Studies on the polyglutamate specificity of thymidylate synthase from fetal pig liver

Studies on the Polyglutamate Specificity of Thymidylate Synthase from Fetal Pig Liver[†]

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ABSTRACT: Thymidylate synthase has been purified 1700-fold from fetal pig livers by using chromatography on Affigel-Blue, DEAE-52, and hydroxylapatite. Steady-state kinetic measurements indicate that catalysis proceeds via an ordered sequential mechanism. When 5,10-methylenetetrahydropteroylmonoglutamate (CH_2-$H_4PteGlu_1$) is used as the substrate, dUMP is bound prior to CH_2-$H_4PTeGlu_1$, and 7,8-dihydropteroylmonoglutamate ($H_2PteGlu_1$) is released prior to dTMP. Pteroylpolyglutamates ($PteGlu_n$) are inhibitors of thymidylate synthase activity and are competitive with respect to CH_2-$H_4PteGlu_1$ and uncompetitive with respect to dUMP. Inhibition constants (K_i values), which correspond to dissociation constants for the dissocation of $PteGlu_n$ from the enzyme–dUMP–$PteGlu_n$ ternary complex, have been determined for $PteGlu_n$ derivatives with one to seven glutamyl residues: $PteGlu_1$, 10 μM; $PteGlu_2$, 0.3 μM; $PteGlu_3$, 0.2 μM; $PteGlu_4$, 0.06 μM; $PteGlu_5$, 0.10 μM; $PteGlu_6$, 0.12 μM; $PteGlu_7$, 0.15 μM. Thus, thymidylate synthase from fetal pig liver preferentially binds pteroylpolyglutamates with four glutamyl residues, but derivatives with two to seven glutamyl residues all bind at least 30-fold more tightly than the monoglutamate. When CH_2-$H_4PteGlu_4$ is used as the one carbon donor for thymidylate biosynthesis, the order of substrate binding and product release is reversed, with binding of CH_2-$H_4PteGlu_4$ preceding that of dUMP and release of dTMP preceding release of $H_2PteGlu_4$. V_{max} and K_m values for dUMP and CH_2-$H_4PteGlu_n$ show relatively little change as the polyglutamate chain length of the substrate is varied. Comparison of the kinetic data obtained in these studies with earlier studies on methylenetetrahydrofolate reductase from pig liver [Matthews, R. G., & Baugh, C. M. (1980) *Biochemistry 19*, 2040–2045] leads us to predict that the partitioning of limiting concentrations of CH_2-$H_4PteGlu_n$ between the reactions catalyzed by thymidylate synthase and methylenetetrahydrofolate reductase will vary with polyglutamate chain length, with hexaglutamyl substrates preferentially being reduced to methyltetrahydrofolate.

Intracellular folate derivatives are present mainly as pteroylpoly-γ-glutamates with 2–10 glutamyl residues (Brown et al., 1974; Eto & Krumdieck, 1981; Foo & Shane, 1982). There is evidence to suggest that the distribution of pteroylpolyglutamates is species dependent (Priest et al., 1981) and may differ with the nature of the folate derivative (Eto & Krumdieck, 1981) and with metabolic conditions inside the cell (Foo & Shane, 1982; Eto & Krumdieck, 1982). Synthesis of a polyglutamate "tail" requires considerable expenditure of cellular energy and presumably results in compensatory advantages to the cell. However, the rationale for formation of long-chain pteroylpolyglutamates remains unclear.

Our laboratory has been examining the binding properties of a series of folate-dependent enzymes for pteroylpolyglutamate derivatives. In addition to the present study on thymidylate synthase, we have examined methylenetetrahydrofolate reductase (Matthews & Baugh, 1980), serine hydroxymethyltransferase (Matthews et al., 1982), and methylenetetrahydrofolate dehydrogenase (Ross et al., 1984). These studies have utilized pteroylpolyglutamate derivatives as *inhibitors* of these enzymes and have determined dissociation constants by kinetic or thermodynamic measurements for a series of inhibitors which differ only in the number of glutamyl residues. All studies have been performed with enzymes isolated from pig liver, to eliminate species differences in polyglutamate specificity. Once the specificity of each enzyme for the length of polyglutamate tail is known and the binding energies associated with the interaction of each glu-

tamyl residue with the enzyme have been determined, we can also determine how significantly binding of the polyglutamate tail affects the kinetic parameters associated with catalysis utilizing pteroylpolyglutamate substrates.

Such studies provide base-line information which can be used to predict the flux of folate metabolites through competing pathways. They are also helpful in predicting the pharmacological and physiological effects that polyglutamylation will have on the inhibition of individual enzymes by folate and antifolate derivatives.

Experimental Procedures

Purification of Thymidylate Synthase. Fetal pigs were obtained from a local slaughterhouse. The highest thymidylate synthase activity was associated with pigs weighing less than 225 g. Enzyme preparation was initiated within 6 h of slaughter, since enzyme activity in the pigs, dissected livers, or homogenates was not stable to storage at either 4 or −20 °C. The livers were dissected out and washed with 0.9% NaCl. They were homogenized in a small Waring blender in 50 mM potassium phosphate buffer, pH 7.5, containing 0.1 M NaCl, 50 mM 2-mercaptoethanol, and 100 μM dUMP.[1] The ratio of buffer to dissected livers in the homogenate was 1 mL of buffer/g of liver. The homogenate was centrifuged at 30000g for 1 h.

