Coenzyme B_{12} Dependent Diol Dehydrase System. Dissociation of the Enzyme into Two Different Protein Components and Some Properties of the Components†

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ABSTRACT: Diol dehydrase from *Aerobacter aerogenes* was dissociated into two dissimilar protein components or subunits, designated components F and S, by chromatography on DEAE-cellulose. Neither component alone possessed any appreciable activity, and the diol dehydrase activity was restored when the two components were combined, indicating that they are subunits of a single enzyme. Both components were also required for inactivation of coenzyme B_{12} by oxygen when incubated aerobically with coenzyme B_{12} in the absence of substrate. These lines of evidence suggest that the activation of the cobalt-carbon bond of the coenzyme moiety is dependent on both components. The more acidic component, component S, was a sulfhydryl protein sensitive to an alkylating agent, iodoacetamide. Sephadex G-25 filtration experiments showed that neither coenzyme B_{12} nor cyanocobalamin was bound by the individual components, F or S—that is, both of them were required for the cobamide binding. The presence of substrate, 1,2-propanediol, in eluting buffer retarded the dissociation of the enzyme, suggesting that the substrate strongly promotes the association of the components. Both components were thermally unstable, and coenzyme B_{12} did not protect them from heat denaturation. As compared to component S, component F was relatively unstable even at 0° to almost the same degree as the apoenzyme. Of the compounds tested, only 20% glycerol showed a marked stabilizing effect on component F.

Diol dehydrase (DL-1,2-propanediol hydro-lyase, EC 4.2.1.28) from *Aerobacter aerogenes* (ATCC 8724) is a coenzyme B_{12}[1] requiring enzyme which catalyzes the conversion of L- or D-1,2-propanediol to propionaldehyde and of 1,2-ethanediol to acetaldehyde (Lee and Abeles, 1963). The coenzyme B_{12} dependent enzymes known at present are divided into two general types (Stadtman, 1971). One type of enzyme appears to consist of similar subunits having sulfhydryl groups and cobamide binding sites on the same subunit (Cannata *et al.*, 1965; Kellermeyer *et al.*, 1964; Kaplan and Stadtman, 1968; Kung and Stadtman, 1971; Goulian and Beck, 1966), and the other type of enzymes is made up of two dissimilar protein moieties (Switzer and Barker, 1967; Suzuki and Barker, 1966; Schneider and Pawelkiewicz, 1966; Stadtman and Renz, 1968; Morley and Stadtman, 1970; Galivan and Huennekens, 1970). In the latter group one of the two protein components binds the cobamide and the other is a sulfhydryl protein; both are required for catalysis of the overall reactions. Diol dehydrase has been generally considered to belong to the former group (Lee and Abeles, 1963; Stadtman, 1971).

During the course of an investigation (Toraya *et al.*, 1971, 1972; Toraya and Fukui, 1972) on the ligand interaction with apodiol dehydrase, we found that this enzyme can be dissociated into two protein fractions by chromatography on DEAE-cellulose. The individual fractions were catalytically inactive, whereas diol dehydrase activity was restored on combination of the two fractions. To obtain new information regarding the ligand interaction with this B_{12} enzyme, a detailed study on the apoenzyme components has been carried out. This report describes the separation of the apoenzyme into two different protein components and some properties of the components.

Materials and Methods

Materials. The crystalline coenzyme B_{12} and CNB_{12} were obtained from Glaxo Ltd., Greenford, U. K. All other chemicals were reagent grade commercial products and were used without further purification. Diol dehydrase apoenzyme was prepared from *Aerobacter aerogenes* (ATCC 8724) by the procedure similar to that of Lee and Abeles (1963), and subjected to chromatography on DEAE-cellulose after dialysis against 0.01 M potassium phosphate buffer (pH 8.0).

Enzyme Assay. The activity of the diol dehydrase apoenzyme was assayed as described previously (Toraya *et al.*, 1971). The protein components (F and S) of diol dehydrase were estimated by the same assay by adding an excess of one component and making the other rate limiting. One unit is defined as the amount of enzyme activity catalyzing the formation of 1 μmol of propionaldehyde/min under the above assay conditions (Lee and Abeles, 1963). Specific activities of the components F and S were determined on the basis of the amount of protein in their fractions. Protein was routinely determined by the procedure of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as the standard.

