EFFECTS OF THE DIASTEREOISOMERS OF METHYLENETETRAHYDROFOLATE ON THE REACTION CATALYZED BY THYMIDYLATE SYNTHETASE*

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SUMMARY: Diastereoisomers of methylenetetrahydrofolate were prepared and shown to have equal and opposite CD spectra. In addition to being inactive in the spectrophotometric assay for Lactobacillus casei thymidylate synthetase, the diastereoisomer having the unnatural configuration at carbon 6, does not promote removal of tritium from 5-(^{3}H)-dUMP. It is a competitive inhibitor of the reaction with a K_I of 5×10^{-5}M.

Thymidylate synthetase (methylenetetrahydrofolate: dUMP C-methyl transferase E.C.2.1.1.b) catalyzes the following reaction: deoxyuridylate + N^5, N^{10}-methylenetetrahydrofolate → thymidylate + dihydrofolate. The reaction is of a special interest because of the unique role of tetrahydrofolate which serves both as the carrier of the single carbon unit and as a reductant and because the enzyme has been considered as a possible target in cancer chemotherapy (1).

Tetrahydrofolate and its derivatives contain a center of asymmetry at C^6 in addition to the α-carbon of the L-glutamate residue. (Fig. 1) The present report concerns the effects of diastereoisomers of tetrahydrofolate on the reaction catalyzed by thymidylate synthetase.

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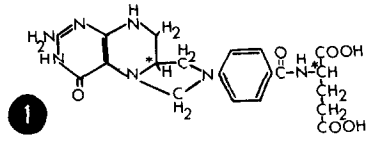
Abbreviations: l-CH_2-THF = natural diastereoisomer of tetrahydrofolate
d-CH_2-THF = unnatural diastereoisomer of tetrahydrofolate



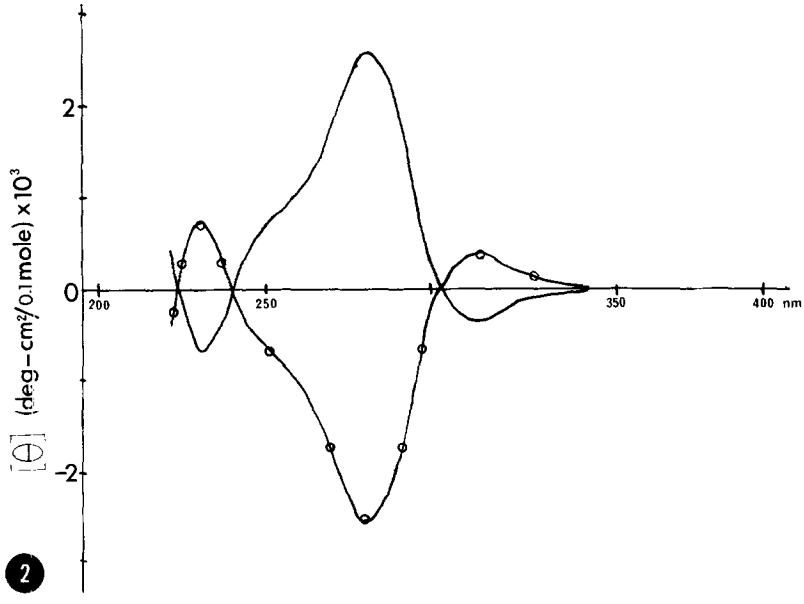
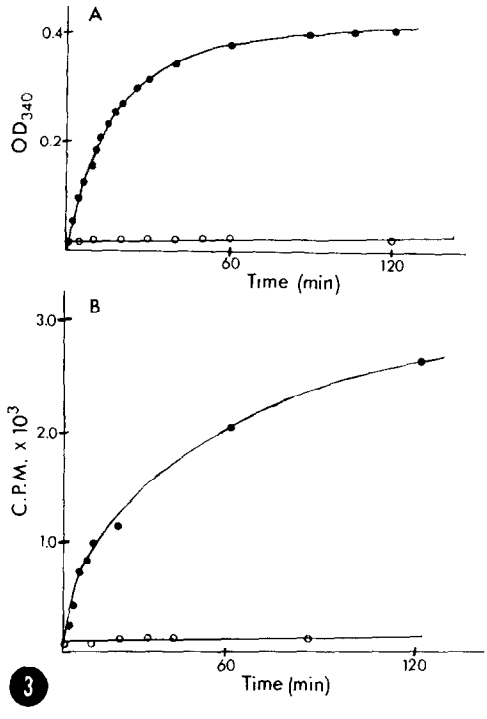


Figure 1 5, 10-Methylenetetrahydrofolate. C^* = asymmetric center at C^6 in the pteridine and the α carbon of the L-glutamate residue.

Figure 2 Circular dichroic spectra of the diastereoisomers of methylene-tetrahydrofolate. The spectra were obtained with a Jasco model J-20 recording spectropolarimeter utilizing a concentration of 2.7×10^{-5} M for each diastereoisomer in a cell of one cm pathlength. l-CH_2-THF (——), d-CH_2-THF (—o—). Buffer: 0.04 M sodium carbonate-bicarbonate, 0.03 M formaldehyde (pH 9.5).

