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Dihydrofolate Synthetase and Folylpolyglutamate Synthetase: Direct Evidence for Intervention of Acyl Phosphate Intermediates†

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ABSTRACT: The transfer of ^{17}O and/or ^{18}O from (COOH-^{17}O or -^{18}O) enriched substrates to inorganic phosphate (P_i) has been demonstrated for two enzyme-catalyzed reactions involved in folate biosynthesis and glutamylation. COOH-^{18}O-labeled folate, methotrexate, and dihydropteroate, in addition to [^{17}O]-glutamate, were synthesized and used as substrates for folylpolyglutamate synthetase (FPGS) isolated from *Escherichia coli*, hog liver, and rat liver and for dihydrofolate synthetase (DHFS) isolated from *E. coli*. P_i was purified from the reaction mixtures and converted to trimethyl phosphate (TMP), which was then analyzed for ^{17}O and ^{18}O enrichment by nuclear magnetic resonance (NMR) spectroscopy and/or mass spectroscopy. In the reactions catalyzed by the *E. coli* enzymes, both NMR and quantitative mass spectral analyses established that transfer of the oxygen isotope from the substrate ^{18}O-enriched carboxyl group to P_i occurred, thereby providing strong evidence for an acyl phosphate intermediate in both the FPGS- and DHFS-catalyzed reactions. Similar oxygen-transfer experiments were carried out by use of two mammalian enzymes. The small amounts of P_i obtained from reactions catalyzed by these less abundant FPGS proteins precluded the use of NMR techniques. However, mass spectral analysis of the TMP derived from the mammalian FPGS-catalyzed reactions showed clearly that ^{18}O transfer had occurred.

The poly(γ-glutamyl) conjugates constitute the predominant intracellular forms of the vitamin folic acid; their physiological roles have been the subject of several recent reviews (Covey, 1980; McGuire & Bertino, 1981; Kisliuk, 1981; Cichowicz et al., 1981; McGuire & Coward, 1984). Conversion to the polyglutamylated form is catalyzed by the enzyme folylpolyglutamate synthetase (FPGS;[1] eq 1). FPGS from two mammalian sources, namely, the hog and rat liver, are available in small quantities but have been well studied (Cichowicz & Shane, 1987; McGuire et al., 1980). In bacteria capable of folate biosynthesis, the step preceding synthesis of poly(γ-glutamates) is the addition of the first glutamate residue to 7,8-dihydropteroate (H_2Pte), catalyzed by dihydrofolate synthetase (DHFS; eq 2). In both *Corynebacterium* (Shane, 1980a,b, 1982) and *Escherichia coli* (Ferone & Warskow, 1981, 1983; Ferone et al., 1983), DHFS and FPGS activities copurify through a number of protein fractionation steps at a constant ratio of specific activities. The bifunctional *E. coli* enzyme has been isolated in large quantities from a transformant containing the cloned FPGS/DHFS gene (*folC*) (Bognar et al., 1985), and the sequence of the *folC* gene has been determined (Bognar et al., 1987). Although kinetic properties and substrate specificities of mammalian and bacterial FPGS differ, all forms of FPGS investigated so far catalyze an ATP-dependent addition of glutamate residues to a variety of folates and antifols (eq 1). It has been postulated,

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[1] Abbreviations: pABA, *p*-aminobenzoic acid; DHFS, dihydrofolate synthetase (EC 6.3.2.12); FPGS, folylpolyglutamate synthetase (EC 6.3.2.17); PteGlu, pteroylglutamic acid (folic acid); H_2Pte, 7,8-dihydropteroic acid; MTX, methotrexate (4-NH_2,10-CH_3PteGlu); TMP, trimethyl phosphate; Tfa, trifluoroacetyl; TFA, trifluoroacetic acid; CPG_1, carboxypeptidase G_1; CPG_2, carboxypeptidase G_2; S value, NMR shift value or $\Delta\delta$.

