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7,8-Dihydropteroyl Oligo-γ-L-glutamates: Synthesis and Kinetic Studies with Purified Dihydrofolate Reductase from Mammalian Sources†

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ABSTRACT: The synthesis of 7,8-dihydropteroyl tri-, penta-, and heptaglutamate has been accomplished by standard solution peptide coupling, followed by dithionite reduction of the pterin moiety. These compounds were tested as substrates for dihydrofolate reductase (EC 1.5.1.3) obtained in highly purified form from four mammalian cell types: human acute myelogenous and acute lymphocytic leukemia cells, a methotrexate resistant murine L1210 leukemia, and erythrocytes from a patient with polycythemia vera treated with methotrexate. In general, the dihydro polyglutamates were as good as, or better substrates (lower K_m, higher V_{max}) than, the corresponding monoglutamate forms. These data, in conjunction with recent evidence demonstrating that intracellular folates exist predominantly as polyglutamate forms, strengthen the concept that folate polyglutamates may be the naturally occurring coenzymes in mammaliantissues.

It is now well accepted that folate coenzymes in most natural sources examined occur as pteroyl derivatives containing one to seven glutamate residues. For example, *Clostridium acidiurici* contains predominantly 5,10-methenyltetrahydropteroyl triglutamate (Curthoys *et al.*, 1972) and yeast contains predominantly 5-methyltetrahydropteroyl heptaglutamate (Pfiffner *et al.*, 1946). Among the mammalian species studied, rat liver has been shown to contain predominantly reduced forms of pteroyl pentaglutamate (Shin *et al.*, 1972; Houlihan and Scott, 1972), and sheep liver folates have been identified as reduced polyglutamate derivatives (Osborne-White and Smith, 1973). Despite this awareness, most of the studies of folate-dependent enzymes have utilized monoglutamate coenzymes, since the appropriate polyglutamate coenzyme derivatives have not been readily available. In addition, most tissues contain highly active conjugase enzymes (γ-glutamyl carboxypeptidases) that, unless removed, make difficult the interpretation of results using the polyglutamyl derivatives. When the activities of oligoglutamyl folate coenzymes have been examined using certain bacterial extracts, they have been shown to have equivalent or greater affinities for the enzymes than the monoglutamate forms (Salem and Foster, 1972). Of interest also has been the elucidation of a B_{12}-independent pathway of methionine biosynthesis in certain microorganisms that requires 5-methyltetrahydropteroyl triglutamate for activity (Taylor and Weissbach, 1973). Although data using folate polyglutamates with mammalian enzymes are limited, one study carried out with serine hydroxymethylase from rabbit liver showed a higher affinity of tetrahydropteroyl triglutamate as compared to tetrahydro monoglutamate (Blakely, 1957). In addition, several groups of investigators (Morales and Greenberg, 1964; Greenberg *et al.*, 1966; Plante *et al.*, 1967) have reported that 7,8-dihydropteroyl triglutamate is a slightly better substrate for dihydrofolate reductase than is 7,8-dihydrofolate.

In this communication, we describe the chemical synthesis of 7,8-dihydropteroyl oligoglutamates, and their activity as substrates for the enzyme dihydrofolate reductase (EC 1.5.1.3) from mouse and human leukemia cells and from human erythrocytes. In addition to providing kinetic data with which to assess the contribution of the substrate glutamate residues, this

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TABLE I: Properties of Blocked and Partially Deblocked Poly-γ-glutamate Derivatives (Solution Synthesis).

Compound	Tlc R_F Solvent System[a] A	B	% Yield[b]	Mp (°C)
Z-L-Glu_2-(O-*t*-Bu)$_3$	0.86	0.71	95	Oil
H-L-Glu_2-(O-*t*-Bu)$_3$	0.36	0.11		Oil
Z-L-Glu_3-(O-*t*-Bu)$_4$[c]	0.85	0.54	97	72–75
H-L-Glu_3-(O-*t*-Bu)$_4$	0.30	0.07		Oil
Z-L-Glu_4-(O-*t*-Bu)$_5$	0.78	0.45	82	72–74
H-L-Glu_4-(O-*t*-Bu)$_5$	0.22	0.04		Oil
Z-L-Glu_5-(O-*t*-Bu)$_6$	0.81	0.26	83	85–90
H-L-Glu_5-(O-*t*-Bu)$_6$	0.17	0.03		Oil
Z-L-Glu_6-(O-*t*-Bu)$_7$	0.78	0.25	95	65–70
H-L-Glu_6-(O-*t*-Bu)$_7$	0.18	0.02		Oil
Z-L-Glu_7-(O-*t*-Bu)$_8$	0.79	0.23	94	75–80
H-L-Glu_7-(O-*t*-Bu)$_8$	0.16	0.01		Oil

