STRUCTURAL REQUIREMENTS OF THE TETRAHYDROPTERIDINE COFACTOR FOR THE SERINE ALDOLASE AND THE THYMIDYLATE SYNTHETASE REACTIONS

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It has long been known that folate.H_4* can function as a coenzyme for serine aldolase, which catalyzes the reaction between glycine and formaldehyde to produce serine, and for thymidylate synthetase, which catalyzes the reaction between deoxyuridylic acid and formaldehyde to produce thymidylic acid. Folate.H_4 is not the form of the tetrahydropteridine coenzyme that occurs in nature; this has recently been indicated to be tetrahydropteridyl-p-aminobenzoyltriglutamate (Rabinowitz and Himes, 1960).

The exact structural elements required for coenzyme activity of the pteridine derivative in the serine aldolase and thymidylate synthetase reactions are not known. In the phenylalanine hydroxylase reaction DMP.H_4, which lacks the p-aminobenzoyl-L-glutamate side chain and thus lacks N^{10}, has been observed to function as a cofactor (Kaufman, 1959; Kaufman and Levenberg, 1959).

There is mounting evidence that "active formaldehyde" is the bridge compound N^5,N^{10}-methylene-folate.H_4 (see Huennekens and Osborne, 1959; Osborne et al., 1960 for references), although the possibility that either N^5- or N^{10}-hydroxymethylfolate.H_4 may function as a coenzyme, at least in some special reactions, has not been excluded.

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* Abbreviations used: Folate.H_4, tetrahydrofolic acid; folate.H_2, dihydrofolic acid; DMP.H_4, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; DMP.H_2, 2-amino-4-hydroxy-6,7-dimethyldihydropteridine.

Experiments were, therefore, undertaken with DMP.H_4 to determine whether this compound could serve as a coenzyme for serine aldolase and thymidylate synthetase. The results showed that it was ineffective, thus establishing that the p-aminobenzoyl group and the N^{10} group are essential for coenzyme activity with the above enzymes.

Experimental

Serine Aldolase. The enzyme preparation used was the second 31-50% ammonium sulfate fraction of Alexander and Greenberg (1956) which produced 0.21 μmoles of serine per mg of protein in 15 minutes under the experimental conditions. Folate.H_4 was prepared by the method of O'Dell et al. (1947) and was stored in 1 M mercaptoethanol (8 mg/ml) under nitrogen at 4°. DMP.H_4 and DMP.H_2 were gifts from the Lilly Research Laboratories and were dissolved in 0.02 M phosphate buffer, pH 7.2, immediately prior to use. The enzyme preparation was pretreated with Dowex 1-Cl^- to remove endogenous pteridines (Wright and Stadtman, 1956).

Each reaction flask contained, in a total volume of 3.0 ml, 0.02 M phosphate buffer ($NaH_2PO_4H_2O$ and $K_2HPO_4 \cdot 3H_2O$), pH 7.2, 100 μmoles of glycine, 20 μmoles of formaldehyde, pyridoxal phosphate (final concentration 5×10^{-5} M), mercaptoethanol (final concentration 1.5×10^{-2} M), 17.5 mg protein, and where indicated, 400 μg of folate.H_4, 200 μg of DMP.H_4 or 200 μg DMP.H_2. The

TABLE I

Inactivity of Dimethylpteridines as Cofactors for Serine Aldolase

Cofactor	Concentration $\times 10^4$ M	Serine Formed[a] (μmoles)
Folate.H_4	3.0	7.4
DMP.H_4	3.5	0[b]
DMP.H_2	3.5	0[b]

[a] Average of two determinations.

[b] Experimental values actually slightly lower than blank values.

enzyme was preincubated with pyridoxal phosphate for 15 minutes at 37°. The reaction flasks were incubated under nitrogen at 37° for 30 minutes and the reaction stopped by the addition of 1.0 ml of 20% trichloroacetic acid to each flask. For blank determinations, the pteridines were added immediately after the addition of trichloroacetic acid. After centrifuging, the supernates were assayed for serine using the method of Alexander and Greenberg (1956). The results are given in Table I.

Thymidylate synthetase. The enzyme was a purified preparation from beef thymus with a specific activity of about 260 (Greenberg et al., 1961). In the experiment reported in Table II, the incubation medium contained 2 μmoles uracildeoxyribose 5'-phosphate, 4.5 μmoles of formaldehyde, 4.5 μmoles of folate.H_4, 3.75 mg of enzyme, 0.05 M veronal buffer, pH 7.6, and varying amounts of DMP.H_4, in a total volume of 1.8 ml. Incubation was for 1 hour at 37° in a Dubnoff shaking metabolator. The thymidylic acid formed was determined by the method of Roberts and Friedkin (1958).

The results obtained in this and other experiments established that DMP.H_4 had no coenzyme activity for thymidylate synthesis and that, on the contrary, it competitively inhibited the effect of folate.H_4 (Table II).

TABLE II

Inhibition of Thymidylate Synthesis by Dimethyltetrahydropteridine

DMP.H_4 concentration, μmoles per 1.8 ml	Thymidylic Acid formed per hour mμmoles	% Inhibition
0	213	
5.0	167	21.5
10.0	117	45

A series of experiments with varied concentrations of folate.H_4 and DMP.H_4 was performed to estimate the Michaelis constants of these compounds.

Values obtained were $K_m = 10^{-6}$ M for folate.H_4 and $K_i = 1.5 \times 10^{-5}$ M for DMP.H_4.

Discussion

The inability of DMP.H_2 to substitute for folate.H_4 was expected since folate.H_2 also has no coenzyme activity with serine aldolase (Blakley, 1959). The inactivity of DMP.H_4 seems to indicate that the p-aminobenzoyl-L-glutamic acid side chain is necessary for activity either because of enzyme specificity for the side chain or because the N^{10} nitrogen is needed to form the methylene bridge pteridine-formaldehyde compound. The inability of formaldehyde to bind with DMP.H_4 as an explanation for its inactivity can very probably be ruled out since Blakley (1959) has shown that formaldehyde binds extensively with 4-hydroxy-6-methyltetrahydropteridine and 2-amino-4-hydroxy-6-methyltetrahydropteridine, both of which are very similar to DMP.H_4.

The results reported here support the hypothesis that N^5,N^{10}-methylene-folate.H_4 is exclusively the active formaldehyde in the serine aldolase and thymidylate synthetase reactions.

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