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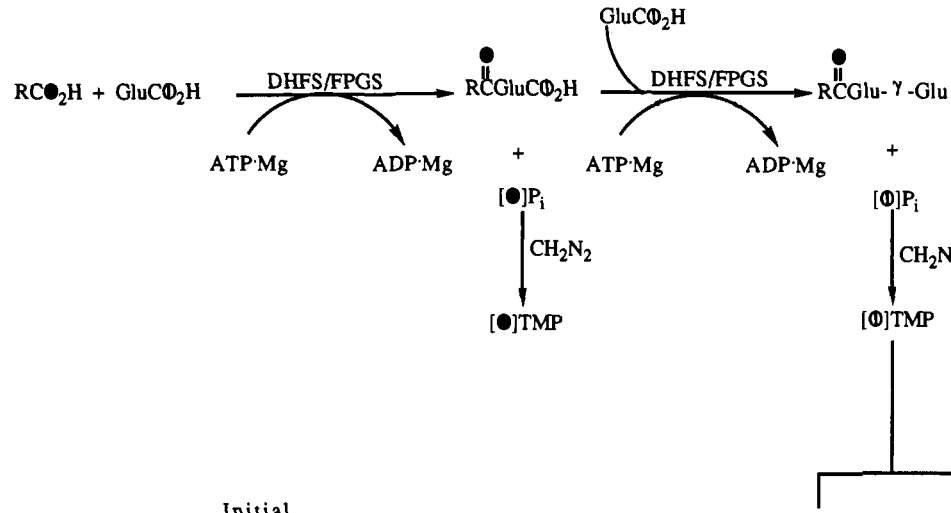
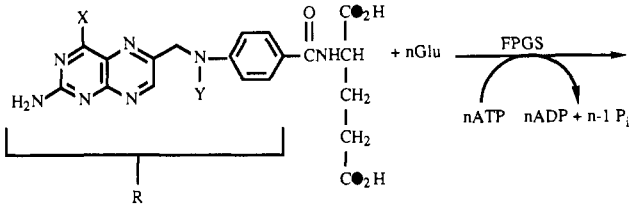
Scheme I



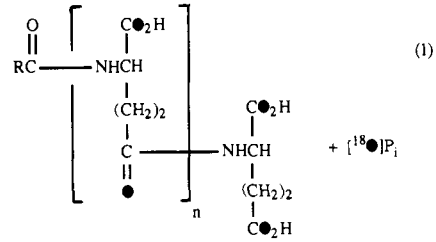
Reactions	Initial Substrate	%●[a]	%Φ	%●[b]
DHFS, FPGS	H_2Pte	46.98	33.72	18.99
FPGS, FPGS	CH_2-H_4PteGlu	64.83	33.72	18.99
FPGS, FPGS	MTX	66.22		

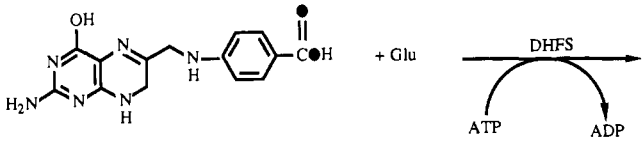
[a]From RC●$_2$H. [b]From GluCΦ$_2$H. Φ = ^{17}O; ● = ^{18}O.

by analogy with the glutamine synthetase reaction, that the FPGS-catalyzed reaction proceeds via an enzyme-bound pteroyl-γ-glutamyl phosphate intermediate (Coward, 1979; Shane, 1980a). A similar benzoyl phosphate intermediate could be envisioned in the DHFS-catalyzed reaction (eq 2).

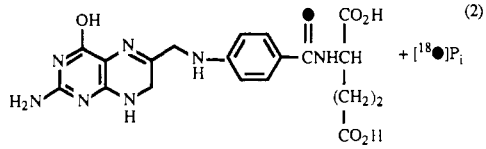


X = OH, Y = H, Folic acid (1)
X = NH_2, Y = CH_3, Methotrexate (2)





H_2 Pteroic acid



We have synthesized [*COOH*-^{18}O]folate (**1**), [*COOH*-^{18}O]methotrexate (**2**), and [*COOH*-^{18}O]pteroate as well as [*COOH*-^{17}O]-L-glutamate for use as mechanistic probes of the FPGS- and DHFS-catalyzed reactions. The proposed intermediacy of acyl phosphates would predict transfer of ^{18}O or ^{17}O from a labeled COOH to inorganic phosphate (P_i) during catalysis. In this paper we report the simultaneous investigation of oxygen transfer in two distinct reactions catalyzed by the bifunctional protein isolated from *E. coli*, and the study of FPGS-catalyzed successive addition of multiple [*COOH*-^{17}O]glutamates to 5,10-methylene-[*COOH*-^{18}O]-5,6,7,8-tetrahydrofolic acid, leading to poly(γ-glutamyl) derivatives (Scheme I). In addition, the antifolate methotrexate has been used in isotope-transfer experiments with both bacterial and mammalian FPGS. ^{17}O and ^{18}O enrichment of the product, P_i, was assessed by mass spectral analysis of the trimethyl ester and, when the more abundant *E. coli* protein was studied, also by observation of the ^{18}O-perturbed ^{31}P NMR spectrum or the ^{17}O NMR spectrum of the ester.