The supernatant was decanted and assayed for thymidylate synthase activity (see below). Affigel-Blue beads (Bio-Rad),

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[1] Abbreviations: CH_2-$H_4PteGlu_n$, 5,10-methylenetetrahydropteroylpolyglutamate with n glutamyl residues; $H_2PteGlu_n$, 7,8-dihydropteroylpolyglutamate with n glutamyl residues; CH_3-$H_4PteGlu_n$, 5-methyltetrahydropteroylpolyglutamate with n glutamyl residues; dTMP, thymidylate; dUMP, deoxyuridylate; HTP, hydroxylapatite; DTT, dithiothreitol; CH_3-H_4folate, 5-methyltetrahydrofolate; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; H_2folate, 7,8-dihyrofolate; FdUMP, 5-fluorodeoxyuridylate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

previously equilibrated with the homogenizing buffer and then allowed to settle in the buffer, were added to the supernatant in the proportion 100 mL of settled beads per 0.22 unit of activity (in μmol min^{-1}). The suspension was stirred overnight at 4 °C. The beads were collected by filtration in a Buchner funnel, and the filtrate was discarded. The beads were resuspended in 50 mM phosphate buffer, pH 7.5, containing 0.1 M NaCl, 50 mM 2-mercaptoethanol, and 100 μM dUMP and collected by filtration. Rinsing was repeated until the absorbance at 280 nm was less than 0.1 when measured vs. a buffer blank. A slurry of beads in the same buffer was poured into a 2.5-cm diameter column. The enzyme was eluted with 1 M NaCl in 50 mM phosphate, pH 7.5, containing 50 mM 2-mercaptoethanol and 100 μM dUMP. The elution was performed at 4 °C, and the column was pumped at a flow rate of 16 mL/h. Fractions, 4 mL, were collected and analyzed for thymidylate synthase activity and for differential absorbance at 280 nm. The active fractions (8–19) were pooled and concentrated in an Amicon concentrator with a PM30 membrane and were then dialyzed overnight in 10 mM phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol.

The dialyzed enzyme was applied to a 2.5 × 10 cm column of DEAE-52 (Whatman), previously equilibrated with 50 mM Tris-HCl buffer, pH 7.8, 20% glycerol, 10 mM 2-mercaptoethanol, and 1 mM EDTA.[1] The column was eluted with a linear gradient of 0–0.1 M KCl in the same buffer, and 5-mL fractions were collected. Active fractions (39–47) were collected, concentrated, and dialyzed overnight in 10 mM phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol.

The enzyme was then applied to a 1.5 × 7 cm Bio-Rad HTP column previously equilibrated with glass-distilled water. The adsorbed enzyme activity was eluted with a 250-mL linear gradient of 0–0.1 M phosphate buffer, pH 7.5, containing 5 mM DTT, and 4-mL fractions were collected. Active fractions (24–38) were pooled and concentrated to an activity of 0.026 μmol min^{-1} (mL of enzyme solution)$^{-1}$. The enzyme solution was clarified by centrifugation, brought to 20% in glycerol, and then stored at −70 °C. Enzyme solutions prepared in this manner showed little or no loss of activity after storage for several weeks.

Methods for Assay of Thymidylate Synthase Activity. Enzyme activity was monitored during the purification of the protein by measuring tritium release from [5-^{3}H]dUMP in the presence of (6*RS*)-CH$_2$-H$_4$folate as initially described by Lomax & Greenberg (1967). Assay mixtures, 0.2 mL, contained 0.1 mL of enzyme and 0.1 mL of an assay cocktail [100 mM Tris-HCl buffer, pH 7.4/50 mM MgCl$_2$/30 mM formaldehyde/200 mM 2-mercaptoethanol/2 mM EDTA/1.25 mM (6*RS*)-H$_4$folate/0.2 M NaF/125 μM [5-^{3}H]dUMP (94 000 dpm/nmol)]. The mixture was incubated for 15 min at 37 °C and then quenched by addition of 1 mL of activated charcoal in 4% perchloric acid (20 g/100 mL). The quenched mixture was incubated for 5 min at 37 °C with frequent shaking and then centrifuged in an Eppendorf microfuge at 15600g for 3 min. An aliquot of the supernatant, 0.3 mL, was added to 5 mL of aqueous counting scintillant (Amersham). The dpm values detected in each sample were corrected for the dpm detected in a control assay, in which the charcoal/perchloric acid quench mixture was added to the enzyme prior to addition of the assay cocktail. Enzyme units are micromoles of ^{3}H released per minute.

For kinetic studies utilizing the purified enzyme a spectrophotometric assay was used. The assay involves measurement of the absorbance changes at 340 nm accompanying the conversion of CH$_2$-H$_4$folate to H$_2$folate (Wahba & Friedkin, 1961). In calculating activities a molar extinction coefficient of 6152 M^{-1} cm^{-1} was used for the absorbance change at 340 nm associated with the conversion of CH$_2$-H$_4$folate to H$_2$folate. This molar extinction coefficient was calculated from the ratios of 340-nm absorbance to peak absorbance of our preparations of CH$_2$-H$_4$folate (340/297 = 0.045) and H$_2$folate (340/282 = 0.267) by using 32 000 M^{-1} cm^{-1} as the molar extinction coefficient for CH$_2$-H$_4$folate at 297 nm (Blakley, 1960a) and 28 400 M^{-1} cm^{-1} as the molar extinction coefficient for H$_2$folate at 282 nm (Blakley, 1960b). Assay mixtures, 1.0 mL, contained approximately 2.6 × 10^{-3} unit of thymidylate synthase (as measured by the tritium release assay)/0.1 M phosphate buffer, pH 6.8/100 μM dUMP/20 μM (6*RS*)-CH$_2$-H$_4$folate. The CH$_2$-H$_4$folate was prepared as a mixture of 0.05 M NaHCO$_3$/2 mM (6*RS*)-H$_4$folate/1.3 mM formaldehyde/50 mM 2-mercaptoethanol and was stored at −20 °C under nitrogen prior to use. Such solutions were stable for several weeks. The assay mixture (with CH$_2$-H$_4$folate and enzyme omitted) was equilibrated with nitrogen in a 1-mL cuvette for several minutes, CH$_2$-H$_4$folate was added, and equilibration with nitrogen was continued for two more minutes. The cuvette was sealed with parafilm and equilibrated in a 25 °C bath for several minutes, and then the blank rate was measured at 340 nm in a spectrophotometer. The assay was initiated by addition of enzyme. Measurements were made on a recorder with an expanded scale (0.1 absorbance full scale) and a spectrophotometer with an optical offset.

Kinetic parameters were evaluated graphically from double-reciprocal plots by using linear regression analysis.

