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THYMIDYLATE SYNTHETASE FROM *DIPLOCOCCUS PNEUMONIAE* PROPERTIES AND INHIBITION BY FOLATE ANALOGS*

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Summary

Thymidilate synthetase (methylenetetrahydrofolate:dUMP *C*-methyltransferase) in crude extract from *Diplococcus pneumoniae* exhibits a partial but variable requirement for Mg^{2+} depending upon the buffer. Optimum Mg^{2+} concentration is between 0.014 and 0.02 M. The optimum pH for activity in a variety of buffers occurred as a broad peak between 7.0 and 7.7. In Tris/acetate buffer, but not in potassium phosphate buffer, the pH optimum was different in the presence and absence of Mg^{2+}. Methylation of uridylate, cytidylate and deoxycytidylate could not be demonstrated over a pH range of 5.0—8.0. The enzyme exhibited an apparent K_m for deoxyuridylate of $3.08 \cdot 10^{-5}$ M and an apparent K_m for L-(+)(—)-5,10-methylene tetrahydrofolate of $2.66 \cdot 10^{-4}$ M. During molecular-sieve chromatography and sucrose density-gradient centrifugation, the enzyme was detectable only as a single catalytically active form of M_r 34 000—38 000. 2,4-Diamino quinazoline antifolates were better competitive inhibitors ($K_i = 3—8 \cdot 10^{-6}$ M) of thymidylate synthetase than 2,4-diamino pteridines ($K_i = 3 \cdot 10^{-5}$ M). 2-Amino-4-hydroxy-quinazolines were the best inhibitors ($K_i = 1.3—2.9 \cdot 10^{-6}$ M). All of the 2,4-diamino quinazolines and pteridines inhibited dihydrofolate reductase from *D. pneumoniae* in a nearly stoichiometric fashion ($K_i = <10^{-10}$ M). The 2-amino-4-hydroxy-quinazolines were poor inhibitors of this enzyme ($K_i = 10^{-5}$ M).

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Abbreviations: dUMP, deoxyuridylate; dTMP, thymidylate; folate-H_2, dihydrofolate; folate-H_4, tetrahydrofolate.

Introduction

Thymidylate synthetase (methylenetetrahydrofolate:dUMP *C*-methyltransferase) catalyzes a reductive methylation of deoxyuridylate [1] by the following reaction;

5,10-methylenefolate-H_4 + deoxyuridylate $\rightleftharpoons$ folate-H_2 + thymidylate

The reaction, which may represent the rate-limiting step in DNA synthesis [2], is inhibited by 5-fluorodeoxyuridylate, the active form of the anti-tumor agent 5-fluorouracil [3,4]. On the basis of more recent evidence [5—8], the enzyme may also be a secondary target for certain folate analogs, presumably involving the binding site for 5,10-methylene folate-H_4.

Both catalytic and physical properties and purification of thymidylate synthethase from a variety of sources have been reported [9—18]. Other studies [4,19—22] have been concerned with the mechanism of the reaction and inhibition by substituted pyrimidine analogs. As part of our own studies [23—27, 42,43] on folate metabolism in *Diplococcus pneumoniae*, we have made a preliminary examination of thymidylate synthetase activity in crude cell-free extracts. The results reported here relate to some of the general catalytic and physical properties of thymidylate synthetase in this organism and includes an analysis of inhibition by a group of folate analogs.

Experimental procedure

Bacterial Strains

The strain of *D. pneumoniae* examined was derived from the wild-type R6 strain obtained from Dr R.D. Hotchkiss, Rockefeller University. Cultural conditions, methods of genetic analysis and the preparation of cell-free extracts have been described [23—29]. *Escherichia coli* B cell preparations were purchased from Calbiochem Inc.

