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Polyglutamyl Derivatives of Tetrahydrofolate as Substrates for *Lactobacillus casei* Thymidylate Synthase†

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ABSTRACT: Tetrahydropteroylpolyglutamates containing up to seven Glu residues were tested as substrates for *Lactobacillus casei* thymidylate synthase. The K_m values decreased from 24 μM for the monoglutamate to 1.8 μM for the triglutamate. Addition of residues 4, 5, 6, and 7 did not decrease the K_m further. When monoglutamate and polyglutamate substrates were simultaneously incubated with the enzyme, the rate observed was characteristic of the polyglutamate even when the monoglutamate concentration was 44 times that of the polyglutamate. Iodoacetamide treatment inhibited the enzyme to the same extent with monoglutamate and polyglutamate substrates. Addition of 0.3 M NaCl doubled the rate obtained with the polyglutamate substrate whereas the rate with the monoglutamate was inhibited 25%. $MgCl_2$ stimulated the reaction only 10% with the polyglutamate substrate compared with 80% stimulation obtained with the monoglutamate. Inhibition by fluorodeoxyuridylate was similar with both mono- and polyglutamate substrates; however, with the phosphonate derivative of fluorodeoxyuridine, the polyglutamate substrate enhanced inhibition 5- to 8-fold.

Tetrahydrofolic acid (H_4PteGlu)[1] is commonly found in tissues in the form of poly(γ-glutamyl) derivatives (Baugh & Krumdieck, 1971). However, most studies on folate requiring enzymes employ H_4PteGu$_1$ as substrate because of its ready availability. Folate enzymes generally show a higher afffinity for the polyglutamates than for H_4PteGlu$_1$ (Baggott & Krumdieck, 1979; Cheng et al., 1975; Coward et al., 1974; Curthoys & Rabinowitz, 1972; Kisliuk et al., 1974; Mackenzie & Baugh, 1980; Matthews & Baugh, 1980). In view of the importance of thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45) in thymine deoxynucleotide biosynthesis and as a target for chemotherapeutic agents (Danenberg, 1977), we undertook a detailed study of the substrate activity of H_4PteGlu derivatives containing one through seven glutamyl residues for *Lactobacillus casei* thymidylate synthase.

Experimental Procedures

Glutamyl derivatives of pteroic acid were prepared by solid-phase peptide synthesis (Krumdieck & Baugh, 1969) and their authenticity was verified as described (Kisliuk et al., 1974). In addition PteGlu$_5$ was analyzed by high-performance liquid chromatography by Dr. Michael Archer (Reed & Archer, 1976) and found to be 94% pure.

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[1] Abbreviations used: PteGlu, folic acid; H_2PteGlu, 7,8-dihydrofolic acid; H_4PteGlu, 5,6,7,8-tetrahydrolfolic acid; CH_2H_4PteGlu, the methylene counterpart; FdUMP, 5-fluoro-2′-deoxyuridylic acid; H_4PteGlu$_{x-y}$, combination of folic acids varying from x to y in degree of polymerization.

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Table I: Effect of Polyglutamate Chain Length and NaCl on the Kinetics of *L. casei* Thymidylate Synthase

no. of Glu residues	highest substrate concn used (μM[a])	K_m (μM)	V_{max} ($10^3\Delta_{OD_{340}}$/min)	$H_4PteGlu_1$ control K_m (μM)	$H_4PteGlu_1$ control V_{max} ($10^3\Delta_{OD_{340}}$/min)	relative V_{max}, $H_4PteGlu_n$/$H_4PteGlu_1$ (%)
			A. Without added NaCl			
2	57	15	24	23	24	100
3	33	1.8	9	32	18	50
4	10	2.1	12	33	36	33
5	10	1.0	11	22	32	34
6	13	1.2	9	15	23	39
7	18	1.8	9	20	22	41
				mean 24 ± 6.5 SD		
			B. With added NaCl, 0.3 M			
1	150	71[b]	18[b]			
2	150	20	24			100
3	150	18	20			111

[a] The points chosen to calculate K_m and V_{max} values were on the linear portions of Lineweaver–Burk plots. When more than 5% of the substrates were utilized, appropriate corrections were applied (Segel, 1975a). [b] Corresponds to the $H_4PteGlu_1$ control run concurrently with $H_4PteGlu_3$ in (A).