Preparation of Folate Substrates and Inhibitors. Pteroylpolyglutamates (PteGlu$_n$) were prepared by solid-phase synthesis (Krumdieck & Baugh, 1969, 1982), purchased from Dr. Charles M. Baugh, and used without further purification. The purity of the pteroylpolyglutamates was checked by high-pressure liquid chromatography using a modification of the procedure described by Schilsky et al. (1983) for the separation of polyglutamate analogues of methotrexate. Approximately 1.5 nmol of PteGlu$_n$ was applied to an Ultrasphere ODS column (0.46 × 25 cm) equilibrated with 30% acetonitrile/70% 5 mM tetrabutylammonium phosphate (Waters PIC A) in glass distilled water. The samples were eluted at 1 mL/min along gradients of 30–50% acetonitrile and 3.5–2.5 mM tetrabutylammonium phosphate in water over 30 min. The column was then eluted isocratically with 50% acetonitrile/50% 5 mM tetrabutylammonium phosphate in water for 15 min. The eluate was monitored at 254 nm. Under these conditions each PteGlu$_n$ derivative chromatographed as a single major peak, and this peak comprised 85–90% of the 254-nm-absorbing material eluting from the column. In particular, contamination by PteGlu$_{n-1}$ was always less than 10%, based on peak height ratios. Under these conditions the following retention times were observed: PteGlu$_n$, 7.0 min; PteGlu$_2$, 12.5 min; PteGlu$_3$, 18.6 min; PteGlu$_4$, 22.0 min; PteGlu$_5$, 24.5 min; PteGlu$_6$, 26.4 min; PteGlu$_7$, 27.9 min.

For studies with CH$_2$-H$_4$PteGlu$_1$, (6*RS*)-H$_4$PteGlu$_1$ was prepared by catalytic hydrogenation of a neutral aqueous solution of PteGlu$_1$ and purified as previously described (Ross et al., 1984). Alternatively, (6*S*)-H$_4$PteGlu$_1$ was prepared by enzymatic reduction of PteGlu$_1$ using dihydrofolate reductase from *L. casei* (Matthews et al., 1982). (6*S*)-H$_4$PteGlu$_n$ derivatives were prepared from PteGlu$_n$ derivatives in the same manner. H$_2$PteGlu$_n$ inhibitors were prepared by dithionite reduction of the corresponding PteGlu$_n$ derivatives as described

Table I. Purification of Thymidylate Synthase from Fetal Pig Liver[a]

step	activity (unit)[b]	protein (mg)[c]	volume (mL)	sp act. (unit/mg)	yield (%)	purification (x-fold)
supernatant from 30000g centrifugation	0.23	12 420	180	1.85×10^{-5}	100	1
eluate from AffiGel Blue	0.045	325	50	1.38×10^{-4}	20	7.5
eluate from DEAE-Sephadex	0.066	45.2	58	1.46×10^{-3}	29	79
eluate from Bio-Rad HTP	0.066	2.1	38	3.14×10^{-2}	29	1700

[a] This preparation utilized 225 g of fetal pig liver, obtained from approximately 30 fetal pigs. [b] The units are micromoles of ^3H released per minute at 37 °C. [c] Protein determinations were made by using Bio-Rad protein assay, according to the manufacturer's directions, and using bovine serum albumin as a standard.

Table II: Product Inhibition of the Thymidylate Synthase Reaction With CH_2-$H_4PteGlu_1$[a] as Folate Substrate[b]

variable substrate	fixed substrate (μM)	inhibitor	inhibition pattern	K_i (μM)
dUMP	CH_2-$H_4PteGlu_1$ (7.2)	dTMP	competitive	$K_{iq} = 20$
CH_2-$H_4PteGlu_1$	dUMP (5)	dTMP	noncompetitive	$K_i(slope) = 60$ $K_{iq} = 20$
CH_2-$H_4PteGlu_1$	dUMP (100)	dTMP	noncompetitive	$K_i(slope) = 1100$ $K_{iq} = 20$
CH_2-$H_4PteGlu_1$	dUMP (10)	$H_2PteGlu_1$	noncompetitive	$K_i(intercept) = 135$ $K_{ip} = 115$
CH_2-$H_4PteGlu_1$	dUMP (100)	$H_2PteGlu_1$	noncompetitive	$K_i(intercept) = 97$ $K_{ip} = 95$
dUMP	CH_2-$H_4PteGlu_1$ (7.2)	$H_2PteGlu_1$	noncompetitive	$K_i(intercept) = 13$
dUMP	CH_2-$H_4PteGlu_1$ (72)	$H_2PteGlu_1$	uncompetitive	$K_{ip} = 90$

[a] Enzymatically reduced $(6R)$-CH_2-$H_4PteGlu_1$ was used for these experiments. With $(6R)$-CH_2-$H_4PteGlu_1$ the K_m for dUMP is 1.7 μM, and the K_m for CH_2-$H_4PteGlu_1$ is 5.2 μM, while when racemic $(6RS)$-CH_2-$H_4PteGlu_1$ is used, the K_m for dUMP is 8 μM, and the K_m for $(6R)$-$CH_2H_4PteGlu_1$ is 8 μM. We infer that $(6S)$-CH_2-$H_4PteGlu_1$ may have some affinity for the dUMP binding site. [b] Plots of the primary data are available to the interested reader on request.

by Matthews & Baugh (1980).

Source and Preparation of Other Reagents. [5-^3H]dUMP was purchased from Amersham and purified on Dowex AG 1-X8 formate columns as described by Lomax & Greenberg (1967). Purified dihydrofolate reductase from *L. casei* was the generous gift of Professor Bruce Dunlap, University of South Carolina. dUMP and dTMP were purchased from Sigma and used without further purification.

Results

Purification of Thymidylate Synthase. The purification procedure utilized to prepare enzyme for kinetic studies is shown in Table I. Certain aspects of this procedure deserve comment. The thymidylate synthase activity in the initial homogenate was very labile if 0.1 M NaCl was omitted from the homogenizing buffer and disappeared with a half-time of about 1 h. With 0.1 M NaCl in the homogenizing buffer, activity in the homogenate was considerably stabilized, with a half-time for inactivation of about 4 h. Addition of phenylmethanesulfonyl fluoride to the homogenate did not protect the enzyme against inactivation. Once the enzyme had been purified by adsorption on Affigel Blue, its lability was greatly decreased and NaCl could be omitted without loss of activity. However, activity disappeared on storage at 4 °C for 4 days unless 5 mM DTT and 20% glycerol were present in the buffer. Inclusion of these agents permitted storage of the enzyme for several weeks at this stage of purification without loss of activity at either 4 or −70 °C. Chromatography on DEAE-52 could be carried out conveniently in 20% glycerol at 4 °C provided that wide, short columns were used. However, reasonable flow rates during chromatography on Bio-Rad HPT at 4 °C could be achieved only if glycerol was removed from the protein prior to application to the column. At this stage of the purification, glycerol was only required to protect the enzyme against inactivation due to freezing.