Assay for thymidylate synthetase activity

A spectrophotometric assay similar to that of Wahba and Friedkin [10,11] was used at 28° C to determine the activity in crude cell-free extracts. Usually 1—2 mg of protein (0.2 ml) was employed in a final volume of 2.4 ml in a 3 ml (1 cm) cuvette. Potassium phosphate at a concentration of 0.05 M (pH 7.4) was routinely used as buffer instead of Tris/HCl. The cofactor, L-(+)-(—)-5,10-methylene tetrahydrofolate, was generated in the reaction mixture with formaldehyde from L-(+)(—)-tetrahydrofolate [10,12]. The concentration of each component in the reaction mixture was 0.02 M 2-mercaptoethanol, 3.0 mM L-(+)(—)-tetrahydrofolate, 0.012 M formaldehyde, 0.02 M $MgCl_2$ and 0.4 mM deoxyuridylate. The reaction rate was constant over a period of 30 min and proportional to the concentration of enzyme within the range of 0.2—3 mg protein/ml. The rate of change in absorbance in the absence of enzyme or in the presence of enzyme when one or another component was omitted was essentially nil. Rates were unchanged when enzyme extract was first dialyzed. One unit of activity represents a change A_{340nm} after 10 min of 0.01 (4 nmol

of folate-H_2 or thymidylate formed, ref. 10). Absorbance measurements were made by simultaneous correction for control values with a Beckman model DB spectrophotometer. When analyzing for substrate activity of nucleotides other than dUMP, two 1 cm cells in a parallel arrangement or a 3 cm cell was used with an incubation period of 20 or 30 min.

Assay for dihydrofolate reductase activity

This enzymatic activity was determined in a manner [28] like that of Osborn and Huennekens [30]. One unit of activity is equal to a change in A_{340nm} of 0.01 (2.6 nmol of folate-H_2 reduced) per min at 25° C. Absorbance measurements were made in a Beckman model DB spectrophotometer.

Solutions and chemicals

The concentration of each buffer was 0.05 M and was supplemented with 2-mercaptoethanol (0.02 or 0.1 M). NADPH was purchased from Calbiochem. Inc. Deoxyuridylate and L-(+)(−)-tetrahydrofolate was purchased from Sigma Chemical. The latter compound was prepared at a concentration of 5 mg/ml in a solution of 1 M 2-mercaptoethanol at pH 7.4 and stored at −20° C usually for only 1 week. Each sample was analyzed spectrophotometrically [10] before use. Dihydrofolate was prepared by the procedure of Blakley [12]. All other chemicals were reagent or analytical grade.

Molecular-sieve chromatography

The details of the procedure used have already been described [31]. A column of packed gel (Sephadex G100, Pharmacia) 1 × 46 cm in dimension was run with a hydrostatic head of 40—45 cm at 4° C or room temperature. The flow rate was maintained at approximately 2 ml per h. One ml fractions (50 enzyme units) were eluted with 0.05 M potassum phosphate buffer (pH 7.4) and 0.01 M or 0.1 M 2-mercaptoethanol. Elution of fractions was also carried out with the reaction mixture for the thymidylate synthetase assay. The column was calibrated with blue dextran 2000 (Pharmacia) and a series of marker proteins [31]. The dihydrofolate reductase and thymidylate synthetase content of each fraction was measured enzymatically. Marker proteins in each fraction were measured separately after elution on the same column by a determination of A_{280nm}.

Sedimentation analysis

Schwarz-Mann ultra-pure sucrose was employed in 5—20% preformed linear gradients for the determination of molecular weight by sucrose density-gradient centrifugation [32]. The SW 27.1 head containing six 18-ml gradients (17 ml of sucrose and 1 ml of sample) was spun at 94 000 × g in the Spinco L2-65B for 40 h. Fractions of 0.6 ml were collected at 4° C using the Buchler auto-densiflow apparatus connected to a Gilson fraction collector. Fractions collected were assayed for dihydrofolate reductase and thymidylate synthetase activities. Marker proteins, run at the same time as the sample in separate gradients, were examined spectrophotometrically at 280 nm in each fraction.

Protein determination

Measurements of protein concentration were made by the method of Lowry et al. [33] using bovine serum albumin as a standard.

Results

General properties

Most crude thymidylate synthetase preparations from *D. pneumoniae* showed a specific activity with dUMP of 2 nmol of product formed/min/mg protein at pH 7.4 and 28° C. Activity exhibited a partial requirement for Mg^{2+}. In Tris/acetate or Tris/HCl buffer, the rate of reaction was almost 60% lower in the absence of 0.02 M $MgCl_2$. Methylation of uridylate, cytidylate and deoxycytidylate could not be demonstrated in this system over a wide range of pH (5.0—8.0). Under the special assay conditions employed (see Experimental procedure) a reaction rate of at least 1—2% of that obtained with dUMP would have been detected. The enzyme exhibited a single broad peak of activity between pH 7.0 and 7.7 in a variety of buffers (Fig. 1). However, some difference in the overall level of activity obtained in each buffer could be demonstrated. The highest level was observed in potassium phosphate buffer, with levels somewhat less in maleate, Tris/HCl and Tris/acetate buffers. Activity in Tris/acetate buffer was approximately 20% less than that seen in potassium phosphate buffer.