Experimental Procedures

Materials. L-[U-^{14}C]Glutamic acid (294 mCi/mmol), [^{3}H]glutamic acid (23.2 or 41.5 Ci/mmol), and [^{32}P]phosphate (carrier free) were obtained from New England Nuclear. Nucleotides and amino acids were obtained from Aldrich. TMP (Gold Label) was from Aldrich. Tris base was obtained from Schwarz/Mann. DE23 and DE52 were purchased from Whatman. EGTA and *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) were obtained from Sigma and Aldrich, respectively. Sodium hydrosulfite (dithionite) was from Mallinckrodt. Bio-Bead SM-4 (20–50 mesh size) was from Bio-Rad. Type GS (0.22-μm pore size) filters were from Millipore. [^{18}O]H_2O (95–99 atom %) and [^{17}O]H_2O (56.4 atom % ^{17}O, 28.7 atom % ^{18}O, and 14.9 atom % ^{16}O) were purchased from Monsanto, while [^{18}O]H_2O (100 atom %) was from ICN Biochemicals. 6-(Bromomethyl)-2,4-diaminopteridine hydrobromide and *p*-(*N*-carbobenzoxy-*N*-methyl)aminobenzoic acid were prepared by Dr. K. C. Tang (Tang & Coward, 1983). 4-NH_2,10-CH_3PteGlu$_2$ and 4-NH_2,10-CH_3PteGlu$_3$, employed as HPLC standards, were gifts from Dr. J. Galivan at the Wadsworth Center for Laboratories and Research, New

York State Department of Health, Albany, NY. Carboxypeptidase G_1 (CPG_1) was from the New England Enzyme Center, Boston, MA, and carboxypeptidase G_2 (CPG_2) was a generous gift from Dr. R. F. Sherwood at the PHLS Centre for Applied Microbiology and Research, Salisbury, Witshire, U.K. All vials used for fraction collection were new and free of P_i. All other glassware used in the enzyme work was rendered P_i free by the procedure of Domanico et al. (1985). Doubly distilled deionized water was used throughout. All other organic reagents, buffers, and inorganic salts were commercially available reagent grade chemicals.

Miscellaneous Procedures. Melting points were determined on a Mel-Temp capillary melting point apparatus and are uncorrected. Hydrogenations were done by using a Parr apparatus. Silica gel plates (60PF254, E. Merck 5735) and cellulose plates (13254, Eastman Kodak 6065) were used for analytical thin-layer chromatography (tlc). The following solvent systems were employed for tlc: (system 1) BuOH:H_2O:pyridine, 15:12:13; (system 2) 5% NH_4HCO_3; (system 3) acetonitrile:H_2O, 9:1. Solvent system 2 was used for cellulose plates, and systems 1 and 3 were used for silica gel plates. Flash chromatography was performed as described by Still et al. (1978).

Synthesis of L-[^{17}O]*Glutamic Acid*, L-[^{18}O]*Glutamic Acid, and Their Di-tert-butyl Esters.* Glutamic acid was enriched with ^{17}O or ^{18}O by the procedure reported by Boyer et al. (1956). The recrystallized product was obtained in 60% yield in each case. A single ninhydrin positive spot with $R_f = 0.38$ for both ^{17}O- and ^{18}O-enriched glutamic acid was seen in tlc system 1. The melting point was 191–192 °C in each case and corresponds to that of glutamate monohydrate. The di-*tert*-butyl esters were prepared as described previously for [^{16}O]Glu(OBu^t)$_2$ (Coward et al., 1974). NMR analysis of [^{18}O]Glu(OBu^t)$_2$: ^1H NMR ($CDCl_3$, TMS) δ 1.45 [s, α-C-$(CH_3)_3$], 1.47 [s, γ-C$(CH_3)_3$], 1.78 and 1.98 (dm, β-CH_2), 2.36 (t, γ-CH_2), 3.33 (t, α-CH); ^{13}C NMR ($CDCl_3$, TMS) δ 29.91 [α-$(CH_3)_3$], 29.96 [γ-$(CH_3)_3$], 32.13 (β-CH_2), 33.8 (γ-CH_2), 56.22 (α-CH), 82.12 (α-O–C), 82.93 (γ-O–C), 174.36 (α-COOH), 176.84 (γ-COOH). When 0.1-Hz line broadening was used, the ^{18}O-induced perturbations were observed on the resonances of the *tert*-butyl quaternary carbons ($S = 0.052$ ppm) and were resolved as shoulders on the carbonyl resonances. CI(+)MS of [^{18}O]Glu(OBu^t)$_2$: Five M + 1 peaks were seen corresponding to 0–4 atoms of ^{18}O incorporated. From the relative intensity of the M + 1 peak corresponding to the $^{18}O_4$ species, the ^{18}O-transfer probability was calculated as discussed under Results. CI(+)MS of [^{17}O]-Glu(OBu^t)$_2$: From the intensities of the M + 1 peaks with m/z 260 and 268 corresponding to the $^{16}O_4$ and $^{18}O_4$ species, the isotope-transfer probability was calculated as described under Results.

