayed for radioactivity. Normally, the sublimations were carried out with a bank of 12 Thunberg tubes on a manifold.

In separating tritiated water from dUMP-5'-H, sublimation of the water was chosen rather than such methods as adsorption of the nucleotide on charcoal or ion exchange resin because it was considered to be more foolproof. For example, in extracts of some organisms or tissues, which might, by side reactions, convert the pyrimidine ring to nonaromatic compounds by reduction, oxidation, or ring cleavage (see Reference 12), the resulting radioactive products would not be expected to adsorb onto charcoal. However, they probably would not sublime together with the water.

Miscellaneous Methods and Chemicals—Radioactive compounds on paper chromatograms were located by an automatic scanner (Vanguard) on a strip cut from the edge of the chromatogram. Aqueous samples containing tritium were measured in a Packard Tri-Carb liquid scintillation spectrometer. The scintillation mixture consisted of a 0.2-ml aqueous sample, 3.0 ml of absolute ethanol, and 7.0 ml of toluene containing 0.4% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene and 0.1% 2,5-diphenylox-
zole. The counting efficiency with this system was 11% for tritiated water. The background counting rate was 35 to 50 cpm.

Protein was measured by the method of Lowry et al. (13) with bovine serum albumin (Fraction V) as a standard uncorrected for water content.

A formaldehyde solution prepared from a 37% solution (Fisher) was standardized gravimetrically by conversion to the formaldehyde derivative. Tetrahydrofolate acid was obtained from Nutritional Biochemicals as a solution in 1 m 2-mercaptoethanol. The concentration of 2-mercaptoethanol was adjusted to 2 mM and this stock solution was stored at −20°C. The concentration of tetrahydrofolate was estimated by measuring the absorbance of dilutions of the stock solution, based on a molar extinction coefficient of 25,000 at 298 nm (14). Dilutions were made in a solution of 0.05 M Tris-Cl, pH 7.4, containing 0.01 M 2-mercaptoethanol and were read immediately. Based on the calculated content of the L, L isomer, approximately 58% of the tetrahydrofolate preparation was reactive in the thymidylate synthetase system.

Deoxyuridine-5-H (1.3 C per mmole) was purchased from Schwarz BioResearch. From the mechanism of synthesis, the tritium was located only in the base. The ratio of the labeling in the hydrogen atoms on positions 5 and 6 was estimated by conversion to 5-bromodeoxyuridine-5-H. From a consideration of the percentage of deoxyuridine-5-H converted to 5-bromodeoxyuridine and of the tritium released into the medium, it was calculated that not more than 30% of the tritium was on C 6, assuming that bromination displaced hydrogen 5 specifically.

Deoxyuridylate was obtained from Calbiochem. Thymidylate and ethylenediaminetetraacetic acid were products of Sigma. The latter two companies were also the source of various other nucleosides and nucleotides used in these studies. 2-Mercaptoethanol was obtained from Eastman.

RESULTS

Requirements for Release of Tritium from Deoxyuridylate-5-3H—

The data in Table I provide evidence that the maximal rate of tritium release is dependent upon all the components of the thymidylate synthetase reaction. When tetrahydrofolate was omitted, no activity above the background of a control containing no enzyme was detectable. In the absence of added formaldehyde, however, the amount of tritium released was 6% of the value for the complete reaction. It is possible that this activity may be accounted for by a small amount of formaldehyde in the tetrahydrofolate preparation. By chemical analysis, these tetrahydrofolate preparations contained 4.2% formaldehyde, on a molar basis.4 Wahba and Friedkin (3) found by the spectrophotometric assay that, on omission of formaldehyde, thymidylate synthetase activity was 5% of the complete system at most stages of purification (15). Table I shows that, when Mg++ was omitted, the rate of release of tritium was reduced to 28% of the control value. To determine whether this residual activity represented a partial enzymatic reaction that released hydrogen 5 of dUMP, the response of this system to a suboptimal level of Mg++ was compared with data obtained under similar conditions with the optical assay. By the spectrophotometric assay method and with an E. coli enzyme fraction purified 45-fold, the rate was reduced to 23% of maximal activity upon omission of Mg++ and to 70% at 6 mM Mg++. The residual activities observed with the tritium release assay were 28 and 54% at 0 and 6 mM Mg++, respectively, when the assay was performed under the conditions of Experiment I, Table I. These values were in reasonable agreement with those obtained in the optical assay and with the results reported by Wahba and Friedkin (15).