The effect of varying concentrations of $MgCl_2$ on thymidylate synthetase activity is shown in Fig. 2. The crude enzyme preparations used during these

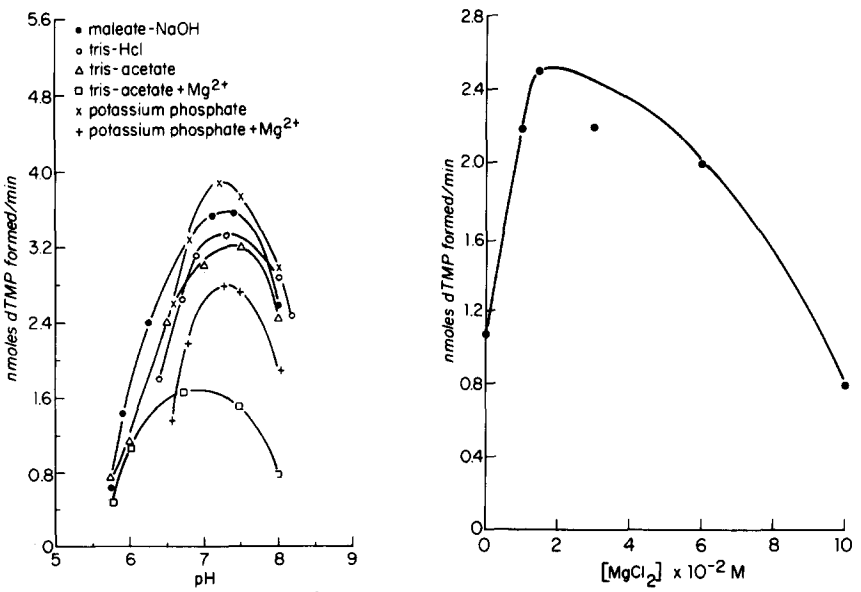

Fig. 1. The effect of pH and Mg^{2+} on the activity of thymidylate synthetase. The buffer concentrations were 0.05 M. The pH was determined for the entire mixture at the start of the reaction. Average of 3 experiments at 28° C.

Fig. 2. The effect of varying concentrations of $MgCl_2$ on the activity of thymidylate synthetase. The buffer used was 0.05 M Tris/acetate. The cell-free extract was dialyzed against 3 changes of buffer before use. Average of 2 experiments at 28° C.

experiments were first exhaustively dialyzed (3 changes of buffer). In Tris/acetate buffer, maximum activation (greater than 2-fold) occurred at a concentration of 0.14 M $MgCl_2$. Further increase in concentration resulted in a diminution of activity. The activation of thymidylate synthetase can be associated with the Mg^{2+} rather than Cl^-, since it has already been shown (Fig. 1) that the activity of this enzyme in Tris/HCl buffer is essentially the same as that observed in Tris/acetate. The extent of the requirement for Mg^{2+}, however, does appear to have some dependence on the buffer used. For instance (Fig. 1), a much smaller effect of 0.02 M $MgCl_2$ was observed with potassium phosphate buffer than that observed in Tris/acetate. Although some of the Mg^{2+} may exist as $MgPO_4$ and remain unavailable to the enzyme, this apparently is not the reason for lack of stimulation at 0.02 M since adding additional Mg^{2+} still did not result in stimulation. The overall level of activity in phosphate buffer in the absence of Mg^{2+} is approximately the same as that observed in Tris/acetate plus Mg^{2+}. Moreover, there is a difference in the pH optimum for activity in Tris/acetate depending on whether or not Mg^{2+} is present.