[a] Systems A, ethyl acetate; B, ethyl acetate–cyclohexane, 1:1; tlc was done on silica gel No. 6061 Eastman chromatogram sheets. [b] Yields represent overall yields of 2 steps: N-deblocking followed by coupling. [c] Calcd for $C_{39}H_{61}N_3O_{12}$: C, 61.32; H, 8.05; N, 5.50. Found: C, 61.19; H, 7.76; N, 5.41.

enzymic conversion makes possible the synthesis of 5,6,7,8-tetrahydropteroyl oligoglutamates of proper chirality for use in studying other folate-dependent reactions.

Materials and Methods

The synthesis of pteroyl oligoglutamates was carried out by conventional solution techniques as described by Meienhofer and coworkers (Meienhofer *et al.*, 1970; Godwin *et al.*, 1972), with minor modifications. Although the solid phase synthetic route (Krumdieck and Baugh, 1969; Baugh *et al.*, 1970) gave the desired pteroyl oligoglutamates in modest yields, extensive chromatographic purification was required to remove pteroic acid, a major contaminant consistently found in the crude products. For solution synthesis, preparation of the required glutamate *tert*-butyl esters *via* a transesterification reaction between the appropriate glutamic acid derivative and *tert*-butyl acetate (Taschner *et al.*, 1961) gave very low yields of impure products in our hands. However, direct esterification of L-glutamate with isobutylene and sulfuric acid (Kajtar and Hollosi, 1970) gave a 77% yield of the α,γ-di-*tert*-butyl glutamate when chloroform was used in place of dioxane as the reaction solvent. Similarly, esterification of Z[1]-L-glutamic acid γ-methyl ester in methylene chloride with isobutylene and sulfuric acid, followed by alkaline hydrolysis of the methyl ester, gave the key blocked monomer, Z-L-glutamic acid α-*tert*-butyl ester (Kovacs *et al.*, 1968). Pteroic acid was prepared from folic acid by enzyme-catalyzed hydrolysis of the glutamate side chain, using carboxypeptidase G_1 (McCullough *et al.*, 1971). The product isolated had spectral and chromatographic properties in accord with those previously reported for pteroic acid prepared by other methods (Levy and Goldman, 1967; Pratt *et al.*, 1968). Physical properties of totally blocked and N-deblocked oligopeptides of glutamic acid are given in Table I. Similar data for three pteroic acid conjugates and their totally blocked synthetic precursors are given in Table II. Melting points of the compounds in Table I vary depending on the method of preparation (Meienhofer *et al.*, 1970), and therefore, thin-layer chromatography (tlc) data were used extensively to establish the homogeneity of the product after each deblocking and coupling reaction. The tlc data of Table II (solvents A and B) for the free pteroyl tri-, penta-, and hepta-γ-glutamates compare favorably with previously reported data obtained using ascending paper chromatography (Godwin *et al.*, 1972).

The reduced 7,8-dihydro forms of the pteroyl tri-, penta-, and heptaglutamates were synthesized by a modification of Friedkin's method for the reduction of folic acid to dihydrofolic acid (Friedkin *et al.*, 1962). Approximately 4 μmol of the oxidized form were passed through a 0.9 × 12 cm column of Sephadex G-15, using water as the eluent, to remove ammonium acetate which resulted from the synthesis of the oxidized polyglutamates. This solution was then adjusted to pH 7.5 with 0.1 N KOH and made 0.2 M with respect to 2-mercaptoethanol. Sodium dithionite was added and stirred vigorously at room temperature in the dark. Spectral changes were followed at each step using the Cary, Model 15, recording spectrophotometer. The known shift in ultraviolet absorbance (Rabinowitz, 1960) from the oxidized (346 nm) to the 7,8-dihydro form (304 nm) was used to monitor the progress of the reduction. Concentrations were calculated using the extinction coefficients for folic acid (λ_{max} 282 nm at p 7.0, ϵ 27.6 × 10^3) (Rabinowitz, 1960), assuming that the polyglutamates have the same spectral properties as the monoglutamate (Godwin *et al.*, 1972). It was found that as the number of glutamates increased, it was necessary to increase the amount of dithionite and the time of incubation to allow the reaction to go to completion. Table III indicates the amount of dithionite and length of time found necessary to obtain maximum reduction to the dihydro form.