Results

Fractionation of Apoenzyme into Components F and S. About 100 units of a partially purified preparation of apodiol dehydrase was dialyzed overnight against 2 l. of 0.01 M potassium phosphate buffer (pH 8.0) and then applied to a column (2.4 × 11 cm) of DEAE-cellulose equilibrated previously with the same buffer. After being washed with 150 ml of the same buffer, the protein was eluted by a linear gradient in which 450 ml of 0.01 M potassium phosphate buffer (pH 8.0) was in the mixing chamber and 450 ml of 0.01 M potassium phosphate

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[1] Abbreviations used are: coenzyme B_{12}, α-(5,6-dimethylbenzimidazolyl)-*Co*-5′-deoxyadenosylcobamide or 5′-deoxyadenosylcobalamin; CNB_{12}, cyanocobalamin.

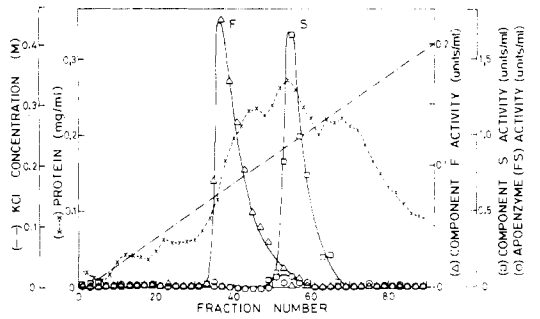

FIGURE 1: Dissociation of diol dehydrase apoenzyme into components F and S by chromatography on DEAE-cellulose. The activities of the apoenzyme (○) and component F (△) were measured in 0.2 ml of each fraction. Component S (□) activity was determined in 0.02 ml of each fraction. Other experimental details are given in the text.

buffer (pH 8.0) with 0.40 M KCl was in the reservoir. The eluate was collected in 10-ml fractions at a flow rate of about 0.7 ml/min. As shown in Figure 1, no fractions alone possessed any significant activity, whereas fractions 35–49 were catalytically active when assayed by adding a certain amount of fraction II (fractions 58–77 were pooled). Similarly, fractions 51–67 restored the enzyme activity in combination with fraction I (fractions 30–49 were pooled). These results indicate that diol dehydrase apoenzyme was resolved by DEAE-cellulose chromatography into two different protein components. Neither of them, by itself, has any appreciable catalytic activity, but together they can be reconstituted into the active apoenzyme complex. The components eluted firstly and secondly from the column will be referred to as components F and S, respectively. Fractions I and II were used as sources of components F and S for the following experiments. Fractions 50–57 were not used because they were contaminated with a trace activity of the apoenzyme complex (FS). Conditions for the complete resolution were rather delicate, since there existed only a small difference in acidity between the two protein components. The presence of substrate, 1,2-propanediol, in the eluting buffer prevented the apoenzyme from dissociation into components (data not shown), suggesting that the substrate plays an important role in association of components F and S.

The effect on the enzyme activity of increasing amounts of

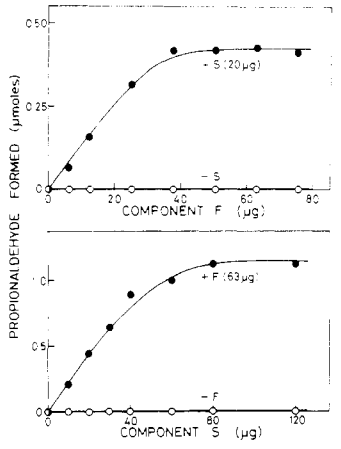

FIGURE 2: Effect of concentration of components F (A) and S (B) on diol dehydrase activity in the presence and absence of a fixed level of the other component. The enzyme activity of the indicated amount of component F or S (○) alone and (●) together with 20 μg of component S or 63 μg of component F was assayed as described in the text. The enzyme reaction was carried out at 37° for 10 min.

TABLE I: Effects of 1,2-Propanediol, Mercaptoethanol, and Coenzyme B_{12} on Stabilities of the Components and the Apoenzyme Complex.[a]

Component(s)	Addition	μmol of Propionaldehyde Formed (% Act. Remaining) after Standing for 2 hr at 0°	37°
Component F	None	0.41 (93)	0.22 (50)
	1,2-Propanediol	0.38 (86)	0.21 (48)
	Mercaptoethanol	0.39 (89)	0.23 (52)
	Coenzyme B_{12}	0.43 (98)	0.24 (55)
Component S	None	0.44 (100)	0.41 (93)
	1,2-Propanediol	0.41 (93)	0.40 (91)
	Mercaptoethanol	0.43 (98)	0.41 (93)
	Coenzyme B_{12}	0.45 (102)	0.43 (98)
Apoenzyme complex (FS)	None	0.44 (100)	0.26 (59)
	1,2-Propanediol	0.41 (93)	0.42 (95)
	Mercaptoethanol	0.44 (100)	0.24 (55)
	Coenzyme B_{12}	0.45 (102)	0.04 (9)
Control		0.44 (taken as 100%)	