The apparent K_m for dUMP and L-(+)(—)-5,10-methylenefolate-H_4 were derived from the double reciprocal plots shown in Fig. 3. The apparent K_m calculated for dUMP, derived within a concentration range of $0.5—5.0 \cdot 10^{-5}$ M, was $3.08 \cdot 10^{-5}$ M. The apparent K_m for L-(+)(—)-5,10-methylenefolate-H_4, using a concentration range of $0.5—5.0 \cdot 10^{-4}$ M, was $2.66 \cdot 10^{-4}$ M. The value for V obtained in each case was essentially the same, 0.371 (with varying concentrations of dUMP) and 0.389 nmol/min/mg protein (with varying concentrations of L-(+)(—)-5,10-methylene folate-H_4).

Molecular weight

An estimation of molecular weight of thymidylate synthetase in cell-free extracts from *D. pneumoniae* was obtained by molecular-sieve chromatography and sucrose density-gradient centrifugation. In both procedures, *D. pneu-*

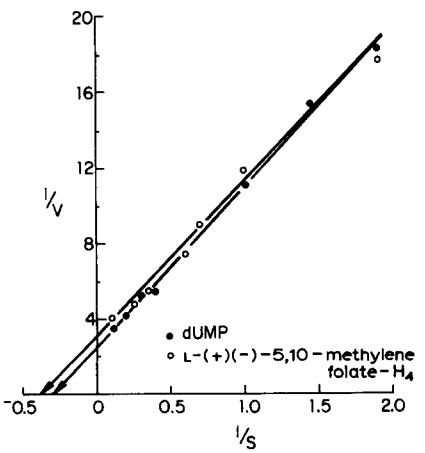

Fig. 3. Double-reciprocal plot of initial reaction velocity and concentration of dUMP and L-(+)(—)-5,10-methylene folate-H_4. v = nmol folate-H_2 formed/min. $S = 10^{-5}$ M deoxyuridylate (dUMP) when L-(+)(—)-5,10-methylene folate-H_4 was present in excess ($3 \cdot 10^{-3}$ M) and 10^{-4} M (L-(+)(—)-5,10-methylene folate-H_2) when dUMP was present in excess ($4 \cdot 10^{-4}$ M). Average of 4 replicate experiments.

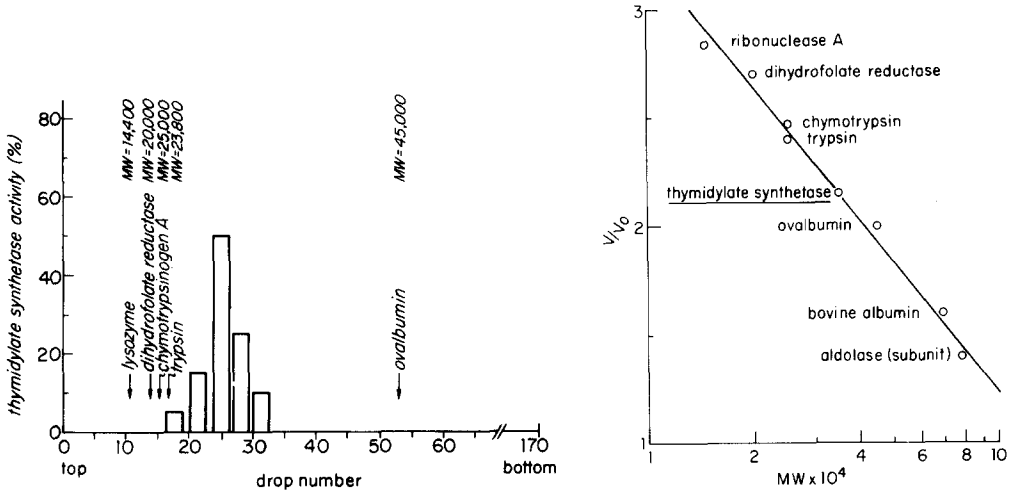

Fig. 4. The relative sedimentation of thymidylate synthetase in a sucrose gradient during ultracentrifugation. The M_r values for the standard proteins are given in the figure. Experimental details are presented in the text. Average of three separate runs.