Characterization of the Kinetic Mechanism Associated with Use of CH_2-$H_4PteGlu_1$. Figure 1 shows a kinetic analysis of the thymidylate synthase reaction using $(6RS)$-CH_2-$H_4PteGlu_1$ as the one-carbon donor. It can be seen that the
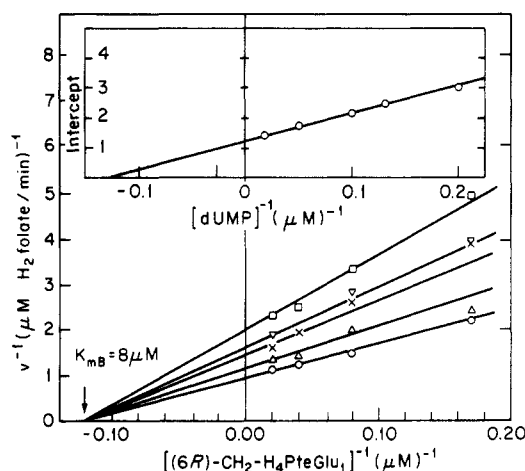

FIGURE 1: Steady-state kinetic measurements with CH_2-$H_4PteGlu_1$ as substrate. A double-reciprocal plot of velocity vs. [$(6R)$-CH_2-$H_4PteGlu_1$] is shown. The folate substrate was added as the racemic mixture, $(6RS)$-CH_2-$H_4PteGlu_1$. The dUMP concentrations were (□) 5, (▲) 7.5, (×) 10, (▽) 20, and (○) 60 μM. Velocities were determined by using the spectrophotometric assay described under Experimental Procedures.

double-reciprocal plots converge, indicative of a sequential mechanism. The K_m for dUMP is 8 μM, as is the K_m for $(6R)$-CH_2-$H_4PteGlu_1$. The order of addition of substrates and of release of products was determined by product inhibition studies, and these experiments are summarized in Table II. The product inhibition studies are indicative of the following kinetic pathway:
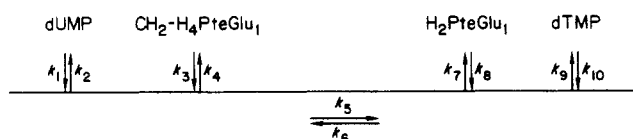
Measurement of the Inhibition Constants Associated with PteGlu_n Inhibitors. The K_i values associated with PteGlu_n inhibitors were measured by using $(6RS)$-CH_2-$H_4PteGlu_1$ as

Table III: Inhibition Constants for PteGlu$_n$ Inhibitors[a]

inhibitor	K_i (μM)	inhibitor	K_i (μM)
PteGlu$_1$	15	PteGlu$_5$	0.100
	6		0.099
PteGlu$_2$	0.38	PteGlu$_6$	0.12
	0.24		0.12
PteGlu$_3$	0.30	PteGlu$_7$	0.19
	0.13		0.10
PteGlu$_4$	0.060		
	0.060		

[a] All assays were conducted with $(6RS)$-CH$_2$-H$_4$PteGlu$_1$ as the folate substrate, under the assay conditions described under Experimental Procedures and employing 100 μM dUMP.

Table IV: Kinetic Parameters Associated with CH$_2$-H$_4$PteGlu$_n$ Substrates[a]

substrate	rel V_{max}	K_m (dUMP) (μM)	K_m (CH$_2$-H$_4$PteGlu$_n$) (μM)
CH$_2$-H$_4$PteGlu$_1$	1.00	1.7	5.2
CH$_2$-H$_4$PteGlu$_2$	0.56	1.7	2.0
CH$_2$-H$_4$PteGlu$_3$	0.39	1.7	1.9
CH$_2$-H$_4$PteGlu$_4$	0.36	1.7	1.9
CH$_2$-H$_4$PteGlu$_5$	0.38	2.3	1.6
CH$_2$-H$_4$PteGlu$_6$	0.43	2.5	1.6
CH$_2$-H$_4$PteGlu$_7$	0.37	2.6	2.1

[a] All assays utilized enzymatically prepared $(6R)$-CH$_2$-H$_4$PteGlu$_n$ derivatives as the folate substrates. As noted in the footnote to Table II, somewhat different K_m values for dUMP and for CH$_2$-H$_4$PteGlu$_1$ are obtained when racemic $(6RS)$-CH$_2$-H$_4$PteGlu$_1$ is used as the substrate. Assay conditions are described under Experimental Procedures. Double-reciprocal plots of velocity vs. [CH$_2$-PteGlu$_n$] and of velocity vs. [dUMP] intersected on the x axis. Thus in all cases, $K_{is} = K_{ms}$ for the first substrate bound.

FIGURE 2: Inhibition of thymidylate synthase by PteGlu$_4$. (A) Double-reciprocal plots of velocity vs. [$(6R)$-CH$_2$-H$_4$PteGlu$_1$], in the presence of 100 μM dUMP. The folate substrate was added as the racemic mixture $(6RS)$-CH$_2$-H$_4$PteGlu$_1$. Concentrations of PteGlu$_4$ inhibitor present were (●) 0, (✕) 0.065, (□) 0.087, (○) 0.108, and (△) 0.152 μM. (B) Double-reciprocal plots of velocity vs. [dUMP], in the presence of 10 μM $(6R)$-CH$_2$-H$_4$PteGlu$_1$ added as the racemic mixture $(6RS)$-CH$_2$-H$_4$PteGlu$_1$. Concentrations of PteGlu$_4$ inhibitor were (○) 0, (✕) 0.07, (□) 0.12, and (△) 0.17 μM.

the folate substrate in each experiment. A typical experiment is shown in Figure 2, with PteGlu$_4$ as the inhibitor. The inhibitor pattern is linearly competitive with respect to CH$_2$-H$_4$PteGlu$_1$ and linearly uncompetitive with respect to dUMP. Such inhibition patterns are expected if PteGlu$_4$ competes with CH$_2$-H$_4$PteGlu$_1$ for the folate binding site on the enzyme–dUMP binary complex. Similar patterns were observed for each PteGlu$_n$ inhibitor, and the K_i values that were calculated from replots such as is shown in the inset to Figure 2A are listed in Table III. In each case, the K_i values correspond to dissociation constants for dissociation of PteGlu$_n$ inhibitor from enzyme–dUMP–PteGlu$_n$ ternary complexes.