[a] Components F (85 μg) and/or S (35 μg) were incubated at 0 or 37° for 2 hr with either of 20 μmol of 1,2-propanediol, 10 μmol of mercaptoethanol, or 15 nmol of coenzyme B_{12}. The activity of the samples was determined by adding the remaining reactants of the usual assay mixture. The enzyme reaction was carried out at 37° for 20 min.

one component in the presence and absence of a fixed level of the other component was examined. Typical enzyme saturation curves are shown in Figure 2. Since component F was relatively unstable as described later, the exact stoichiometric ratio has not as yet been established.

Stabilities of Components F and S. Table I summarizes the effects of 1,2-propanediol, mercaptoethanol, and B_{12} coenzyme on stabilities of the apoenzyme and its components. Component F was relatively unstable and lost about 50% of the activity after being incubated at 37° for 2 hr. None of the substrate, mercaptoethanol, and coenzyme could stabilize this component. The half-life of the component F activity at 0–5° was only about 3–4 days. On the other hand, component S was a relatively stable protein, and less than 10% of the original activity was lost in 2 hr at 37°. When both components were combined, the stability of the apoenzyme complex was influenced by that of the less stable component (F). However, in clear contrast to component F alone, the apoenzyme complex (FS) was markedly stabilized by the substrate. These data may suggest that both components are necessary for the substrate binding. Remarkable inactivation of apoenzyme complex by incubating with coenzyme B_{12} in the absence of substrate will be discussed later in detail.

Table II shows the effects of storage conditions on the remaining activities of component F and the apoenzyme complex (FS). Addition of glycerol at a concentration of 20% protected component F markedly.

Thermal Stabilities of Components F and S. Figure 3 depicts thermal stabilities of the components and the apoenzyme complex at different temperatures. Component F was very heat labile, and more than 80% of the activity was lost when heated

TABLE II: Effects of Storage Conditions on Activities of Component F and the Apoenzyme Complex Remaining after 9 Days.[a]

Temp (°C)	Addition	μmol of Propionaldehyde Formed (% Act. Remaining) Component F	Apoenzyme Complex
0–5	None	0.00 (0)	
0–5	20% 1,2-propanediol	0.27 (31)	
0–5	20% glycerol	0.86 (100)	
−18	None	0.56 (65)	0.54 (63)
−18	2% 1,2-propanediol		0.90 (105)
Control (before storage)		0.86 (taken as 100%)	0.86 (taken as 100%)

[a] Component F (63 μg) was stored under various conditions in the presence and absence of component S (20 μg). After 9 days, the activity of the samples was determined by adding the remaining reactants of the usual assay mixture. The enzyme reaction was carried out at 37° for 10 min.

at 50° for 5 min. Component S was also thermally unstable, and more than 50% of the activity was lost at 50°. In contrast, the apoenzyme complex was more stable than either component, suggesting that more rigid conformation resulted from the complex formation. Coenzyme B_{12} could not protect either component from heat denaturation at 50 and 60° (data not shown).

Requirements for Inactivation of Coenzyme B_{12} by Oxygen. When the apoenzyme of diol dehydrase is incubated aerobically with coenzyme B_{12} in the absence of substrate, the enzyme-bound coenzyme reacts with oxygen and the modified coenzyme causes the rapid inactivation of the enzyme by forming the undissociable complex with the apoprotein (Lee and Abeles, 1963; Wagner *et al.*, 1966). This inactivation process involves irreversible dissociation of the cobalt-carbon σ bond of the coenzyme B_{12} which is activated by the interaction with the apoenzyme. The ability of the enzyme to inactivate the coenzyme may be closely related to the catalytic process. Figure 4 illustrates the effects of components F and/or S on inactivation of the coenzyme B_{12} by oxygen. Although neither component alone caused O_2 inactivation of the coenzyme, marked inactivation by oxygen took place when the two components were combined. Together with the requirements for catalytic activity, this result suggests that both components are necessary for activation of the cobalt-carbon linkage of the coenzyme moiety.