After reduction had gone to completion, the solution was adjusted to pH 8.5 with 0.1 M KOH. Buffer, 2-mercaptoethanol, and NaCl were also adjusted to that of the starting column concentrations. This solution was then chromatographed on a 0.9 × 25 cm DEAE-Sephadex A-25 column which had been equilibrated with 5 mM Tris-HCl (pH 8.5) containing 50 mM 2-mercaptoethanol and 0.2 M NaCl. Previous to application of the sample, a portion of the equilibration buffer was collected for use as a spectral blank when monitoring the column effluent. A gradient consisting of 250 ml of 5 mM Tris-HCl (pH 8.5) containing 50 mM 2-mercaptoethanol and 0.2 M NaCl, and 250 ml of 5 mM Tris-HCl (pH 8.5) containing 50 mM 2-mercaptoethanol and 0.7 M NaCl in the mixing chamber were employed. Fractions of 2.5 ml were collected and their ultraviolet spectra recorded in order to locate the product. A schematic representation of the elution profiles of the 7,8-dihydropteroyl polyglutamates is shown in Figure 1. Percentage of recovery varied from 82.5% for the monoglutamate; 40% for the triglutamate; 26.5% for the pentaglutamate; and 56.8% for the heptaglutamate. It should be emphasized that the solution synthesis of oxidized pteroyl poly-γ-glutamates precludes any contamination of the product by oligomers of shorter chain length than desired. Therefore, we can be confident of the number of glutamate residues in each of the reduced pteroyl oligoglutamates of Figure 1.

Enzyme Source. Dihydrofolate reductase was isolated from four mammalian cell types. Peripheral blast cells of a patient with untreated acute myelogenous leukemia (AML) were obtained from peripheral blood. An enlarged spleen of a patient with acute lymphatic leukemia (ALL) was obtained 4 hr after

[1] Abbreviations used are: Z, carbobenzyloxy; AML, acute myelogenous leukemia; ALL, acute lymphatic leukemia; MTX, methotrexate; Tfa, trifluoroacetyl.

TABLE II: Properties of Pteroyl Oligo-γ-glutamates and Their Blocked Derivatives.

Compound	Tlc R_F Solvent System[a] A	B	C	D	% Yield
Folic acid[b]	0.35	0.44			
Pteroic acid[b]	0.09	0.11	0.13	0.01	89
N^2,N^{10}-Bis-Tfa-pteroic acid[c]	0.86[d]	0.80[d]	0.68[d]	0.72[d]	64
N^2,N^{10}-Bis-Tfa-pteroyl-γ-Glu_3(O-*t*-Bu)$_4$	0.01[d]	0.01[d]	0.75[d]	0.78[d]	69
Pteroyl-γ-Glu_3[e]	0.72	0.69	0.03	0.03	43
N^2,N^{10}-Bis-Tfa-pteroyl-γ-Glu_5(O-*t*-Bu)$_6$	0.03[d]	0.03[d]	0.88[d]	0.75[d]	48
Pteroyl-γ-Glu_5[e]	0.88	0.82	0.02	0.02	37
N^2,N^{10}-Bis-Tfa-pteroyl-γ-Glu_7(O-*t*-Bu)$_8$	0.03[d]	0.03[d]	0.89[d]	0.77[d]	73
Pteroyl-γ-Glu_7[e]	0.94	0.93	0.02	0.02	79

[a] Eastman cellulose chromatogram sheets (No. 6064) were used for tlc in systems A, 5% NH_4HCO_3; and B, 5% Na_2HPO_4. For systems C, methanol, and D, butanol–acetic acid–water (4:1:1), silica gel (Eastman No. 6061) was used. [b] Folic acid and pteroic acid are included for comparison. [c] Calcd for $C_{18}H_{10}F_6N_6O_5$: C, 42.90; H, 1.99; N, 16.70. Found: C, 42.48; H, 2.53; N, 16.61. [d] Fluorescent spot. All Tfa derivatives gave fluorescent spots. Figures represent the R_F of the major spots in each system. Some fluorescent impurity was also present as a trace tailing contaminant in each system. [e] Final deblocking of the Tfa group was accomplished with 1 N NH_4OH.