Measurement of the Kinetic Parameters Associated with CH$_2$-H$_4$PteGlu$_n$ Substrates. Table IV summarizes the relative values for V_{max} and the K_m values for dUMP and CH$_2$-H$_4$PteGlu$_n$ obtained from kinetic analyses of the series of $(6R)$-CH$_2$-H$_4$PteGlu$_n$ substrates. These experiments were all run on the same day with the same preparation of enzyme. Despite the very large differences in affinity of the enzyme
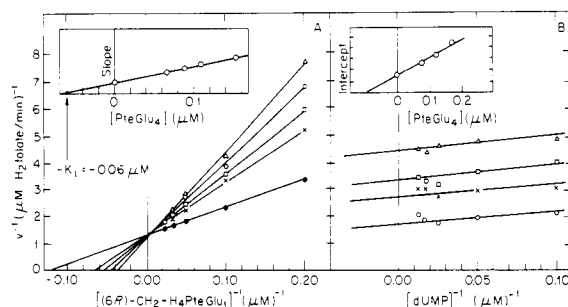
for PteGlu$_n$ inhibitors, the variation in V/K with polyglutamate chain length is very small. One possible explanation for these discrepancies would be that longer chain polyglutamate substrates result in a change in order of addition of substrates or of release of products so that the rate constants contained in these kinetic parameters are not comparable for all substrates. In order to look for changes in order of addition of substrates and/or of product release, we have performed product inhibition studies using CH$_2$-H$_4$PteGlu$_4$ as the folate substrate and H$_2$PteGlu$_4$ and dTMP as product inhibitors. The results of these studies are summarized in Table V. These inhibition studies are consistent with the following mechanism:
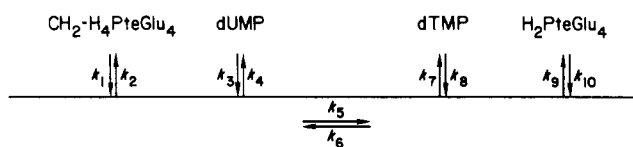
Comparison with the kinetic scheme obtained by using CH$_2$-H$_4$PteGlu$_1$ as the folate substrate indicates that use of the polyglutamate substrate has resulted in changes in the order of addition of substrates and of release of products. Similar product inhibition studies with CH$_2$-H$_4$PteGlu$_2$ as substrate using H$_2$PteGlu$_2$ and dTMP as product inhibitors suggest that with this substrate also the folate substrate is bound first and the folate product is released last.

When the inhibition patterns associated with inhibition by PteGlu$_4$ are examined with CH$_2$-H$_4$PteGlu$_4$ as the folate substrate, PteGlu$_4$ is linearly competitive with respect to CH$_2$-H$_4$PteGlu$_4$ but exhibits a noncompetitive pattern with respect to dUMP. These inhibition patterns are also consistent with CH$_2$-H$_4$PteGlu$_4$ binding prior to dUMP. Furthermore,

Table V: Product Inhibition of the Thymidylate Synthase Reaction with CH$_2$-H$_4$PteGlu$_4$ as Folate Substrate[a,b]

variable substrate	fixed substrate (μM)	inhibitor	inhibitor pattern	K_i (μM)
CH$_2$-H$_4$PteGlu$_4$	dUMP (10)	H$_2$PteGlu$_4$	competitive	$K_{iq} = 3.3$
dUMP	CH$_2$-H$_4$PteGlu$_4$ (7.2)	H$_2$PteGlu$_4$	noncompetitive	K_i(slope) = 15
				$K_{iq} = 3.1$
dUMP	CH$_2$-H$_4$PteGlu$_4$ (72)	H$_2$PteGlu$_4$	noncompetitive	K_i(slope) = 45
				$K_{iq} = 1.2$
dUMP	CH$_2$-H$_4$PteGlu$_4$ (7.2)	dTMP	noncompetitive	K_i(intercept) = 400
				$K_{ip} = 320$
dUMP	CH$_2$-H$_4$PteGlu$_4$ (64)	dTMP	noncompetitive	K_i(intercept) = 222
				$K_{ip} = 220$
CH$_2$-H$_4$PteGlu$_4$	dUMP (10)	dTMP	noncompetitive	K_i(intercept) = 50
CH$_2$-H$_4$PteGlu$_4$	dUMP (100)	dTMP	uncompetitive	$K_{ip} = 320$

[a] Enzymatically reduced $(6R)$-CH$_2$-H$_4$PteGlu$_4$ was used for these experiments. [b] Plots of the primary data are available to the interested reader on request.
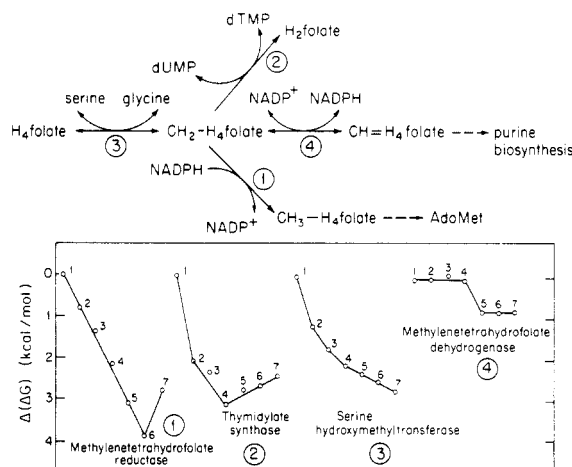
FIGURE 3: Semilogarithmic plots of $\Delta(\Delta G)$ associated with the binding of folate inhibitors vs. the number of glutamyl residues on the folate inhibitor. (1) For methylenetetrahydrofolate reductase, $\Delta(\Delta G)$ values were calculated from K_i values determined kinetically for inhibition of enzyme by $H_2PteGlu_n$ inhibitors in the presence of saturating NADPH and varied CH_2-$H_4PteGlu_1$ (Matthews & Baugh, 1980). (2) For thymidylate synthase, $\Delta(\Delta G)$ values were calculated from the data in Table III. (3) For serine hydroxymethyltransferase, $\Delta(\Delta G)$ values were calculated from spectrophotometric determinations of the K_d values for dissociation of CH_3-$H_4PteGlu_n$ from E-CH_3-$H_4PteGlu_n$-glycine ternary complexes (Matthews et al., 1982). (4) For methylenetetrahydrofolate dehydrogenase, $\Delta(\Delta G)$ values were calculated from K_i values determined kinetically for inhibition of the enzyme by $PteGlu_n$ inhibitors in the presence of saturating $NADP^+$ and varied CH_2-$H_4PteGlu_1$ (Ross et al., 1984).