Synthesis of [*COOH-*^{18}O]*Folate* (**1**). [^{18}O]PteGlu was synthesized by coupling of the N^{10}-Tfa derivative of pteroic acid and [^{18}O]Glu(OBu^t)$_2$ by the mixed anhydride coupling procedure of Meienhofer et al. (1970; Coward et al., 1974) with the following important modification. The crude product mixture from the coupling reaction containing N^{10}-Tfa-Pte-[^{18}O]Glu(OBu^t)$_2$ was dissolved with heating in EtOAc:i-PrOH (1:4) and purified by silica flash chromatography. EtOAc:i-PrOH (6:4) was used to elute the product. An off-white powder was obtained after drying (60% yield from coupling step): mp >220 °C (dec). A single fluorescent spot with $R_f = 0.66$ was seen by tlc in solvent system 3.

N^{10}-Tfa-Pte[^{18}O]Glu(OBu^t)$_2$ was deblocked with TFA and 0.1 N NaOH as described by Plante et al. (1967). The crude product was purified by chromatography on a 1 × 29 cm, DEAE-cellulose (DE52) column equilibrated with 15 mM NH_4HCO_3, pH 7.75, and eluted with a linear gradient ranging from 15 mM to 1 M NH_4HCO_3, pH 7.75 (total volume 500 mL). The material eluting at 765 mM NH_4HCO_3 accounted for the major UV absorbance and had spectral properties consistent with those reported for folic acid (Blakley, 1969). Lyophilization of the folate-containing fractions gave a hygroscopic, fluffy yellow solid (30% overall yield from the coupling step). A single UV-positive spot was seen with $R_f = 0.48$ in tlc system 2: λ_{max} (0.1 N NaOH) 254, 282, 363 nm; ^1H NMR (D_2O + NaOD, pH 13, referenced to external TMS standard) δ 1.36 (m, β-CH_2), 1.59 (t, γ-CH_2), 3.6 (q, α-CH), 3.91 (s, C9-CH_2), 6.16 (d, H3′, H5′), 7.0 (d, H2′, H6′), 7.93 (s, H7); ^{13}C NMR (DMSO-d_6 + 2 drops of 40% NaOD, TMS) δ 28.12 (β-C), 32.24 (γ-C), 45.84 (C-9), 53.26 (α-C), 111.25 (C-3′, C-5′), 121.94 (C-4′), 127.7 (C-4a), 128.45 (C-2′, C-6′), 147.97 (C-6), 148.29 (C-7), 150.46 (C-1′), 150.5 (C-8a), 155.37 (C-2), 156.1 (C-4), 165.28 (C-7′), 174.93 (α-COOH), 175.74 (γ-COOH). FAB(+)MS: Five M + 1 peaks were seen in approximately the same proportions as in the intermediate [^{18}O]Glu(OBu^t)$_2$. HPLC analysis of **1**, using the system described by Cashmore et al. (1980), revealed a single component (purity 95%) with a retention time (12.7 min) identical with that of authentic folic acid.

Synthesis of [*COOH-*^{18}O]*Methotrexate* (**2**). [^{18}O]MTX was synthesized according to the method described by Piper and Montgomery (1977). Purification on DEAE-cellulose (Galivan et al., 1985) gave [^{18}O]methotrexate (**2**) in 26% overall yield from the coupling of 6-(bromomethyl)-2,4-diaminopteridine hydrobromide and [*COOH-*^{18}O]methylaminobenzoyl Glu(OBu^t)$_2$: λ_{max} (0.1 N NaOH) 257, 302, 370 nm; (0.1 N HCl) 244, 307 nm. HPLC analysis was performed by isocratic elution with 15 mM phosphate buffer, pH 3.3, and a flow rate of 1 mL/min using the column and other conditions described under Analytical Procedures. Compound **2** exhibited a retention time of 7.35 min, identical with that of authentic MTX, and was judged to be >99% pure: ^{13}C NMR (D_2O/NH_4OH, pD 7.9, TMS) δ 28.97 (β-C), 34.41 (γ-C), 49.71 (C-9), 55.93 (α-C), 111.96 (C-3′, C-5′), 121.01 (C-4′), 122.27 (C-4a), 128.87 (C-2′, C-6′), 148.21 (C-6, C-7), 149.42 (C-1′), 151.88 (C-8a), 153.66 (C-2), 163.25 (C-4), 169.2 (C-7′), 178.97 (α-COOH), 182.18 (γ-COOH). FAB(+)MS of **2** showed a family of isotopic isomers with 0–4 ^{18}O's incorporated in a ratio consistent with that observed for the intermediate [^{18}O]Glu(OBu^t)$_2$.