$H_4PteGlu_1$ having the natural configuration at C-6 (Fontecilla-Camps et al., 1979) was prepared by incubating 2.8 mM $H_2PteGlu_1$, 50 mM Tris, 1 mM EDTA, 10 mM glucose 6-phosphate, 16 μM NADP, 0.2 M 2-mercaptoethanol, 0.15 unit/mL glucose-6-phosphate dehydrogenase, and 0.75 unit/mL dihydrofolate reductase for 1 h at 37 °C at p H 7.4 (Mathews & Huennekens, 1960). One unit of glucose-6-phosphate dehydrogenase catalyzes the formation of 1 μmol of NADPH/min at pH 7.4 and 30 °C (Sigma Chemcial Co., type XV). One unit of dihydrofolate reductase catalyses the formation of 1 μmol of $H_4PteGlu$/h at pH 7.4 and 30 °C. The reductase was prepared from methotrexate-resistant *L. casei* grown at the New England Enzyme Center (Blair et al., 1972; Kisliuk et al., 1974).

$H_4PteGlu_{2-7}$ having the natural configuration at C-6 were prepared by incubating the corresponding PteGlu derivatives at 0.1 mM with 50 mM sodium acetate, 0.4 mM glucose 6-phosphate, 0.01 mM NADP, 0.1 M 2-mercaptoethanol, and 0.025 unit/mL glucose-6-phosphate dehydrogenase at pH 5.5 and 37 °C for 20 min, after which dihydrofolate reductase was added to a level of 6.5 units/mL followed by incubation for 90 min more. The ΔOD_{340} for the conversion of PteGlu to $H_4PteGlu$ was obtained by subtracting the final OD_{340} from the OD_{340} at the start of the 90-min incubation. On the basis of an extinction coefficient of 7200 at 340 nm, the conversion of PteGlu to $H_4PteGlu$ was 76%. At the end of the incubation the pH of the mixture was adjusted to 7.4 with KOH and the concentration of 2-mercaptoethanol brought to 0.2 M.

$H_4PteGlu_{1-7}$ were purified by chromatography on a 2 × 8 cm DEAE-cellulose column (OH^- form) eluted with a gradient generated by having 600 mL of 5 mM Tris, 0.2 M NaCl, and 0.2 M 2-mercaptoethanol, pH 7.4, in the mixing chamber and 600 mL of the same buffer with 1.0 M NaCl in the reservoir. Fractions of 5 mL were collected and their absorption was monitored at 298 nm. Fractions containing significant amounts of $H_4PteGlu$ were pooled and lyophilized. $H_4PteGlu_1$ was dissolved in 5–10 mL of 0.07 M NH_4HCO_3 and 0.2 M 2-mercaptoethanol, pH 7.4, for desalting. It was applied to a 2.4 × 58 cm Sephadex G-25 column and eluted with the solution buffer. Fractions containing $H_4PteGlu_1$ were lyophilized to remove NH_4HCO_3. Under these conditions NaCl eluted before $H_4PteGlu_1$. $H_4PteGlu_{2-7}$ were dissolved in 0.005 M NH_4HCO_3 and 0.2 M 2-mercaptoethanol, pH 7.4, and then applied to a 2.4 × 58 cm Sephadex G-10 column and eluted with the solution buffer. $H_4PteGlu_{2-7}$ eluted immediately after the void volume and were followed by NaCl (Shin et al., 1972). The products were 100% utilized in the thymidylate synthase reaction. When one started with 45 μmol of $H_4PteGlu_1$ and 25 μmol of $H_4PteGlu_{2-7}$ the overall yields were 55% and 40%, respectively.

In our earlier study (Kisliuk et al., 1974) we converted folylpolyglutamates to their corresponding dihydro derivatives by dithionite treatment before incubating them with dihydrofolate reductase. This method was satisfactory for $PteGlu_{1-3}$, but with $PteGlu_{4-6}$ the overall yield was 20% rather than 40% due to cleavage to (*p*-aminobenzoyl)Glu_{4-6} and a pteridine. In the present study we prepared all of the $H_4PteGlu$ polyglutamates by the enzymatic procedure described above which was suggested to us by Dr. E. J. Pastore. By use of this procedure a significant amount of unidentified material absorbing at 298 nm eluted from the DEAE-cellulose column before the $H_4PteGlu$ derivatives.