Linearity of Release of Tritium with Enzyme Concentration—

Fig. 2 shows the dependence of tritium release upon enzyme concentration. The rate of tritium release was linear with the amount of enzyme up to approximately 0.4 unit/0.5 ml of incubation mixture, a value consistent with data obtained in the optical assay under similar conditions. Correcting the specific

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4 Y.-C. Yeh and G. R. Greenberg, unpublished data.
activity of dUMP-3H for the 3H estimated to be at position 6 (see "Methods"), the rate of tritium release in the linear portion of the curve becomes 4.4 mpatoms per min per ml of enzyme. This value corresponds to 80% of the activity determined by the optical assay. While there is an uncertainty about the exact level of tritium at position 6 of the dUMP-3H employed because of the calculation involved and because it is not known whether bromination labilizes the hydrogen atom on C-6, the figure of 80% represents an upper limit. If all of the tritium of dUMP-3H were on C-5, the value would be 56%. This uncertainty has been superseded by later studies with specifically labeled dUMP-5-3H (see "Discussion").

Effect of pH on Relative Rates of Dihydrofolate Formation and Tritium Release.—The rates of dihydrofolate formation and tritium release were compared at different pH values throughout the range from 6.0 to 9.0 (Fig. 3). The relative rates of tritium release and dihydrofolate formation were affected similarly by the range from 6.0 to 9.0 (Fig. 3). The relative rates of tritium release and dihydrofolate formation were affected similarly by the range from 6.0 to 9.0 (Fig. 3). The relative rates of tritium release and dihydrofolate formation were affected similarly by the range from 6.0 to 9.0 (Fig. 3).

DISCUSSION

Displacement of tritium from dUMP-5-3H appears to be the most sensitive assay for thymidylate synthetase presently available. It is possible that the sensitivity of the method may be limited only by the specific activity of the labeled substrate. The method offers the advantages of simplicity as well as a low blank. There is no interference with the reaction either from oxidation of the starting tetrahydrofolate derivative or reduction of dihydrofolate, or by phosphatase action on dTMP. The major source of the blank is the presence of tritiated water in the dUMP-3H solutions. This labeled water arises either by slow dissociation or by degradation of the dUMP. Ordinarily, this blank value can be reduced merely by lyophilizing the dUMP-3H sample.

All of the components of the enzymatic system are necessary for maximum activity when the tritium displacement procedure is used, and the requirements are very similar to those of the optical assay (3, 15). Thus, in the absence of formaldehyde, the value obtained by tritium release was 6.4% of that of the complete system. When Mg++ was omitted from the reaction mixture, the rate of tritium release was reduced to approximately 28 to 28% of that of the control value. The rates in the absence of formaldehyde or of added Mg++ agree well with the values obtained by the optical assay and with the earlier findings (3, 15).

Thymidylate synthetase activity as measured by the tritium release assay is always less than the activity measured by the optical assay. For example, in Fig. 2, partially purified phage-induced thymidylate synthetase showed an activity by tritium release which was estimated to be a maximum (see "Results") of 80% of the activity obtained by the optical assay. Actually, subsequent studies with thymidylate synthetase from E. coli and from partial revertants of a thymine-dependent mutant, E. coli B3, with the use of specifically labeled dUMP-5-3H have shown activities by the tritium release assay which were from 26 to 64% of those obtained by the spectrophotometric determination. While this difference must be attributed to an isotope effect, it appears to vary with the nature of the enzyme source. The factors bringing about this variation are not clearly understood. Naturally, the ratio would determine the absolute value obtained by the tritium release method, and it must be accepted that such a variation would represent a drawback. However, within a given set of conditions the ratio appears to be quite constant. For example, an effort to demonstrate differences in this ratio by variation of the pH showed no apparent alteration (Fig. 3). In these experiments it was considered that an increase in the ratio, tritium released to dihydrofolate formed, might occur if the thymidylate synthetase reaction consisted of two steps and a significant concomitant exchange with water took place. Nevertheless, another study now has shown that an exchange of hydrogen 5 of dUMP with the protons of water does in fact occur at a rate that is approximately 5 to 10% of the over-all forward reaction.

The idea of using 3H displacement into water as an assay of a reaction has, of course, a great number of possibilities. With the use of dCMP-5-3H this method has been applied to the assay of dCMP hydroxymethylase with excellent results (17). In contrast to the thymidylate synthetase reaction, only tetrahydrofolate is required for the release of tritium from dCMP-5-3H. Formaldehyde is unnecessary, and the release is, in fact, an enzyme-catalyzed exchange reaction with water. The discovery of the exchange reactions catalyzed by thymidylate synthetase and dCMP hydroxymethylase has led to more definitive statements of the mechanisms of the reactions.

The simple procedure described here has been in general use in this laboratory for several years. It has been possible to measure the activities of crude extracts of a number of slightly leaky thymine-dependent mutants of E. coli and of a series of partial revertants of a thymine-requiring organism. Crude extracts of such organisms may have activities of 0.002 unit per mg of pro-
tein and less, about 500-fold lower than that of the wild type organism. Assay of such extracts by either the spectrophotometric method or the radioisotope chromatography procedures is precluded because of the limitation of sensitivity or because of the high blank rates in the absence of dUMP.

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