Fig. 5. The determination of the molecular weight for thymidylate synthetase by molecular-sieve chromatography on Sephadex G100. Methods are outlined in the text. Average of 6 replicate experiments at 4° C.

moniae dihydrofolate reductase, which was also enzymatically detectable in crude extracts, was utilized as an internal standard. The elution on G100 and the sedimentation behavior of dihydrofolate reductase (M_r = 20 000, refs 31,-34) allows a convenient comparison between thymidylate synthetase and a known protein within the same sample. This is particularly useful during the centrifugation analysis, since the sedimentation rate in sucrose may be influenced by the interaction among proteins in a concentrated sample. The relative sedimentation rates in a linear sucrose gradient for thymidylate synthetase and several marker proteins are given in Fig. 4. Approximately 50 enzyme units (1 ml of extract) were added to the gradient. All of the enzymatically detectable enzyme sedimented as a single peak (recovery = 95% of original) and a value of 37 680 was derived for the approximate molecular weight. This value was obtained by the method of Martin and Ames [32] using known molecular weight values and relative sedimentation rates for the marker proteins (Fig. 4). The elution characteristics of thymidylate synthetase and a group of marker proteins on Sephadex G100 are compared in Fig. 5. Fifty enzyme units (1 ml of extract) were added to the column. All of the thymidylate synthetase, which could be enzymatically detected (recovery = 85%), eluted as a single peak with an estimated M_r between 34 000 and 35 000.

The value for the molecular weight of thymidylate synthetase determined by both procedures was in good agreement. However, the average value derived (approx. 36 000) is about half of the value reported [15—17,36] for active forms of other microbial thymidylate synthetase enzymes. One possible explanation for the apparent lower value in the present study is that the enzyme exists in a dissociated form during both analytical procedures, with reassocia-

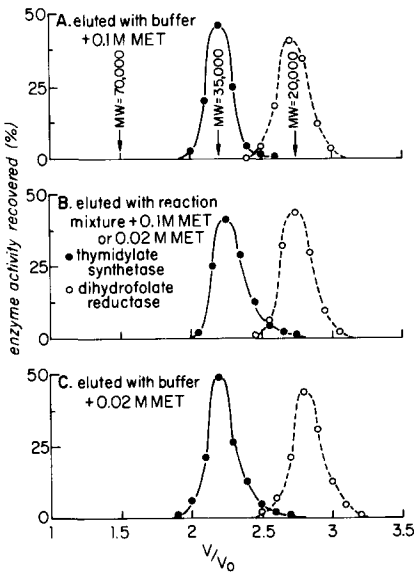

Fig. 6. Molecular-sieve chromatography of thymidylate synthethase with different eluting solutions. Elution of enzyme was carried out with 0.05 M potassium phosphate buffer (pH 7.4) with 0.1 M 2-mercaptoethanol (MET) (A) or 0.02 M 2-mercaptoethanol (C), or reaction mixture with either 0.02 M or 0.1 M 2-mercaptoethanol (B). The composition of the reaction mixture for measuring thymidylate synthetase activity is given in the text. Average of 2 or 3 experiments at 4° C.

tion occurring under conditions of the assay for activity. Consequently, we compared the elution properties of thymidylate synthetase activity on G100 at 4° C with both the original buffer (0.05 M potassium phosphate) and with the reaction mixture. Both solutions were supplemented with 0.1 M 2-mercaptoethanol. The results are shown in Fig. 6, where it can be seen that the elution characteristics of this enzyme in each case was the same. The elution of thymidylate synthetase was also compared with 0.02 M 2-mercaptoethanol in both buffer and reaction mixture (Fig. 6), since this is the concentration normally used in the reaction mixture. Again, no difference was observed. These chromatographic runs were also repeated at room temperature with the same result.

In a control experiment, a cell-free extract from *E. coli* strain B was also chromatographed on the same G100 column. In this case, the enzyme activity was eluted (0.05 M potassium phosphate plus 0.01 M 2-mercaptoethanol) as two peaks (total recovery = 89%). Approximately 25% of the total activity recovered was eluted at a volume (v/v_0 = 1.4) equivalent to M_r 70 000. The remaining 75% eluted as M_r 35 000 (v/v_0 2.1). The same elution pattern was obtained when enzyme was eluted with reaction mixture (as above). Moreover, when portions from each peak were rechromatographed and eluted with reaction mixture, each fraction eluted as a single peak with the same elution volume as the original fraction.