Thymidylate synthase was obtained from methotrexate-resistant *Lactobacillus casei* as described previously (Kisliuk et al., 1974) and was 50% pure. In addition to $H_4PteGlu$ derivatives, the standard assay system (Wahba & Friedkin, 1962) contained 12 mM CH_2O, 21 mM $MgCl_2$, 40 μM dUMP, 0.11 M 2-mercaptoethanol, 40 mM Tris, and 0.8 mM EDTA at pH 7.4.

Results

$H_4PteGlu_{1-7}$ were tested as substrates for *L. casei* thymidylate synthase. The K_m values obtained from Lineweaver–Burk plots are given in Table I. The most striking change occurs between $H_4PteGlu_2$ and $H_4PteGlu_3$, where the K_m decreased 8-fold. Addition of Glu residues 4, 5, 6, and 7 did not further decrease the K_m. V_{max} values for the polyglutamate substrates relative to the monoglutamate were obtained by performing a control with $H_4PteGlu_1$ with each polyglutamate substrate under the same conditions. The V_{max} values for $H_4PteGlu_1$ and $H_4PteGlu_2$ were the same, whereas that for $H_4PteGlu_3$ decreased by 50%. With $H_4PteGlu_4$, the V_{max} was lower still, but addition of Glu residues 5, 6, and 7 caused little additional change. $H_4PteGlu_3$ showed an unusual substrate saturation curve which leveled off at higher concentrations (Figure 1C) rather than approaching V_{max} asymptotically as occurred with $H_4PteGlu_1$ (Figure 1A). Substrate saturation curves obtained with $H_4PteGlu_{4-7}$ (data not shown) were

Table II: Effect of Simultaneous Incubation of $H_4PteGlu_1$ and $H_4PteGlu_6$ on Thymidylate Synthase Activity

expt	$H_4PteGlu_1$ (μM)	rate[a]	$H_4PteGlu_6$ (μM)	rate[a]	$H_4PteGlu_1$ plus $H_4PteGlu_6$ rate[a]	calculated rate[a,b] I	calculated rate[a,b] II
1			20	7.3			
2			30	7.2			
3	110	18.5	20		7.7	8.8	7.5
4	220	18.2	20		8.6	10.0 (9.8)[c]	7.9 (7.8)
5	440	17.2	20		7.7	12.8 (11.9)	10.0 (9.3)
6	880	15.2	20		8.5	15.7 (12.9)	13.6 (11.2)

[a] $10^3\Delta OD_{340}$/min. [b] I, calculated assuming that each substrate reacts at the same rate as when present singly. II, calculated assuming that occupation of one subunit by $H_4PteGlu_6$ induces the other subunit to react at the rate observed with $H_4PteGlu_6$ (7.3) even when it is occupied with $H_4PteGlu_1$. [c] Rates in parentheses are corrected for the inhibition at high $H_4PteGlu_1$ concentrations observed in experiments 4, 5, and 6. The percent of $H_4PteGlu_1$ (designated A) and $H_4PteGlu_6$ (designated B) present on the respective subunits was estimated by assuming that in experiment 5 AA = 25, AB = 50, BB = 25 (see Results; AB and BA are assumed to be identical). It can then be calculated that the percents expected in experiments 3, 4, and 6 are (3) AA = 3.1, AB = 21.9, BB = 75; (4) AA = 6.25, AB = 37.5, BB = 56.25; (6) AA = 56.25, AB = 37.5, BB = 6.25.