the measured K_i for $PteGlu_4$ binding to the free enzyme (measured with CH_2-$H_4PteGlu_4$ as substrate and 100 μM dUMP) is 0.35 μM, which is substantially higher than the K_i associated with $PteGlu_4$ binding to the enzyme-dUMP binary complex (0.06 μM, measured with CH_2-$H_4PteGlu_1$ as substrate and 100 μM dUMP). Clearly $PteGlu_4$ binds both free enzyme and the enzyme-dUMP binary complex. However, the relatively simple inhibition patterns we observe with this inhibitor suggest that such inhibition studies are relatively insensitive to a small amount (less than 20%) of inhibitor binding to an alternate enzyme form.

Discussion

The values for inhibition constants of $PteGlu_n$ inhibitors shown in Table III can be used to calculate the change in free energy $\Delta(\Delta G)$ associated with the binding of each of a series of polyglutamate inhibitors. In Figure 3, these free energy changes associated with the binding of folylpolyglutamates to thymidylate synthase are compared with similar profiles obtained for other folate-dependent enzymes from pig liver in earlier studies from our laboratory. We note that the folate-dependent enzymes differ markedly in their affinity for folylpolyglutamates as compared to folylmonoglutamates, and they differ also in the chain length of folylpolyglutamate which is preferentially bound. Such comparisons suggest that the chain length of folylpolyglutamate derivatives may indeed play a role in determining how these derivatives will be metabolized, as originally suggested by Baggott & Krumdieck (1979). Two of the enzymes that have been studied, serine hydroxymethyltransferase and methylenetetrahydrofolate dehydrogenase, catalyze reactions that are thought to be maintained at equilibrium in the cytoplasm and presumably play little role in the direction of one-carbon units to various metabolic pathways. The other two enzymes, thymidylate synthase and methylenetetrahydrofolate reductase, determine the flux of one-carbon units through their respective pathways, e.g., into thymidylate biosynthesis or regeneration of AdoMet

Table VI: Partitioning of Limiting Concentrations of CH_2-$H_4PteGlu_n$ Substrates between the Reactions Catalyzed by Methylenetetrahydrofolate Reductase

|	relative flux		
substrate	methylenetetra-hydrofolate reductase[a]	thymidylate synthase[b]	flux ratio
CH_2-$H_4PteGlu_1$	1	1	1
CH_2-$H_4PteGlu_2$	2.4	1.4	1.7
CH_2-$H_4PteGlu_3$	7.2	1.1	6.5
CH_2-$H_4PteGlu_4$	15.5	1.0	15.5
CH_2-$H_4PteGlu_5$	11.6	1.2	9.7
CH_2-$H_4PteGlu_6$	27.1	1.3	20.8
CH_2-$H_4PteGlu_7$	5.5	0.9	6.1

[a] Data calculated from Matthews & Baugh (1979) assuming a cytoplasmic NADPH concentration of 200 μM. [b] Data calculated from Table IV of this paper assuming a cytoplasmic dUMP concentration of 60 μM (Jackson, 1978).

via CH_3-$H_4folate$ and methionine, and catalyze reactions that are essentially irreversible in vivo. In Table VI we have attempted to calculate the effect of the polyglutamate chain length of CH_2-$H_4folate$ on the relative rates of incorporation of the methylene group into dTMP or into CH_3-$H_4folate$. These calculations assume a cytoplasmic concentration of NADPH of about 200 μM (Conway et al., 1983) and a cytoplasmic dUMP concentration of about 60 μM [calculated from the data of Jackson (1978) assuming 0.68 mL of water/10^9 cells]. The calculations further assume limiting concentrations of CH_2-$H_4folate$ (V/K conditions). Under these conditions, it can be seen that the ratio of fluxes through these two competing pathways is quite dependent on the polyglutamate chain length of CH_2-$H_4PteGlu_n$, so that if the flux ratio through the two pathways is set equal to 1 for the monoglutamate, it increases to 20.8 for the hexaglutamate, with polyglutamates being preferentially reduced to CH_3-$H_4folate$. Thus, under conditions of limiting CH_2-$H_4folate$, chain length is expected to be an important determinant of flux.

Our studies also suggest that one should be very cautious about drawing inferences about substrate *affinities* from comparisons of V_{max}/K_m with folate substrates of varying polyglutamate chain length, particularly for ordered sequential mechanisms. Our observation that increased affinity of thymidylate synthase for folylpolyglutamates leads to reversals in the order of substrate binding and product release means that the kinetic constants contained in V_{max}/K_m change with polyglutamate chain length and hence that these values can not be directly compared.

Previous studies of thymidylate synthase from a number of sources have suggested an ordered mechanism in which dUMP binding precedes CH_2-$H_4PteGlu_1$ binding (Lorenson et al., 1967; Dolnick & Cheng, 1977; Danenberg & Danenberg, 1978; Daron & Aull, 1978; Bisson & Thorner, 1981). Evidence in support of an ordered mechanism in which dUMP binding precedes CH_2-$H_4PteGlu_1$ binding was also obtained from equilibrium dialysis measurements with the enzyme from *L. casei* (Galivan et al., 1976b). Studies have also been performed with enzyme from a variety of sources that examined the effect of substrate polyglutamate chain length on V_{max} and the K_m values for CH_2-$H_4PteGlu_n$. Thus, the yeast enzyme exhibits a 10-fold decrease in K_m when CH_2-$H_4PteGlu_5$ is compared with CH_2-$H_4PteGlu_1$ (Bisson & Thorner, 1981); the enzyme from human blast cells shows a 15-fold decrease in K_m when the same two substrates are compared (Dolnick & Cheng, 1978), and the enzyme from *L. casei* shows decreases in both V_{max} (3-fold) and the K_m for CH_2-$H_4folate$ (15-fold) (Kisliuk et al., 1981). The enzyme from calf thymus