TABLE III: Binding of Coenzyme B_{12} by Separate Components, F or S.[a]

Component Incubated with Coenzyme B_{12}	Sp Act. of Component (Units/mg) − Coenzyme	+ Coenzyme	Complex Formation (%)
Component F	0.00	0.49	0
Component S	0.00	2.52	0

[a] A mixture, containing component F (338 μg) or S (141 μg), 600 μmol of 1,2-propanediol, 200 nmol of coenzyme B_{12}, 34 μmol of potassium phosphate buffer (pH 8.0), and 200 μmol of KCl, in a total volume of 3.4 ml, was incubated at 37° for 15 min, and then subjected to gel filtration on Sephadex G-25 (fine) equilibrated previously with 0.05 M potassium phosphate buffer (pH 8.0) containing 0.05 M KCl and 0.10 M 1,2-propanediol (Toraya *et al.*, 1971). After being eluted with the same buffer, the specific activity of component F or S in the protein-containing fractions was determined as described in the text, with (+) or without (−) exogenously added coenzyme B_{12}.

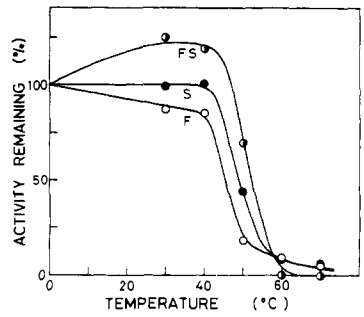

FIGURE 3: Thermal stabilities of component F (○), component S (●), and the apoenzyme complex (FS) (◑) at different temperatures. Components F (85 μg) and/or S (35 μg) were heated for 5 min at the indicated temperatures with 200 μmol of 1,2-propanediol. After the heat treatment, the activity was determined by adding the remaining reactants of the usual assay mixture. The enzyme reaction was carried out at 37° for 10 min.

Cobamide Binding. The diol dehydrase holoenzyme and apoenzyme–coenzyme B_{12} analog complexes are not resolved into the apoenzyme and B_{12} compounds when subjected to gel filtration on Sephadex G-25 using a potassium phosphate buffer containing both KCl and 1,2-propanediol (Toraya *et al.*, 1971, 1972). This procedure was applied to test the ability of the individual components to bind coenzyme B_{12} or its analog. As shown in Table III, neither component alone bound coenzyme B_{12} in such a manner that the coenzyme could not be removed by Sephadex G-25 gel filtration. Since no appreciable inhibition was observed after gel filtration (Table IV), even CNB_{12}, a potent cobamide inhibitor, was not bound by the individual components. These facts indicate that both components F and S were absolutely required for the binding of coenzyme B_{12} or its analog. The association of the components into the apoenzyme complex may generate the cobamide binding site.

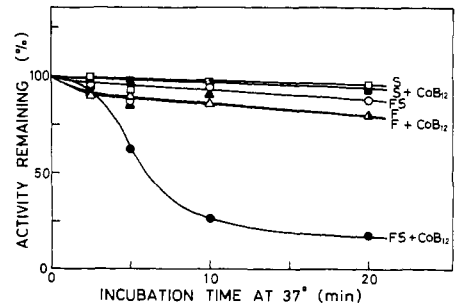

FIGURE 4: Effects of components F and/or S on inactivation of coenzyme B_{12} by oxygen. Components F (85 μg) and/or S (35 μg) were aerobically incubated with or without 15 nmol of coenzyme B_{12} at 37° for the indicated time. The activity was determined by adding the remaining reactants of the usual assay mixture. The enzyme reaction was carried out at 37° for 10 min.

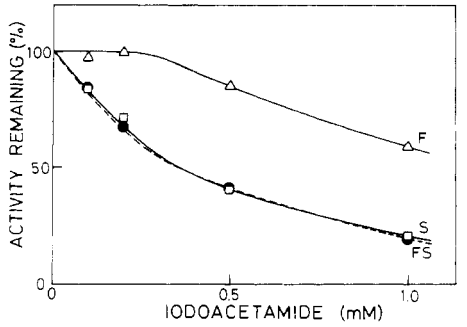

FIGURE 5: Effects of iodoacetamide treatment on activities of component F (△), component S (□), and the apoenzyme complex (●). Components F (63 μg) and/or S (60 μg) were preincubated with the indicated concentrations of iodoacetamide at 37° for 10 min. Then the residual iodoacetamide was destroyed by reaction with an excess of mercaptoethanol (10 mM) at 37° for 20 min. Finally the activity was determined by adding the remaining reactants of the usual assay mixture. The enzyme reaction was carried out at 37° for 12 min.