the patient expired and served as the source for the ALL enzyme. Erythrocytes were obtained from 500 ml of heparinized blood from a patient with polycythemia vera 7 days after treatment with 25 mg of methotrexate (Bertino *et al.*, 1970). L1210 leukemia cells from a methotrexate resistant strain (L1210/MTX) were harvested from 100 mice 7 days after inoculation of each mouse with 10^6 cells. After cells were lysed by freeze–thawing three times, the extracts were centrifuged at 37,000*g* for 30 min. The supernatant solutions were clarified by adjusting the pH to 5.0 and centrifuging (37,000*g*, 10 min). The latter supernatant solution was adjusted to pH 6.0 and the enzymes were purified by affinity chromatography, using MTX bound to Sepharose (Makulu *et al.*, 1973). As eluted from the column, the enzymes had a specific activity of 46 μmol $min^{-1}mg^{-1}$ (pH 7.5, 37°), and were greater than 95% pure as estimated by titration with methotrexate (Perkins *et al.*, 1967). These preparations were totally free of conjugase (γ-glutamyl carboxypeptidase) activity (Krumdieck and Baugh, 1970).

Assay of Enzyme Activity. The assay of enzyme activity using the nonreduced folate forms, as well as the assay using the dihydropteroyl polyglutamates, was carried out spectrophotometrically, measuring the decrease in absorbancy that occurs when either the pteroyl or dihydropteroyl moiety is converted to tetrahydropteroyl form, and NADPH is converted to NADP. For conversion of the oxidized folate ϵ 18.3×10^3 (Zakrzewski *et al.*, 1966), and for the dihydro forms, ϵ 12×10^3 (Bertino *et al.*, 1965), were used. The low enzyme activity and low substrate concentrations required the use of the expanded 0–0.5 scale of the Gilford spectrophotometer. The assay contained, in a final volume of 1 ml: Tris-HCl buffer (pH 7.5), 100 μmol; KCl, 150 μmol; NADPH, 0.1 μmol; and sufficient enzyme to give an absorbance change with the oxidized pteroyl monoglutamate of 0.005 unit/min. The reaction was initiated by adding the pteroyl oligoglutamate last, to give a final reaction mix of pH 7.78 ± 0.05. Appropriate blanks minus substrate were run. When the dihydropteroyl compounds were used, only one-tenth the enzyme activity was needed. In order to be able to compare kinetic data obtained using the nonreduced pteroyl oligoglutamates with those obtained using the dihydro derivatives, all enzyme assays were carried out at pH 7.8, even though the pH optimum for nonreduced forms is 6.0.

TABLE III: Reaction Conditions for Reduction of Pteroyl Oligoglutamates.[a]

	Moles of $Na_2S_2O_4$/mole	Length of time (min)
$Pte(Glu)_1$	27.3	30
$Pte(Glu)_3$	118.0	60
$Pte(Glu)_5$	137.0	75
$Pte(Glu)_7$	193.0	90

[a] Pte, pteroyl.

Results and Discussion

Despite the high affinity of the dihydropteroyl compounds

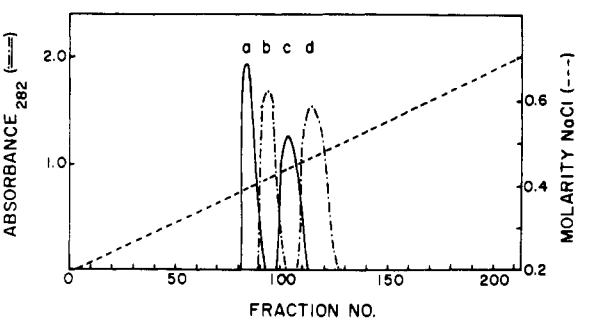

FIGURE 1: Schematic representation of the elution of 7,8-dihydropteroyl γ-glutamates on DEAE-Sephadex A-25 as described in the text: (a) monoglutamate, (b) triglutamate, (c) pentaglutamate, (d) heptaglutamate.