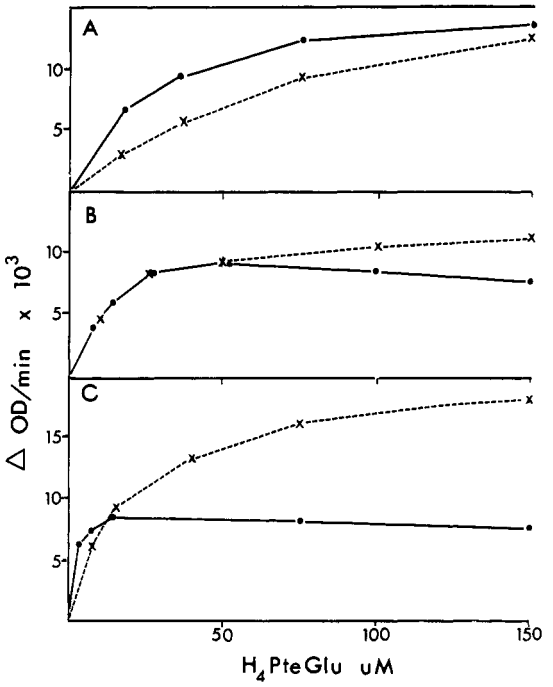

FIGURE 1: Substrate saturation curves for thymidylate synthase in the absence (●) and presence (×) of 0.3 M NaCl. (A) $H_4PteGlu_1$; (B) $H_4PteGlu_2$; (C) $H_4PteGlu_3$

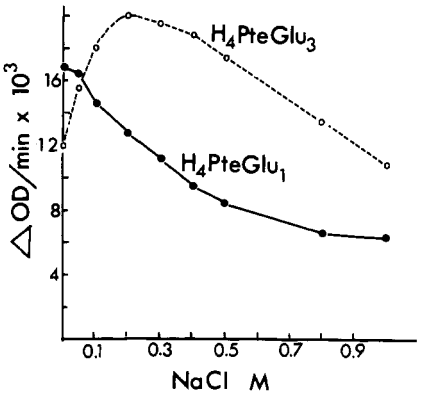

FIGURE 2: Effect of NaCl concentration on the rate of the thymidylate synthase reaction with 44 μM $H_4PteGlu_1$ (●) and 44 μM $H_4PteGlu_3$ (○) as substrates.

similar to those obtained with $H_4PteGlu_3$. With $H_4PteGlu_2$ some inhibition was observed at higher substrate concentrations (Figure 1B).

When two substrates compete for the same active site under conditions such that their $[S]/K_m$ ratios are equal and both [S] values are saturating, the expected rate is the average of the rates obtained with each substrate alone (Segel, 1975b). Since the K_m value for $H_4PteGlu_1$ is 20 times that for $H_4PteGlu_6$ (Table I), we expected that simultaneous incubation, with the former at 20 times the concentration of the latter, would result in a rate equal to the average of the rates obtained with each substrate alone; that is, each substrate would have equal access to the enzyme. However, even when $H_4PteGlu_1$ was present at 44 times the concentration of $H_4PteGlu_6$ (Table II), the rate was close to that observed with $H_4PteGlu_6$ alone, indicating that in the presence of $H_4PteGlu_6$, $H_4PteGlu_1$ has little access to the active site.

Accurate K_m values are essential to interpret the results of Table II. Our calculations were made from the straight-line portions of Lineweaver–Burk plots in which the substrate concentrations ranged from $1.4K_m$ to $10K_m$. In addition, we repeated the K_m determination for $H_4PteGlu_4$ using a radioactive assay (Roberts, 1966) with half the enzyme concentration used in Table I. The substrate range in this instance was $0.33K_m$ to $9.4K_m$. This assay yielded a K_m of 2.0 μM, in good agreement with the value reported in Table I. The K_m values were corrected when more than 5% of the substrate was utilized (Segel, 1975a). The uncorrected K_m values were $H_4PteGlu_3$ 2.2 μM, $H_4PteGlu_4$ 4.2 μM, $H_4PteGlu_5$ 2.5 μM, $H_4PteGlu_6$ 1.9 μM, and $H_4PteGlu_7$ 2.0 μM. The K_m value necessary to accommodate the observed rate of 8.5 in Table II, experiment 6, would be less than 0.6 μM.