Effect of Sulfhydryl Inhibitor. It has been reported that diol dehydrase is a sulfhydryl enzyme, and that the apoenzyme is highly sensitive to mercurial or alkylating reagents (Lee and Abeles, 1963; Toraya and Fukui, 1972). It is of interest to know which of the two components or both of them are susceptible to sulfhydryl inhibitors. Figure 5 shows the effect on catalytic activity of the treatment of the components with various concentrations of an alkylating agent, iodoacetamide. At any concentrations tested, component S was inactivated by this treatment to almost the same degree as the apoenzyme complex, whereas component F was relatively insensitive. These data suggest that component S has an alkylating agent-sensitive sulfhydryl group(s) which is essential for catalysis.

Discussion

Experimental data reported here demonstrate that diol dehydrase consists of two dissimilar protein components, both of which are required for catalysis. Starch gel electrophoresis indicates that about 90% of the protein of a highly purified preparation of this enzyme is present in a single component that contains all of the enzyme activity (Lee and Abeles, 1963). Since the buffers used for starch gel electrophoresis contained the substrate (2% 1,2-propanediol), the resolution of the enzyme into protein fractions might be retarded.

TABLE IV: Binding of CNB_{12} by Separate Components, F or S.[a]

Component	Sp Act. of Component (Units/mg)		Complex Formation (%)
	Preincubated without CNB_{12}[b]	Preincubated with CNB_{12}	
F	0.56	0.55	2
S	2.55	2.51	2

[a] Preincubation with CNB_{12} and Sephadex G-25 filtration of each component were performed as described in Table III, except that CNB_{12} was used in the place of coenzyme B_{12}. The specific activity of component F or S in the protein-containing fractions was determined as described in the text with added coenzyme B_{12}. [b] Samples preincubated without CNB_{12} were treated in a similar manner as controls.

Through a Sephadex G-25 filtration method (Toraya *et al.*, 1971), it was concluded that neither component alone bound coenzyme B_{12} and CNB_{12}; both were necessary for the cobamide binding. The following two observations offer additional evidence in support of this conclusion: (a) both components were absolutely required for the inactivation of tlhe coenzyme B_{12} by oxygen when incubated aerobically with the coenzyme in the absence of substrate; (b) addition of coenzyme B_{12} did not increase the heat stabilities of the individual components at 50 or 60°, although the apoenzyme acquires much greater stability to heat by forming a tight complex with CNB_{12} (Toraya *et al.*, 1972). Thermal labilities of both components are in clear contrast with glycerol dehydrase, one of whose components is reported to show relatively high thermostability (Schneider *et al.*, 1966). In the cases of other cobamide-dependent enzymes except for glycerol dehydrase (Schneider and Pawelkiewicz, 1966), the one protein moiety, by itself, binds cobamide and is usually obtained as a pink protein. An effect of the sulfhydryl component of glutamate mutase system is to increase the affinity of the cobamide component for coenzyme B_{12} (Switzer and Barker, 1967). Component S showed almost the same sensitivity to iodoacetamide as the apoenzyme complex, indicating that component S has a sulfhydryl group(s) essential for catalysis. Since the substrate not only promotes the association of the components but also facillitates the binding of B_{12} compounds by this enzyme (Toraya *et al.*, 1971, 1972), the association of the components into the active apoenzyme may be necessary for the generation of the cobamide binding site. Dissociation and reassociation of the components, influenced by the substrate, may control both the enzyme activity and the level of coenzyme B_{12} which would be available for other cobamide-dependent systems in this microorganism.

Similar results have been obtained with glycerol dehydrase (Schneider and Pawelkiewicz, 1966; Schneider *et al.*, 1966). Addition of potassium or ammonium ion has been reported to promote reassociation of the subunits of glycerol dehydrase (Schneider *et al.*, 1966). This may also be the case for diol dehydrase.

In contrast with component S, component F was relatively unstable to nearly the same extent as the apoenzyme of diol dehydrase, whereas 1,2-propanediol stabilized the latter only. This may suggest that both components are necessary also for the substrate binding. A high concentration of glycerol (20%) showed a remarkable stabilizing effect on component F, which may be useful for purification of the component.

It is not clear why unbalanced activities of components F and S were recovered from the DEAE-cellulose column. Two explanations may be offered for this finding. First, it may be assumed that the activities of both components *in vivo* are well balanced, and that component F is predominantly inactivated *in vitro* because of its instability. A second explanation would be that the enzyme activity is regulated by control of the component F concentration. Further purification and more detailed study of the components of diol dehydrase would provide valuable information not only regarding the ligand interaction with the B_{12} enzyme but also regarding the mechanism of action of vitamin B_{12} coenzyme.

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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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