Synthesis of [*COOH-*^{18}O]*Pteroic Acid.* [^{18}O]Pteroic acid was prepared as follows: KH_2PO_4 (340 mg) was dissolved in 9.5 mL of H_2O, and the pH was adjusted to 11.1 with NaOH. $ZnCl_2$ (10 mM, 0.1 mL) was added, and the solution was lyophilized. The residue was redissolved in 0.75 mL of [^{18}O]H_2O (95–99%) and stirred at room temperature for 3 h in a closed flask to prevent contamination with atmospheric moisture. The buffer was relyophilized; 10 mL of [^{18}O]H_2O (100% enriched) and 300 mg of folic acid (0.68 mmol) were added with stirring to give a solution with pH 7.42. CPG_2 (100 μL containing 80 units of activity) was lyophilized on a vacuum centrifuge (Speed-Vac). The pellet was redissolved in 50 μL of [^{18}O]H_2O (95–99%), allowed to equilibrate at room temperature for 20 min in a closed container, and relyophilized. This procedure was repeated with another 25 μL of [^{18}O]H_2O (95–99%) in order to allow exchange of enzyme-associated H_2O with ^{18}O-enriched H_2O. The enzyme was dissolved in 3 × 50 μL of [^{18}O]H_2O (95–99% enriched) and added to the folate solution. The reaction mixture was stirred overnight at ambient temperature and protected from light and atmospheric moisture. The thick yellow precipitate was sepa-

rated as described previously (McCullough et al., 1971). A dull amorphous powder was obtained (185.6 mg, 0.59 μmol) in 87% yield. A single UV-positive spot was seen by tlc with R_f = 0.1 (vs 0.48 for folic acid) in system 2: λ_{max} (0.1 N NaOH, pH 13) 256, 276, 365 nm; ^{1}H NMR (DMSO-d_6, TMS) δ 4.49 (s, C9-CH_2), 6.65 (d, H3′, H5′), 6.93 (br s, N, 10-NH), 7.67 (d, H2′, H6′), 8.66 (s, C7-H); ^{13}C NMR (DMSO-d_6 + 2 drops of 40% NaOD, TMS) δ 111.35 (C-2′, C-6′), 127.83 (C-4′), 128.89 (C-4a), 130.97 (C-3′, C-5′), 146.36 (C-6), 146.79 (C-7), 149.59 (C-1′), 157.22 (C-8a), 164.72 (C-2), 171.88 (C-4), 172.29 (C-7′).

Zn/TFA Cleavage of [COOH-^{18}O]Pteroic Acid. [^{18}O]-Pteroic acid (2 mg) was cleaved at the C-9,N-10 bond by the method of Baugh et al. (1979). [^{18}O]pABA was purified by chromatography on a 1.5 × 12 cm DEAE-cellulose (DE52) column equilibrated with 10 mM NH_4HCO_3, pH 7.7; elution was by a step gradient (100 mL of 10 mM NH_4HCO_3 and then 100 mL of 100 mM NH_4HCO_3, both at pH 7.7). pABA eluted toward the end of the 100 mM wash, and the pABA-containing fractions were pooled and lyophilized to give an off-white powder. CI(+)MS revealed the presence of molecular ion peaks corresponding to [^{16}O]pABA and [^{18}O]pABA in 6.04%:93.96% relative abundance.

Reduction of [COOH-^{18}O]Pteroic Acid. [^{18}O]Dihydropteroic acid was prepared just before use. [^{18}O]Pteroic acid (48 mg) was dissolved in 3 mL of 0.5 N NaOH and 12 mL of 10% sodium ascorbate. Dithionite (500 mg) was added slowly over 30 min with stirring. The reaction was terminated by cooling in an ice bath, and the solution was acidified to pH 3 with 5 N HCl to precipitate [^{18}O]H_2Pte. The precipitate was washed three times with 1 mM HCl containing 10 mM β-mercaptoethanol. The pellet was then suspended in 50 mM Tris-HCl, pH 9.5, containing 100 mM β-mercaptoethanol. The concentration of the solution was calculated by using a molar extinction coefficient of 22.4 × 10^3 at 282 nm (Blakley, 1969).