The presence of NaCl greatly altered the kinetics of *L. casei* thymidylate synthase (Table IB). With $H_4PteGlu_1$ as substrate, addition of 0.3 M NaCl increased the K_m 3-fold but the V_{max} remained the same; with $H_4PteGlu_2$ both K_m and V_{max} were unaltered, but with $H_4PteGlu_3$ the K_m was increased 10-fold and the V_{max} doubled. The effect of NaCl on the shape of the substrate saturation curves is shown in Figure 1. With $H_4PteGlu_1$ the rate was inhibited whereas with $H_4PteGlu_2$ the rate was stimulated. Inhibition is observed at low $H_4PteGlu_3$ concentrations, but as its concentration increased, stimulation occurred (Figure 1C).

The effect of NaCl concentration on the reaction rates at saturating substrate concentration is shown in Figure 2. With $H_4PteGlu_3$ maximal stimulation occurred at 0.2 M NaCl. Higher NaCl concentrations caused inhibition. With $H_4PteGlu_1$ only inhibition was observed.

Iodoacetamide reacts with thymidylate synthase in a molar ratio of 1:1 and inactivates the enzyme 55% when assayed in the presence of mercaptoethanol (Leary et al., 1975). This result was interpreted to indicate that iodoacetamide inacti-

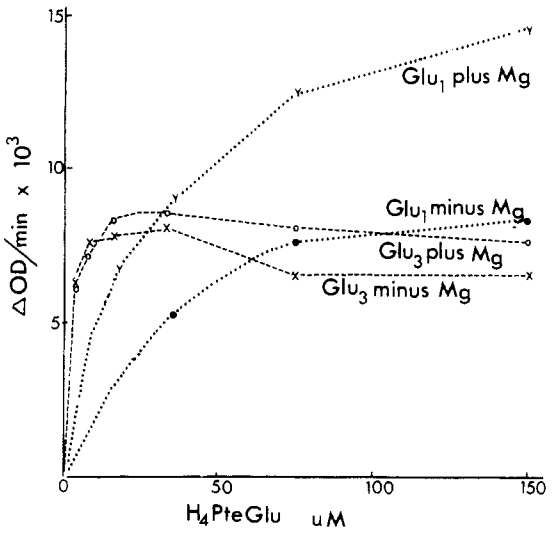

FIGURE 3: Effect of 0.02 M $MgCl_2$ on the rate of the thymidylate synthase reaction with $H_4PteGlu_1$ and $H_4PteGlu_3$ as substrates.

vates only one of the two subunits of the enzyme dimer (Dunlap et al., 1971; Maley et al., 1979a; Danenberg & Danenberg 1979). We repeated the iodoacetamide inactivation and then tested $H_4PteGlu_1$ and $H_4PteGlu_3$ as substrates at 150 μM. With both compounds 58% inactivation occurred, suggesting that the remaining active subunit reacted with both $H_4PteGlu_1$ and $H_4PteGlu_3$.

The K_m for dUMP with 20 μM $H_4PteGlu_3$ as the cosubstrate was 1.7 μM (data not shown). The K_m value reported for dUMP with $H_4PteGlu_1$ as the cosubstrate is 5.2 μM (Dunlap et al., 1971; Crusberg el al., 1970). Thus the polyglutamate substrate enhanced the affinity of the enzyme for dUMP.

$MgCl_2$ enhanced the rate of *L. casei* thymidylate synthase reaction with $H_4PteGlu_1$ as substrate (Crusberg et al., 1970). With $H_4PteGlu_3$ as substrate, stimulation by $MgCl_2$ was greatly reduced (Figure 3); at low levels of $H_4PteGlu_3$ no rate enhancement was observed whereas at high levels the stimulation was only 10%. With $H_4PteGlu_1$ the rate enhancement caused by $MgCl_2$ was 60–80% at all substrate concentrations tested.

The inhibition of *L. casei* thymidylate synthase by FdUMP increased with increased incubation time and with increased $H_4PteGlu_1$ concentration (Santi & McHenry, 1972). In the present experiments we examined the effect of polyglutamate chain length, NaCl concentration, and preincubation time on the inhibition caused by FdUMP and its phosphonate derivative in which the 5′-oxygen is replaced by a methylene group (Montgomery et al., 1979). We found that, without preincubation, $H_4PteGlu_1$ at 150 μM (6 times its K_m) was more effective than $H_4PteGlu_3$ at 20 μM (11 times its K_m) at promoting inhibition by FdUMP whereas after a 10-min preincubation inhibition was the same with both $H_4PteGlu$ derivatives (Table III). Addition of 0.2 M NaCl enhanced the inhibition caused by FdUMP with $H_4PteGlu_6$ as substrate 5-fold but did not alter the inhibition with $H_4PteGlu_1$ as substrate. With the phosphonate derivative of FdUMP, similar levels of inhibition were obtaine with $H_4PteGlu_1$ and $H_4PteGlu_3$ at zero time but with preincubation the phosphonate derivative became 5–8-fold more inhibitory with $H_4PteGlu_3$ as compared with $H_4PteGlu_1$.