MATERIALS AND METHODS

Tetrahydrofolate was prepared by catalytic hydrogenation of folic acid in neutral aequeous solution (2). 5,10-methylenetetrahydrofolate was prepared by the method of Osborn et al. (3), and the resolution of the diastereoisomers achieved utilizing the procedure developed by Kaufman et al. (4). Recrystallized homogeneous thymidylate synthetase was prepared from methotrexate resistant Lactobacillus casei as described (5). 5-(^{3}H)-dUMP was purchased from New England Nuclear, Boston, Massachusetts. Enzyme assays were carried out using either the spectrophotometric assay of Wahba and Friedkin (6) or the radioactive assay of Lomax and Greenberg (7).



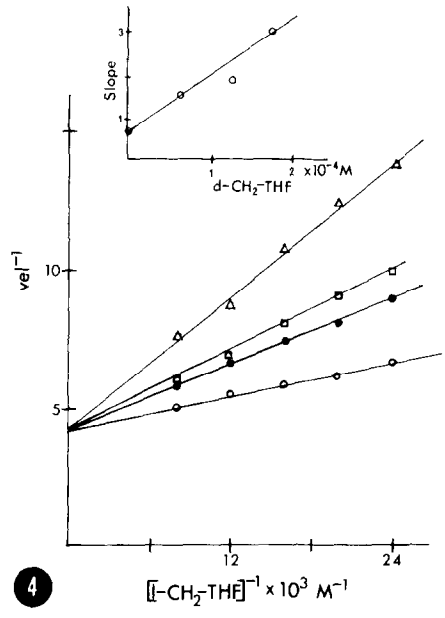


Figure 3 Comparison of the ability of the diastereoisomers of 5,10-methylenetetrahydrofolate to serve as substrates in the reaction catalyzed by thymidylate synthetase. A Spectrophotometric assay; B Radioactive assay. l-CH_2-THF (●), d-CH_2-THF (o). Additional components of the reaction mixture were: 2-mercaptoethanol 0.11 M, formaldehyde 0.012 M, $MgCl_2$ 0.021 M and dUMP 4 x 10^{-5}M.

Figure 4 Effect of d-CH_2-THF on the reaction catalyzed by thymidylate synthetase. Concentrations of d-CH_2-THF: zero (o), 6.3 x 10^{-5}M (●), 1.21 x 10^{-4} M (□), 1.78 x 10^{-4} M (△). dUMP concentration 1.3 x 10^{-5}M.

Insert: secondary plot of slopes versus concentration of d-CH_2-THF.

RESULTS AND DISCUSSION

The circular dichroic spectra of the diastereoisomers of methylenetetrahydrofolate are shown in Figure 2. l-CH_2-THF exhibits a large positive peak centered at 283 nm, [0] = 2.59 x 10^3 deg-cm^2/decimole, and two negative peaks of lower ellipticity at 230 and 312 nm. d-CH_2-THF exhibits peaks at these same wavelengths of equal ellipticity with opposite signs.

The individual diastereoisomers were tested for their ability to serve as substrates

in the reaction catalyzed by thymidylate synthetase using both the spectrophotometric and radioactive assays at 30°. Each diastereoisomer was tested at 1.5×10^{-4} M. A minus enzyme control was used as a blank and readings at 340 nm taken at the indicated times. The radioactive assays were carried out under the same conditions except that the incubation volume and amount of enzyme was one-half that of the spectrophotometric assay. Aliquots were withdrawn at the indicated times and the amount of tritium released into water determined (7). Zero time controls were withdrawn before the addition of enzyme. As shown in Figure 3 d-CH_2-THF shows no activity when assayed by either the spectrophotometric or radioactive assay procedures.

A stereospecific requirement for l-CH_2-THF has also been shown for the thymidylate synthetases of Streptococcus faecalis (8) and Escherichia coli (9). Further studies with the former system (10) compared the K_m obtained for l-CH_2-THF with that for dl-CH_2-THF. The K_m for the dl mixture was 10% greater than twice that found for l-CH_2-THF. This difference was not considered large enough to cause significant errors in a kinetic study.

We observed with pure L. casei thymidylate synthetase that the rate of the reaction with dl-CH_2-THF at 3×10^{-4}M was 75% that obtained with 1.5×10^{-4}M l-CH_2-THF (11). The same difference in rates was observed whether the l-CH_2-THF was prepared by resolution of dl-CH_2-THF (4) or by an enzymatic procedure employing dihydrofolate, NADPH and L. casei dihydrofolate reductase (12). The lower rate obtained with dl-CH_2-THF appeared not to be due to an impurity since chromatography of dl-THF on DEAE-cellulose (12) yielded material giving the same rate as untreated material.

This inhibition was further investigated by varying the concentration of the l-CH_2-THF at fixed levels of d-CH_2-THF. Figure 4 shows a reciprocal plot of the data obtained in this experiment. The d-CH_2-THF is a competitive inhibitor of the reaction with respect of l-CH_2-THF. The K_i value, calculated from a secondary plot of slope versus concentration is 5.0×10^{-5}M. The K_m for l-CH_2-THF under the same conditions is 1.5×10^{-5}M. This value was calculated from a plot of intercepts versus l-CH_2-THF

at various dUMP concentrations. The details will be published elsewhere.

When a competitive inhibitor is present in a sample of substrate a reciprocal plot lies above and is parallel to a reciprocal plot for substrate not containing the inhibitor (13, 14). That this is true for dl-CH_2-THF and l-CH_2-THF was shown in separate experiments with L. casei thymidylate synthetase.

Thus d-CH_2-THF is not an inert ingredient of the standard assay mixture of thymidylate synthetase (6). d-CH_2-THF has also been reported to be an inhibitor of the reaction catalyzed by E. coli CH_2-THF dehydrogenase (15).

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