Synthesis of [^{17}O]- and [^{18}O]TMP. Labeled TMP were synthesized by the method of Abbott et al. (1979): ^{17}O NMR ($CDCl_3$, unreferenced): a doublet was observed with J_{PO} of 149 Hz, which is in close agreement with that in the literature [156 Hz by Lowe et al. (1979), 154 Hz by Tsai et al. (1980), and 154 Hz by Sammons et al. (1983)]; ^{31}P NMR ($CDCl_3$, referenced to external H_3PO_4 standard at 0 ppm) a single peak with both [^{18}O]- and [^{17}O]TMP was observed at 2.54 ppm. With 8-Hz line broadening, the ^{17}O effect was manifest in [^{17}O]TMP, and poorly resolved P–^{17}O coupling was seen.

Analytical Procedures. CPG_1 and CPG_2 were assayed as described by McCullough et al. (1971) and Sherwood et al. (1985), respectively. HPLC analysis, unless specified otherwise, was done on a microparticulate anion-exchange (Whatman Partisil SAX, 10 μm) column as previously described (Cashmore et al., 1980; Shane, 1982). NMR spectra were recorded on a Varian XL-200 instrument operating at 200, 80.98, and 50.3 MHz for ^{1}H, ^{31}P, and ^{13}C, respectively. FAB-MS analysis was done by Marion Kirk at The Southern Research Institute, Birmingham, AL.

^{31}P NMR. The following parameters were employed to observe ^{18}O effects on ^{31}P NMR. Proton-decoupled spectra were obtained by using a 90° pulse width, 600-Hz sweep width, and the transmitter offset at 100 Hz. A delay time of 8 s and an acquisition time of 4 s were employed. Approximately 5000 scans were recorded for each spectrum. The spectra were referenced to an external H_3PO_4 standard (containing EGTA). The samples contained 6 mM EGTA in a final volume of 2.5 mL of $CDCl_3$. The approximate concentration of the samples of biosynthetic origin was 3–6 mM.

Mass Spectroscopy. A Hewlett-Packard Model 5987A gas chromatograph–mass spectrometer (GC–MS) in the electron ionization mode was used at Rensselaer Polytechnic Institute for the analysis of trimethyl phosphate (TMP) of biosynthetic origin. Isotopic analysis was performed by total ion monitoring of the effluent of a prebaked 25 × 0.2 mm fused silica capillary column. The injection port temperature was 200 °C. The column was maintained at 45 °C for 3 min, and the temperature was then increased in increments of 30 °C/min. Under these conditions TMP exhibited a retention time of 4.1 min. Some of the runs were repeated at The University of Michigan in the electron ionization mode on a Finnigan 4500 quadrupole GC–MS with an Incos data reduction system. A DB-5 fused silica capillary column 30 × 0.32 mm was used. The injection port temperature was 200 °C. The following temperature regimen was employed: 50 °C for 1 min; raised in increments of 20 °C/min. Under these conditions TMP exhibited a retention time of 4.3 min.

Enzymology. The bifunctional *E. coli* enzyme (Bognar et al., 1987), FPGS from hog liver (Cichowicz & Shane, 1987), and FPGS from rat liver (McGuire et al., 1980) were purified as previously described. Reaction mixtures for *E. coli* FPGS/DHFS contained Tris-HCl, pH 9.75 (100 mM), [^{17}O]- or [^{16}O]glutamic acid (5 mM), ATP (1 mM), $MgSO_4$ (5 mM), KCl (100 mM), DTT (5 mM), DMSO (2 mL), enzyme (ca. 1 mg, 0.83 IU), and [^{18}O]H_2Pte (1 mM), [^{18}O]H_2PteGlu (1 mM), or [^{18}O]MTX (1 mM) in a total volume of 20 mL. The reactions with [^{18}O]H_2PteGlu and [^{18}O]H_2Pte (expt 1, Tables I and II) involved in situ conversion of H_2PteGlu to H_4PteGlu via enzyme-catalyzed reduction and included *Lactobacillus casei* dihydrofolate reductase (3.5 IU) and NADPH (1 mM). Additionally, aqueous CH_2O (ca. 12 mM) was added to the reaction involving [^{18}O]H_2PteGlu to facilitate the nonenzymatic conversion to the preferred *E. coli* FPGS substrate, CH_2-H_4PteGlu (expt 2, Tables I and II). The mixtures were incubated at 37 °C for 1 h (MTX) or 6 h ([^{18}O]H_2Pte and [^{18}O]H_2PteGlu). Under these conditions, the following amounts of product were formed with the three substrates: 18.9 μmol ([^{18}O]H_2Pte), 18.5 μmol ([^{18}O]H_2PteGlu), and 18.7 μmol (MTX). The specific activity of the enzyme (measured by using H_2Pte as substrate) was 50 μmol h^{-1} (mg of protein)$^{-1}$. [^{3}H]Glutamic acid was diluted to give 1 μCi/mL, and 0.03 μCi was added to each reaction mixture, except that containing MTX. Aliquots of 0.5 mL were removed from each tube at the end of the incubation to estimate the extent of the reaction and product distribution, and the remainder was kept frozen at −20 °C until workup.