Discussion

Our results illustrate the profound influence of polyglutamate chain length on the activity of *L. casei* thymidylate synthase. The largest change in K_m occurred on adding Glu_3. Addition of residues 4, 5, 6, and 7 produced little further change. The number of Glu residues actually found in folates in *L. casei* varies from four to nine depending on the level of folate in the growth medium, higher folate concentrations leading to fewer Glu residues per folate (Scott, 1976). Since addition of Glu residues 4, 5, 6, and 7 did not alter substrate activity (Table I), it appears that alterations in glutamate chain length are not important in regulating thymidylate synthase activity in vivo.

Table III: Effect of Polyglutamate Chain Length, Preincubation Time, and NaCl Concentration on the Inhibition of Thymidylate Synthase by Fluorinated Pyrimidine Deoxynucleotides[a]

inhibitor	preincubation time (min)	concn for 50% inhibition (μM) 150 μM $H_4PteGlu_1$	20 μM $H_4PteGlu_3$
	Experiment 1		
FdURP	0	0.11	1.8
	10	0.05	0.07
FdUR-phosphonate	0	1000	1500
	10	26	3
	40	4.6	1.0
	60	3.6	0.7
	Experiment 2		50 μM $H_4PteGlu_6$
FdURP	0	0.11	0.28
+ 0.2 M NaCl	0	0.10	0.06

[a] In each instance the reaction was initiated by the addition of dUMP.

We considered the possibility that the formation of dihydropolyglutamates during the course of the reaction might explain the atypical substrate saturation curve observed with polyglutamate substrates (Figure 1C). $H_2PteGlu_{3-6}$ are known to be potent inhibitors of the enzyme when $H_4PteGlu_1$ is the substrate (Kisliuk et al., 1974). However, the maximum concentration of dihydro derivatives formed under our assay conditions, which were designed to obtain initial rates, was never larger than 2.5 μM. Additional experiments[2] showed that it takes 25 μM $H_2PteGlu_6$ to inhibit the reaction 50% with 35 μM $H_4PteGlu_6$ as substrate. Therefore the formation of dihydro derivatives cannot explain the saturation curve in Figure 1C.

When $H_4PteGlu_1$ and $H_4PteGlu_6$ were incubated with thymidylate synthase simultaneously, the observed rate was close to that with $H_4PteGlu_6$ alone even when $H_4PteGlu_1$ was present in great excess (Table II). Possible explanations are the following: (1) The K_m for $H_4PteGlu_6$ may actually be lower than 1.2 μM (Table I) due to product or substrate inhibition. Product inhibition was not a factor because the initial rates were constant over the 2-min assay interval employed. Substrate inhibition was not a factor because the K_m values were calculated from substrate concentrations below those causing the curve to level off (Figure 1C). (2) The presence of $PteGlu_6$ on one subunit of the enzyme dimer may impede the rate of $H_4PteGlu_1$ turnover on the other subunit. However, even if it is assumed that occupation of one subunit with $H_4PteGlu_6$ induces the other subunit to react at the rate obtained with $H_4PteGlu_6$, although being occupied by $H_4PteGlu_1$, the calculated rates are not as low as the observed rates (Table II, column b). (3) $CH_2H_4PteGlu_6$ may play a role in the removal of dTMP and $H_2PteGlu_6$ from the enzyme.

[2] Y. Gaumont and R. L. Kisliuk, unpublished observations.

The angle at which the polyglutamate tail of $H_2PteGlu_6$ protrudes from the enzyme may allow access of $CH_2H_4PteGlu_6$, which, after initial binding via its own polyGlu tail, extrudes the reaction products. $H_4PteGlu_1$ would be ineffective in this regard, leading to poor affinity for the enzyme.