Scheme I
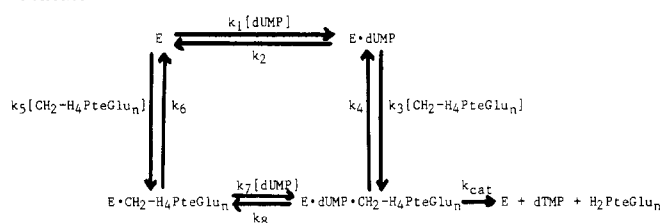
shows no change in K_m with polyglutamate chain length (Dwivedi et al., 1983). Thus, in the cases which have been examined relatively small changes in kinetic parameters have been observed as the polyglutamate chain lengths of CH_2-H_4folate substrates were varied, in agreement with our own results. Previous investigators have not reported changes in order of substrate addition or product release, although our findings were presaged by the observation of Galivan et al. (1976a) that while CH_2-$H_4PteGlu_1$ is only bound to thymidylate synthase from *L. casei* when dUMP or a pyrimidine analogue is present, CH_2-$H_4PteGlu_5$ forms binary complexes with the enzyme in the absence of a pyrimidine.

In contrast, both we and others have documented much larger changes in the *affinity* of the enzyme for folate derivatives as polyglutamate chain length is varied. Friedkin and co-workers (1975) isolated a naturally occurring inhibitor of thymidylate synthase from *Escherichia coli* and showed that it was 5-formyl-H_4Pte-Glu_5. They then determined I_{50} values of 5-CHO-$H_4PteGlu_n$ inhibitors and showed that the hexaglutamyl derivative was 200-fold more potent as an inhibitor than the monoglutamate, with the largest changes in potency being associated with addition of the second and third glutamyl residue to the inhibitor. Kisliuk et al. (1979) observed a 375-fold decrease in I_{50} associated with inhibition by $PteGlu_5$ as compared to that of $PteGlu_1$ by using enzyme from *L. casei*. Priest & Mangum (1981) have examined the effect of polyglutamate chain length on the rate of formation and equilibrium position of enzyme–FdUMP–CH_2-$H_4PteGlu_n$ ternary complexes with thymidylate synthase from *L. casei*. They reported a 25-fold increase in the rate of formation of the complex as the polyglutamate chain length was increased from one to five glutamyl residues. They also found that long-chain CH_2-$H_4PteGlu_n$ derivatives were bound preferentially, with the biggest difference in incorporation seen when CH_2-$H_4PteGlu_1$ and CH_2-$H_4PteGlu_2$ were competing for a binding site in the ternary complex. Fernandes & Bertino (1980) showed that polyglutamate derivatives of methotrexate and H_2folate, when binding with FdUMP to form ternary complex with enzyme from L1210 cells, led to greatly decreased K_d values for dissociation of FdUMP from the ternary complex as compared to the corresponding monoglutamates. We suggest that our observation that polyglutamate substrates cause reversals in the order of substrate binding and product release may prove to be a common phenomenon occurring with thymidylate synthase enzymes from a variety of sources.

The observation of reversal of substrate binding and product release with polyglutamate substrates also has implications for the formation and breakdown of inhibitory ternary complexes with FdUMP. Danenberg & Danenberg (1978) suggested that increasing concentrations of CH_2-H_4folate, resulting for instance in response to administration of folinic acid, might block dissociation of FdUMP from ternary complex and enhance the toxicity of FdUMP under these conditions. These conclusions were based on studies of the dissociation of E–FdUMP–CH_2-$H_4PteGlu_1$ ternary complex formed with the enzyme from *L. casei* which showed that CH_2-$H_4PteGlu_1$

dissociation preceded that of FdUMP. We would suggest, however, that the order of dissociation is probably reversed with complexes formed with naturally occurring folate polyglutamates, so that cofactor concentration would not affect the rate of dissociation of FdUMP in the cell.

The reversal of preferred order of substrate binding when steady-state kinetics with CH_2-$H_4PteGlu_1$ or CH_2-$H_4PteGlu_2$ as the folate substrate are compared implies that the ratio of rate constants for release of dUMP (k_8) and CH_2-$H_4PteGlu_n$ (k_4) from the ternary complex undergo sufficient change to go from preferred release of CH_2-$H_4PteGlu_1$ to preferred release of dUMP (see Scheme I). Under the assumption that a preferred order mechanism requires *at least* a 5-fold difference in the off constants for the two substrates dissociating from the ternary complex, this change would require at least a 25-fold decrease in the off constant for CH_2-$H_4PteGlu_2$ release from the ternary complex (k_4) relative to that of CH_2-$H_4PteGlu_1$. Our inhibition data suggest a 33-fold increase in the affinity of thymidylate synthase for $PteGlu_2$ relative to $PteGlu_1$ or just enough change in affinity to lead to the observed change in substrate binding order. The fact that the order of product release also changes suggests that the off constant for release of $H_2PteGlu_2$ product from the ternary enzyme–product complex also decreases at least 25-fold relative to the rate constant for release of monoglutamate product. For substrates with two or more glutamyl residues the K_m for the folate substrate is approximately equal to the dissociation constant for binding of the substrate to free enzyme, since for all these substrates $K_{ma} = K_{ia}$. Inspection of Table IV will indicate little change in the K_{ia} values for folate polyglutamates with more than two glutamyl residues. The small differences in K_i values observed when $PteGlu_n$ inhibitors with more than two glutamyl residues bind to the E–dUMP binary complex must therefore be reflected in ligand synergism or in the rates of individual catalytic steps rather than in the binding of the folate substrate to the free enzyme. The decrease in relative V_{max} values observed with polyglutamate substrates is also consistent with increasingly slow and rate-limiting product dissociation. Thus, our studies are fully consistent with the major effect of the binding energy of the second glutamyl residue being exerted on the off constant for dissociation of folate substrate from either binary or ternary complexes (k_6 and k_4) and on the off constants for dissociation of product from the binary or ternary enzyme product complexes with little or no effect on ligand synergism. The weaker binding energies associated with more distal glutamyl residues do not appear to affect dissociation of folate substrate directly and presumably lead to small perturbations in ligand synergism or in the catalytic rates of individal catalytic steps.