Inhibition studies

The inhibition of thymidylate synthetase and dihydrofolate reductase in crude extracts of *D. pneumoniae* by a series of pteridine and quinazoline analogs is compared in Table I. The 2,4-diamino pteridines (aminopterin and

TABLE I

INHIBITION OF DIHYDROFOLATE REDUCTASE AND THYMIDYLATE SYNTHETASE FROM *D. PNEUMONIAE* BY FOLATE ANALOGS

Assays for dihydrofolate reductase and thymidylate synthetase are discussed in the materials and methods. Samples were preincubated for 4 min with drug prior to making measurements. Values for K_i were derived from data obtained from double-reciprocal plots in 3 replicate experiments.

Compound	Basic ring structure	substituents				$K_i \cdot 10^{-6}$ M	
		4	5	10	17	Thymidylate synthetase	Dihydrofolate reductase
Aminopterin	pteridinyl	NH_2	—	—	glutamyl	34.7	<0.0001
Amethopterin	pteridinyl	NH_2	—	CH_3	glutamyl	28.4	<0.0001
Deaza-aminopterin	quinazolinyl*	NH_2	—	—	glutamyl	7.5	<0.0001
Quinaspar	quinazolinyl	NH_2	—	—	aspartyl	4.4	<0.0001
Methasquin	quinazolinyl	NH_2	CH_3	—	aspartyl	2.9	<0.0001
SK 28, 758	quinazolinyl	OH	—	CH_3	aspartyl	1.5	>0.0020**
SK 28, 757	quinazolinyl	OH	—	—	aspartyl	1.3	10.0
SK 24, 815	quinazolinyl	OH	—	—	glutamyl	2.9	>0.0020**

* Quinazolinyl = 5,8-deaza-pteridinyl.

** The K_i for these compounds could not be determined with dihydrofolate reductase because of contamination with 2,4-dimino derivatives which amounted to 5—10% as determined by enzyme titration [35] and bio-autographic analysis (Mehta, B.M., personal communication).

amethopterin) and quinazolines (deaza-aminopterin, quinaspar and methasquin) examined were all potent inhibitors of dihydrofolate reductase. The K_i for inhibition by these analogs ($<10^{-10}$ M) can only be estimated since the enzyme was actually titrated in a stoichiometric manner [35]. Inhibition with glutamyl and aspartyl derivatives of the quinazoline class was similar. The 2-amino-4-hydroxy quinazolines examined were relatively poor inhibitors of dihydrofolate reductase, although a K_i value could be obtained for only SK 28 757 ($K_i = 10^{-5}$ M) since both 28 758 and 24 815 were contaminated with 2,4-diamino quinazolines. Inhibition of thymidylate synthetase by the 2,4-diamino pteridines and quinazolines was considerably less than that seen with dihydrofolate reductase. Although the data were not given, the kinetics of inhibition obtained in a Lineweaver-Burk double-reciprocal plot were linear competitive with L-(+)(—)-5,10-methylene folate-H_4. Inhibition by the pteridines (aminopterin and amethopterin) was the least effective. Values for apparent K_i (calculated from the equation, for competitive inhibition, K_i = [drug]/-(K_p/K_m) — 1) were in excess of 10^{-5} M. The presence of a methyl group at N^{10} (amethopterin) had no significant effect. Values for the quinazolines were 5- to 10-fold lower. The aspartyl forms were more effective than the glutamyl forms and substitution of a methyl group at position 5 appeared to enhance inhibition. In contrast to that observed with dihydrofolate reductase, the 2-amino-4-hydroxy quinazolines as a group were the best inhibitors of thymidylate synthetase. The apparent K_i for inhibition for both aspartyl forms (SK 28, 757, SK 28, 758) approached 10^{-6} M. The presence of a methyl group at N^{10} appeared to have no effect, but the replacement of the aspartyl moiety by glutamyl (SK 24, 815) seemed to diminish inhibition. Values for apparent K_i of thymidylate synthetase inhibition for the two impure 2-amino-4-hydroxy derivatives (SK 24, 815, SK 28, 758) are reasonable estimates since the impurities are related 2,4-diamino derivatives (see Table I), which have already been associated with somewhat lower levels of inhibition.

Discussion

The requirements for thymidylate synthetase activity in *D. pneumoniae* resemble those reported [10—12,15—18] for the same enzyme from other bacteria. Like enzymes from *E. coli* [10,11] and *Lactobacillus casei* [15,17], the magnitude of the requirements for Mg^{2+} by the *D. pneumoniae* enzyme depends upon the buffer used in the reaction mixture. In the case of the *D. pneumoniae* enzyme, however, the partial requirement for Mg^{2+} in Tris/acetate buffer seems related to a shift in pH optimum to the alkaline side when the ion is present. A slightly alkaline pH optimum for *D. pneumoniae* thymidylate synthetase in the presence of Mg^{2+} has also been reported [10,12] for enzymes from both *E. coli* and *Streptococcus faecium* var. *durans*.