Explanation 3 is in accord with studies (Kisliuk et al., 1979) showing that inhibition of *L. casei* thymidylate synthase by $PteGlu_1$ and $PteGlu_3$ is noncompetitive when $CH_2H_4PteGlu_1$ is the substrate but competitive when $CH_2H_4PteGlu_6$ is the substrate. Similarly tetrahydrohomofolate is a noncompetitive inhibitor vs. $H_4PteGlu_1$ (Crusberg et al., 1970) but is competitive vs. $H_4PteGlu_3$.[2] These results suggest that a dUMP inhibitor–enzyme complex is formed (Lockshin & Danenberg, 1979) which is dissociated more readily in the presence of $H_4PteGlu_3$ than in the presence of $H_4PteGlu_1$.

NaCl concentration is an important variable in studies of thymidylate synthase kinetics. NaCl antagonizes the inhibition caused by polyglutamate derivatives of PteGlu (Kisliuk et al., 1974), methotrexate (Kisliuk et al., 1979), and 5-formyl-$H_4PteGlu$ (Friedkin et al., 1975). This is consistent with the NaCl-induced increase in K_m values seen with H_4Ptepolyglutamate substrates (Table I, Figure 1), indicating that NaCl lowers the affinity of the polyglutamates for the eznyme. The concomitant increase in V_{max} suggests that looser binding in the presence of NaCl enables the polyglutamate substrate and product to change places on the enzyme more readily.

NaCl at 0.2 M also stimulates *L. casei* dihydrofolate reductase (Dann et al., 1976). NaCl stimulation of dihydrofolate reductase and thymidylate synthase may reflect a common regulating mechanism since both enzymes are involved in dTMP formation (Wahba & Friedkin, 1962).

With $H_4PteGlu_1$ as substrate, $MgCl_2$ stimulates the activity of thymidylate synthases derived from *E. coli* (Wahba & Friedkin, 1962), Coliphage T_2 (Maley et al., 1979b), *S. faecium* (Blakley & McDougall, 1962), *D. pneumoniae* (McCuen & Sirotnak, 1975), and *L. casei* (Figure 3). However $MgCl_2$ is without effect on the activity of thymidylate synthases from calf thymus (Horinishi & Greenberg, 1972), chick embryo (Lorenson et al., 1967), Ehrlich ascites cells (Reyes & Heidelberger, 1965), L1210 murine leukemia cells (Livingston et al., 1968), and human leukemia cells (Lockshin et al., 1979). Mg^{2+} inhibits thymidylate synthase activity in extracts of *Aedes aegypti* (Jaffe & Chrin, 1979). The results of the present study, which show very little activation of the *L. casei* enzyme by $MgCl_2$ when $H_4PteGlu_3$ is the substrate, indicate that Mg^{2+} does not play a role in regulating thymidylate synthase activity in *L. casei* cells where polyglutamates are the substrates.

The interaction of nucleotides with the enzyme differs with polyglutamate substrates as compared with $H_4PteGlu_1$. The K_m for dUMP is lowered 3-fold in the presence of $H_4PteGlu_3$. The inhibitory action of fluorinated pyrimidine analogues is also altered with polyglutamate substrates, the effects varying with NaCl concentration and preincubation time. The most striking effect is the enhanced inhibition of the enzyme by the phosphonate analogue of FdUMP in the presence of $H_4PteGlu_3$ (Table III).

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Multiple Species of Mammalian *S*-Adenosylmethionine Synthetase. Partial Purification and Characterization†