References

Baggott, J. E., & Krumdieck, C. L. (1979) *Biochemistry 18*, 1036–1041.

Bisson, L. F., & Thorner, J. (1981) *J. Biol. Chem. 256*, 12456–12462.

Blakley, R. L. (1960a) *Biochem. J. 74*, 71–82.

Blakley, R. L. (1960b) *Nature (London) 188*, 231–232.

Brown, J. P., Davidson, G. E., & Scott, J. M. (1974) *Biochim. Biophys. Acta 343*, 78–88.

Conway, J. G., Kauffman, F. C., & Thurman, R. G. (1983) *J. Biol. Chem. 258*. 3825–3831.

Danenberg, P. V., & Danenberg, K. D. (1978) *Biochemistry 17*, 4018–4024.

Daron, H. H., & Aull, J. L. (1978) *J. Biol. Chem. 253*, 940–945.

Dolnick, B. J., & Cheng, Y.-C. (1977) *J. Biol. Chem. 252*, 7697–7703.

Dolnick, B. J., & Cheng, Y.-C. (1978) *J. Biol. Chem. 253*, 3563–3567.

Dwivedi, C. M., Kisliuk, R. L., & Baugh, C. M. (1983) in *Folyl and Antifolyl Polyglutamates* (Goldman, I. D., Chabner, B. A., & Bertino, J. R., Eds.) pp 65–74, Plenum Press, New York.

Eto, I., & Krumdieck, C. L. (1981) *Anal. Biochem. 115*, 138–146.

Eto, I., & Krumdieck, C. L. (1982) *Life Sci. 30*, 183–189.

Fernandes, D. J., & Bertino, J. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5663–5667.

Foo, S. K., & Shane, B. (1982) *J. Biol. Chem. 257*, 13587–13592.

Friedkin, M., Plante, L. T., Crawford, E. J. & Crumm, M. (1975) *J. Biol. Chem. 250*, 5614–5621.

Galivan, J. H., Maley, F., & Baugh, C. M. (1976a) *Biochem. Biophys. Res. Commun. 71*, 527–534.

Galivan, J. H., Maley, G. F., & Maley, F. (1976b) *Biochemistry 15*, 356–362.

Hillcoat, B. L., Nixon, P. F., & Blakley, R. L. (1967) *Anal. Biochem. 21*, 178–189.

Jackson, R. C. (1978) *J. Biol. Chem. 253*, 7440–7446.

Kisliuk, R. L., Gaumont, Y., Baugh, C. M., Galivan, J. H., Maley, G. F. & Maley, F. (1979) in *Chemistry and Biology of Pteridines* (Kisliuk, R. L., & Brown, G. M., Eds.) pp 431–435, Elsevier/North-Holland, New York.

Kisliuk, R. L., Gaumont, Y., Lafer, E., Baugh, C. M., & Montgomery, J. (1981) *Biochemistry 20*, 929–934.

Krumdieck, C. L., & Baugh, C. M. (1969) *Biochemistry 8*, 1568–1572.

Krumdieck, C. L., & Baugh, C. M. (1980) *Methods Enzymol. 66*, 523–529.

Lomax, M. I. S., & Greenberg, G. R. (1967) *J. Biol. Chem. 242*, 1302–1306.

Lorenson, M. Y., Maley, G. F., & Maley, F. (1967) *J. Biol. Chem. 242*, 3332–3344.

Matthews, R. G., & Baugh, C. M. (1980) *Biochemistry 19*, 2040–2045.

Matthews, R. G., Ross, J., Baugh, C. M., Cook, J. D., & Davis, L. (1982) *Biochemistry 21*, 1230–1238.

Priest, D. G., & Mangum, M. (1981) *Arch. Biochem. Biophys. 210*, 118–123.

Priest, D. G., Happel, K. K., Mangum, M., Bednarek, J. M., Doig, M. T., & Baugh, C. M. (1981) *Anal. Biochem. 115*, 163–169.

Ross, J., Green, J., Baugh, C. M., MacKenzie, R. E., & Matthews, R. G. (1984) *Biochemistry 23*, 1796–1801.

Schilsky, R. L., Jolivet, J., Bailey, B. D., & Chabner, B. A. (1983) in *Folyl and Antifolyl Polyglutamates* (Goldman, I. D., Chabner, B. A., & Bertino, J. R., Eds.) pp 247–257, Plenum Press, New York.

Comparison of DNA Binding Properties of Activated, Covalent and Noncovalent Glucocorticoid Receptor–Steroid Complexes from HTC Cells[†]

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ABSTRACT: Several differences in the interaction with DNA of noncovalent vs. covalent glucocorticoid receptor–steroid complexes are described. HTC cell glucocorticoid receptors were incubated under cell-free conditions with the potent reversible glucocorticoid dexamethasone and with the irreversible antiglucocorticoid dexamethasone 21-mesylate to yield noncovalent and covalent complexes, respectively. Using DNA immobilized on cellulose, we found that the noncovalent dexamethasone complexes were activated (by dilution in pH 8.8 buffer at 0 °C) to a DNA binding species 2-fold faster than were covalent dexamethasone 21-mesylate labeled complexes. The affinity of activated, noncovalent dexamethasone complexes for DNA in an equilibrium binding assay was 2-fold higher than that of the activated, covalent dexamethasone 21-mesylate complexes. This conclusion was supported by the observations in a DNA–cellulose pellet assay that covalent receptor–steroid complex binding to DNA was disrupted by lower NaCl concentrations than was noncovalent complex binding. The same studies of DNA binding at various NaCl concentrations failed to provide evidence that glucocorticoid receptor–steroid complex binding to DNA is a multistep process. These quantitative distinctions in the DNA binding properties of covalent and noncovalent receptor–steroid complexes represent the first physicochemical differences between the complexes of antiglucocorticoid and glucocorticoid steroids and may partially account for their divergent biological properties.

Antiglucocorticoids which bind to glucocorticoid receptors are potentially useful probes of the mechanism of action of glucocorticoid steroids. Unfortunately this line of research has been severely limited by the facts that most antiglucocorticoids (1) have a low affinity for, and a rapid rate of dissociation from, the receptor protein and (2) interact with other receptor proteins (Samuels & Tomkins, 1970; Raynaud et al., 1980; Svec & Rudis, 1982). We therefore have been