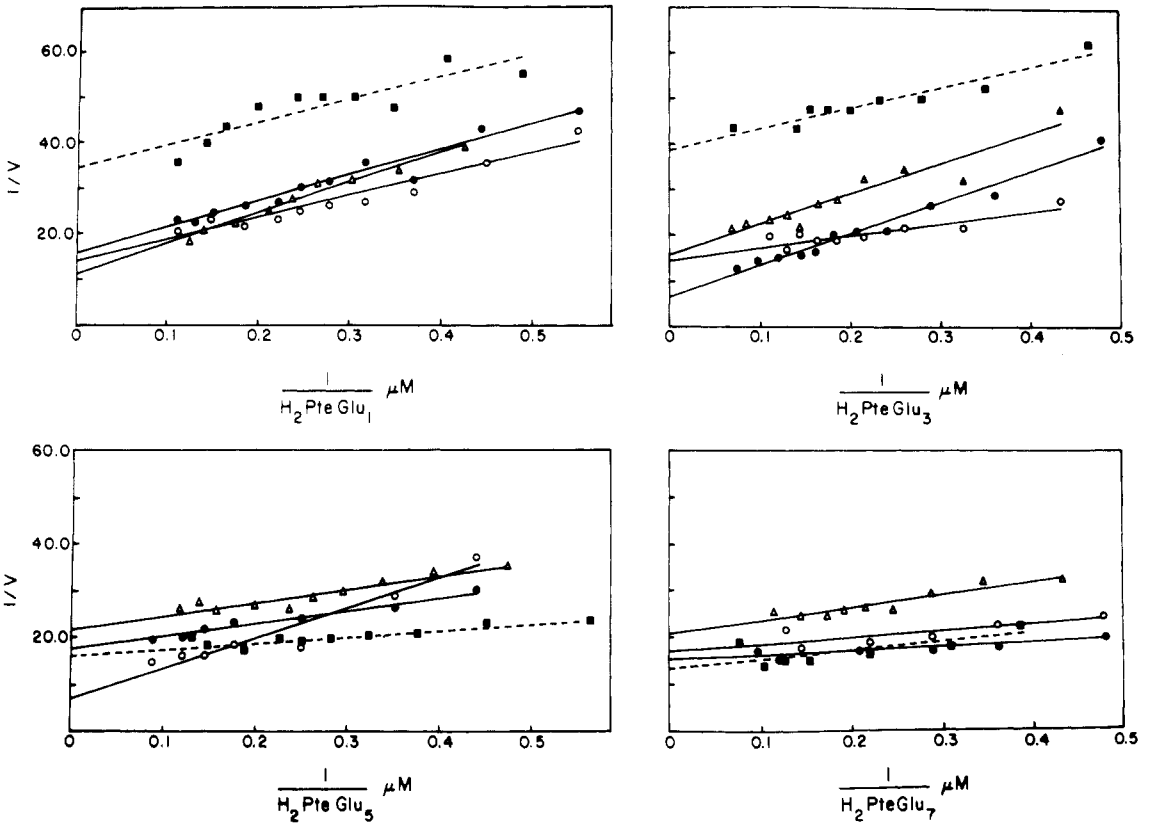

FIGURE 2: Kinetic data for the reduction of 7,8-dihydropteroyl oligo-γ-glutamates by dihydrofolate reductase from acute myelogenous leukemia (AML), ●; acute lymphatic leukemia (ALL), ○; erythrocytes, △; and L1210 leukemia cells from a methotrexate resistant strain (L1210/MTX), ■. Velocity is in units of ΔOD_{340} per 5 min.

for the dihydrofolate reductase preparations studied, reproducible K_m values could be obtained by expanding the recorder scale and working with low levels of enzyme activity. The best fit lines from the data were plotted according to the method of Lineweaver and Burk, using an unweighted least-squares program on an IBM 1130 computing system (Figure 2). Each point on the graph represents an average of at least two determinations. Substrate concentrations of greater than 15 μM were not plotted. At this level and higher, using the dihydropteroyl compounds, substrate inhibition was noted. From these plots, K_m and V_{max} values for the oxidized (Table IV) and dihydropteroyl oligoglutamate substrates (Table V) were obtained. All kinetic constants were determined in at least two separate experiments. V_{max} values were highly reproducible (deviation of less than 10% from the mean); reproducibility of K_m values for the dihydropteroyl oligoglutamates were within the same error limits. In the case of the oxidized pteroyl oligoglutamates, somewhat poorer reproducibility (less than 20%) was oserved in the K_m determination.