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ABSTRACT: Two species of *S*-adenosylmethionine (*S*-Ado-Met) synthetase (EC 2.5.1.6) exist in rat liver cytosol and a distinct species of the enzyme exists in kidney cytosol. *S*-Ado-Met synthetases α and β in rat liver cytosol have been partially purified about 200- and 80-fold, respectively. The apparent molecular weight estimated by gel filtration and the sedimentation coefficient are 210000 and 9 S for *S*-Ado-Met synthetase α and 160000 and 5.5 S for *S*-Ado-Met synthetase β. Both enzymes absolutely require Mg^{2+} and K^+ for the activity and are completely inhibited by *p*-(chloromercuri)-benzoate. Kinetic studies indicate that *S*-Ado-Met synthetases α and β exhibit negative cooperativity with low $S_{0.5}$ (ligand concentration required for half-maximal velocity) for L-methionine (17 μM) and ATP (0.5 mM) and positive cooperativity with much higher $S_{0.5}$ values ($S_{0.5}$ (L-methionine) = 0.5 mM, $S_{0.5}$ (ATP) = 2 mM), respectively. The cryoprotectants dimethyl sulfoxide and glycerol markedly lower the $S_{0.5}$ values of *S*-Ado-Met synthetase β without significant effect on V_{max}. A single species of *S*-Ado-Met synthetase has been purified about 1000-fold from rat kidney cytosol. The kidney enzyme, termed *S*-Ado-Met synthetase γ, has an apparent molecular weight of 190000 and a sedimentation coefficient of 7.5 S and is resistant to the inhibition by *p*-(chloromercuri)benzoate. *S*-Ado-Met synthetase γ exhibits slightly negative cooperativity with an apparent $S_{0.5}$ value for L-methionine of 6 μM and for ATP of 70 μM.

S-Adenosylmethionine synthetase [ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6] catalyzes the formation of *S*-adenosylmethionine (*S*-Ado-Met)[1] which is the methyl donor for transmethylation reactions as well as the propylamine-group donor in the biosynthesis of polyamines (Lombardini & Talalay, 1970; Cantoni, 1975; Raina & Jänne, 1975). We have been studing the properties of rat *S*-Ado-Met synthetase and the regulation of *S*-Ado-Met biosynthesis under several conditions, including induction of DNA and RNA syntheses. *S*-Ado-Met synthetase from yeast has been purified to homogeneity and characterized in detail (Chiang & Cantoni, 1977). In mammals, however, only the hepatic enzyme has been partially purified (Cantoni & Durell, 1957; Pan & Tarver, 1967; Lombardini et al., 1970; Liau et al., 1977), and little formation is available on precise characteristics of the enzyme. Recently, evidence for the existence of two distinct species of *S*-Ado-Met synthetase has been reported in rat liver (Liau et al., 1977; Hoffman & Kunz, 1977; Okada et al., 1979), but the purification of each of the two enzymes in rat liver and their kinetic and molecular properties have not yet been established. One of the two enzyme species in rat liver is strikingly stimulated by Me_2SO at a low concentration (25 μM) of L-methionine and the other is only slightly activated (Hoffman & Kunz, 1977; Okada et al., 1979). The less Me_2SO-stimulated enzyme and the Me_2SO-stimulated enzyme were tentatively termed *S*-Ado-Met synthetases α and β, respectively (Okada et al., 1979). In contrast to the rat liver enzymes, the enzyme activities in the cytosol from rat brain, heart, and kidney are reported to be slightly inhibited by Me_2SO (Hoffman & Kunz, 1977). From kinetic evidence using the crude enzyme preparation of rat and human livers, Liau et al. (1979a,b) have reported the existence of three isozymes of *S*-Ado-Met synthetase which are termed low-K_m, intermediate-K_m, and high-K_m isozymes according to their K_m values for L-methionine.

As will be described in this paper, *S*-Ado-Met synthetase activity in crude extracts from various nonheptatic tissues examined similarly responded to tripolyphosphate, an intermediate of the enzyme reaction, as well as to Me_2SO. Therefore, we compared hepatic *S*-Ado-Met synthetases α and β with the enzyme from kidney having the highest specific activity of the nonhepatic tissues examined. We wish to describe some molecular and catalytic properties of *S*-Ado-Met synthetases α and β in rat liver and of the kidney enzyme, tentatively termed *S*-Ado-Met synthetase γ.

Materials and Methods

Chemicals. L-[*methyl*-^{3}H]Methionine (8.7 Ci/mmol) and [2-^{3}H]ATP (16 Ci/mmol) were obtained from Radiochemical Centre (Amersham, England). Potassium tripolyphosphate, spectroquality Me_2SO, and poly(ethylene glycol) 6000 were

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[1] Abbreviations used: *S*-Ado-Met, *S*-adenosylmethionine; Me_2SO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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