The assay mixture for the hog liver FPGS contained Tris-HCl, pH 9.5 (100 mM), $MgSO_4$ (5 mM), ATP (1 mM), KCl (20 mM), β-mercaptoethanol (100 mM), [^{18}O]MTX (1 mM), glutamate (5 mM), and 4 × 10^{-3} units of enzyme in a total volume of 5 mL. The reaction mixtures were incubated for 2 h at 37 °C and terminated by storage at −20 °C until workup. Under these conditions, with MTX as substrate, 280 nmol of product was formed.

For the rat liver FPGS assays, the following components were used: ATP (2.5 mM), pH 7.5, $MgCl_2$ (10 mM), [^{18}O]MTX (100 μM), Tris-HCl, pH 8.85 (100 mM), β-mercaptoethanol (100 mM), [^{3}H]glutamate (5 mM; 1100 cpm/nmol), and 0.22 mL (0.44 mg) of enzyme in a total volume of 2.75 mL. The 200-fold purified enzyme had a specific activity of 43 nmol of [^{3}H]Glu incorporated h^{-1} (mg of protein)$^{-1}$ under standard conditions. A 1.5-mL portion of enzyme was freshly dialyzed in 50 mM Tris-HCl, pH 8.85, containing 10% glycerol, 250 mM KCl, and 50 mM β-mercaptoethanol. The enzyme was dialyzed against 1 L of buffer

Table I: Product Distribution and Total Glutamylation for *E. coli* Bifunctional Enzyme-Catalyzed Reactions

expt	substrate	product distribution[a] (%)					glutamylation[b]
		Glu_0	Glu_1	Glu_2	Glu_3	Glu_4	
1[c]	[*COOH*-^{18}O]H_2Pte	9.2[d]	3.6 (3.8)	76.0 (75.8)	11.1 (11.2)		1.89
2[c]	[*COOH*-^{18}O]CH_2-H_4PteGlu		4.9[d]	6.1 (6.3)	88.4 (88.0)	0.6 (0.8)	1.85
3	[*COOH*-^{18}O]MTX		5.75[d]	66.2	28.0		1.22

[a] Distribution data are based on HPLC analysis using absorbance to quantitate the amount of material present in each peak. The numbers in parentheses are derived from radioactivity measurements to quantitate the distribution of [^{3}H]glutamate in each peak. [b] Total moles of glutamate incorporated per mole of pteroyl substrate. [c] [^{17}O]Glutamate was employed in these experiments. [d] Unmetabolized substrate.

for 6 h and against fresh buffer for an additional 10 h; 0.22-mL aliquots of enzyme were then removed and added to each reaction. The reaction mixtures were incubated for 3 h at 37 °C and terminated by placing in ice. Under these conditions, with MTX as substrate, 23.5 nmol of product was formed. Aliquots (250 μL) were removed from each reaction for product analysis by HPLC, and the remainder was stored at −20 °C until workup.

Estimation of Glutamate Incorporation and Product Distribution. The extents of the reactions and product distribution (Table I) were determined as follows. The reactants and products were separated on a DEAE-cellulose anion-exchange column (see below), and the purified folyl products were converted to $pABAGlu_n$ via the azo derivatives and quantitated as described by Foo et al. (1980). The $NaBH_4$ and $HgCl_2$ steps were omitted. Analysis of methotrexate polyglutamates was carried out by product isolation and direct spectral quantitation of the DEAE-cellulose effluents and their identity confirmed by HPLC analysis.

Purification of P_i from Enzymatic Reactions. The detailed protocol employed for the purification of P_i is described below.