The apparent Michaelis constant (K_m) for dUMP ($3.08 \cdot 10^{-5}$ M) and L-(+)(—)-5,10-methylenefolate-H_4 ($2.66 \cdot 10^{-4}$ M) calculated for thymidylate synthetase are greater than those reported [10—12, 15—18] for other microbial enzymes. Values for K_m reported were generally in the range of $4 \cdot 10^{-6}$ M for dUMP and $5—30 \cdot 10^{-6}$ M for L-(+)(—)-5,10-methylenefolate-H_4. It should be

pointed out that values reported by other workers were obtained with partially purified or purified enzyme preparations. Still, it is difficult to say to what extent, if any, this difference might relate to the kinetic values obtained in the current study. Since wild-type and available mutant strains of *D. pneumoniae* are extremely poor sources of thymidylate synthetase activity, an attempt at purification was not feasible at this time.

The results of molecular-sieve chromatography and sucrose density-gradient centrifugation revealed the presence of only one catalytically active form of thymidylate synthetase with a M_r of 34—38 000. This is about half the size reported for the enzyme from *L. casei* [15,17], *E. coli* [36] and Ehrlich carcinoma [37]. Thymidylate synthetase isolated from chicken embryo extracts has an M_r of 58 000 [14]. Recent findings [16,17] showing the dissociation of thymidylate synthetase from *L. casei* into two subunits of 35 000 each suggest a possible basis for the interpretation of this difference. The *D. pneumoniae* enzyme may, in fact, exist within the cell as a dimer (M_r = ~ 70 000). However, since the conditions under which the determinations for molecular weight were made during this study (particularly the ionic content of the buffer) are essentially the same as those used by others [14,15—17,36,37], it would have to be concluded that the dimer form of the *D. pneumoniae* enzyme, if it exists, is unusually unstable under the conditions of isolation used here. In any event, it is interesting to find that the *D. pneumoniae* enzyme which exists in a low-molecular weight form (approx. 36 000) is catalytically active.

The related results obtained with *E. coli* B extracts are of interest in two respects. The elution of a substantial portion of this enzyme activity as M_r 70 000 suggests that a dimer form does have an elution volume expected for this G100 column and that enzyme eluting at a v/v_0 of 2.1—2.2 is probably a monomer. These results also suggest, however, that this latter form of the enzyme (like late eluting *D. pneumoniae* enzyme) is catalytically active. The exact significance of these findings with both enzymes in view of prior results will require further study. However, the notion of a catalytically active monomer is not, in itself, contradictory to data presented in such reports, since subunits (approx. 35 000) have been identified [16,17], but only under conditions not permitting measurement of catalytic activity.

The 2,4-diamino quinazolines appear to be as effective as the 2,4-diamino--pteridines as inhibitors of dihydrofolate reductase from *D. pneumoniae*. On the other hand, the 2-amino-4-hydroxy-quinazolines, like folate [38], were poor inhibitors of this enzyme. The results comparing pteridine and quinazoline inhibition of dihydrofolate reductase are similar to those reported from our own laboratory and elsewhere with the same enzyme from other bacteria [8,39,40] and avian [8] and mammalian [40,41] sources. The inhibition of thymidylate synthetase by a variety of other folate derivatives has also been reported [5—8]. Only a previous study [8] compares the inhibition of thymidylate synthetase by quinazoline and pteridine analogs. In this study involving thymidylate synthetase from *E. coli*, 2,4-diamino quinazoline antifolates were found to be better inhibitors by at least one order of magnitude than the corresponding 2,4-diamino pteridine. Also, 2-amino-4-hydroxy-quinazolines

were even more effective inhibitors by an additional order of magnitude. Our results would appear to confirm the general conclusion based on these findings, although some stereochemical differences exist between the analogs used in each case. The results of these studies, as did earlier studies [5,8], have interesting therapeutic implications and suggest the possibility for further exploitation of this enzymic site.

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