TABLE IV: Michaelis Constants and V_{max} Values for Oxidized Pteroyl Oligo-γ-L-glutamates.[a]

Enzyme Source	Pte(Glu)$_1$	Pte-(Glu)$_3$	Pte(Glu)$_5$	Pte-(Glu)$_7$
	A. K_m Values (μM)			
L1210/MTX	20	81	120	21
Erythrocyte	47	52	28	28
AML	74	33	49	39
ALL	73	18	16	36
	B. V_{max} Values (μmol hr^{-1} mg^{-1})			
L1210/MTX	7.6	29	15	9.0
Erythrocyte	3.6	12	4.8	3.4
AML	1.5	1.4	2.2	1.5
ALL	9.6	24	10.0	5.0

[a] Pte, pteroyl.

Nonreduced Forms as Substrates. The L1210 and the erythrocyte enzyme had approximately the same affinity for the polyglutamate forms as pteroyl monoglutamate. The human leukemia enzymes, in particular the ALL, had higher affinity (lower K_m values) for the polyglutamate coenzymes as compared to pteroyl monoglutamate. When V_{max} values were compared for three of the four enzymes studied (L1210/MTX, erythrocyte, and ALL), the pteroyl triglutamate gave a significantly higher rate than the other forms. The penta- and heptaglutamates gave rates approximately the same as the monoglutamate.

Dihydropteroyl Glutamates as Substrates. As we anticipated from previous studies, the K_m values obtained for dihydropteroyl glutamate were an order of magnitude less than for the pteroyl glutamate for all of the enzymes. This relationship also held for the dihydropteroyl oligoglutamates tested, with differences noted between enzymes. For example, the K_m values obtained for the L1210/MTX enzyme were the lowest obtained for all of the folate forms for any enzyme; the values were approximately equivalent for each of the dihydro forms. The erythrocyte enzyme had a smaller K_m value for the dihydropteroyl penta- and heptaglutamate coenzymes as compared to the mono- and triglutamate coenzymes. For the AML enzyme a decreased affinity was noted with the triglutamyl coen-

TABLE V: Michaelis Constants and V_{max} Values of 7,8-Dihydropteroyl Oligo-γ-L-glutamates.

Enzyme Source	H_2Pte-$(Glu)_1$	H_2Pte-$(Glu)_3$	H_2Pte-$(Glu)_5$	H_2Pte-$(Glu)_7$
	A. K_m Values (μM)			
L1210/MTX	1.4	1.2	0.8	1.3
Erythrocyte	5.9	4.2	1.3	1.3
AML	3.6	10.0	1.5	0.6
ALL	3.5	1.8	9.3	0.8
	B. V_{max} Values (μmol hr^{-1} mg^{-1})			
L1210/MTX	190	170	403	496
Erythrocyte	285	202	145	153
AML	60	146	56	65
ALL	480	447	953	386

[a] H_2Pte, 7,8-dihydropteroyl.

zyme as compared to the monoglutamate, whereas the penta and hepta again showed an increased affinity for the enzyme. The ALL enzyme differed from the AML enzyme in that the pentaglutamate coenzyme demonstrated the largest K_m value of the four polyglutamates tested. The lowest affinities were obtained with the tri- and heptaglutamates. Thus, the human enzymes studied all have different K_m profiles for the dihydro compounds; further investigations of differences in structure or properties of these enzymes are in progress.

V_{max} Values. When V_{max} values were compared for the enzymes using the dihydro forms as substrates, higher activities were noted with the penta and hepta coenzymes as compared to the mono- and triglutamate forms for the L1210 enzyme. The highest V_{max} values noted with the AML and ALL enzyme were with the triglutamyl and pentaglutamyl forms, respectively.

The data presented strengthen the concept that polyglutamate forms of folate coenzymes may be the functional intracellular folate forms. With only two exceptions, the tri, penta, and hepta coenzymes had at least the same, and usually a greater, affinity for the four enzymes studied. Measurement of folate forms in erythrocytes (Noronha and Aboobaker, 1963), L1210 cells (Nixon *et al.*, 1973), and human leukemia cells (Swendshed *et al.*, 1951) has established presumptive evidence that the majority of the folate coenzymes present are in the polyglutamate form. Since plasma contains potent conjugase activity, it would appear that folates are transported to these cells at the monoglutamate level (presumably 5-methyltetrahydrofolate), and that folate polyglutamates are synthesized within the cell.

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