(*A*) *Anion-Exchange Chromatography.* Columns of DE-23 (2.6 × 40 cm and 1 × 29 cm) equilibrated with 50 mM NH_4HCO_3, pH 8.0, were used to purify the components from reactions run on a 20- and 5-mL (or less) scale, respectively. Reaction mixtures containing reduced pteroyl compounds were purified at 4 °C. Samples were spiked with $^{32}P_i$ (ca. 1 μCi) to enable monitoring of the product P_i. The reactions in which labeled glutamate had not been added (MTX with *E. coli* FPGS and with hog liver FPGS) were spiked with [^{14}C]-glutamate (ca 0.5 μCi). The diluted samples were loaded onto the columns, the sample containers washed with 2 × 4 mL (bifunctional enzyme, B), 4 × 0.5 mL (hog liver FPGS, H), or 3 × 0.5 mL (rat liver FPGS, R) of 50 mM NH_4HCO_3 (pH 8.0), and the washes applied to the columns. Fractions were collected at a flow rate of ca. 0.9 mL/min. The column was washed with 4 × 150 mL (B) or 150 mL (H and R) of 50 mM NH_4HCO_3 (pH 8.0) and eluted with a linear gradient ranging from 50 to 260 mM NH_4HCO_3 (pH 8.0) for the columns run at ambient temperature and from 80 to 220 mM NH_4HCO_3 (pH 8.0) for columns run at 4 °C. A total volume of 2 L (B) or 550 mL (H and R) was used to elute all products except polyglutamates of MTX from the column. A step gradient with the following buffer (NH_4HCO_3, pH 8.0) sequence was employed to elute the di- and triglutamate derivatives of methotrexate: 260 mM NH_4HCO_3 (100 mL), 310 mM (200 mL), 360 mM (250 mL), and 410 mM (500 mL). Fractions were analyzed for [^{14}C]- or [^{3}H]glutamate content by liquid scintillation counting and for [^{32}P] content by Cerenkov radiation. The nucleotides, folates, and methotrexate derivatives were monitored spectrally between 200 and 450 nm. Glutamate, P_i, AMP, ADP, ATP, and methotrexate eluted from the column in this order by using the gradients mentioned above. All peaks were well resolved. The $^{32}P_i$-containing fractions were pooled and lyophilized. Lyophilization was repeated until no salt remained. Other fractions of interest (e.g., ADP, MTX/folate, and polyglutamate products) were also pooled and lyophilized.

(*B*) *Millipore Filtration.* Particulate impurities present in the lyophilized material from step A were removed by filtration of the reconstituted P_i solution through a GS-Millipore filter of 0.22-μm pore size. The filtrate was lyophilized.

(*C*) *Bio-Bead Adsorption Chromatography.* This step was included to remove contaminating plasticizers that severely limited mass spectral analysis when present. The origin of these nonpolar impurities was probably the plastic reservoir and tubing employed during the column chromatography. Bio-Bead (type SM-4) was washed as recommended by the supplier. The P_i sample was dissolved in 20 mL of H_2O and passed through the column (6 × 0.5 cm) at a flow rate of ca. 1 mL/min. P_i was eluted with an additional 40 mL of H_2O, and the eluant was lyophilized.

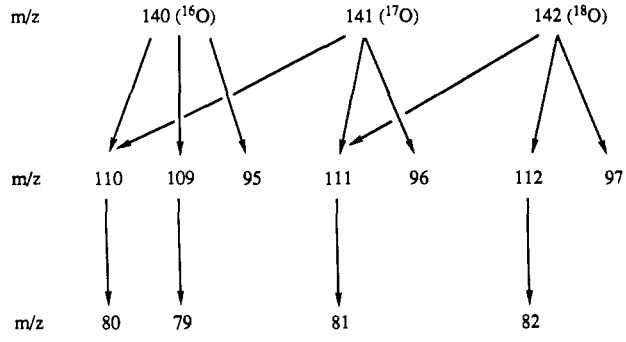
Methylation of Biosynthetic P_i with CH_2N_2. P_i was methyl esterified by titration with diazomethane. This was cautiously prepared from diazald by steam distillation as recommended by the supplier. The sides of the lyophilization flask containing P_i were washed with a few drops of 95% EtOH followed by the addition of 5 μL of concentrated HCl and the ethereal diazomethane solution. When bubbling of the solution stopped, another 5 μL of concentrated HCl and fresh diazomethane were added. Excess solvent was removed by a stream of dry N_2.

RESULTS

As a probe for possible acyl phosphate intermediates in the enzyme-catalyzed, ATP-dependent reactions studied in this work, we used ^{17}O- and/or ^{18}O-enriched substrates to determine if oxygen transfer occurred from the carboxyl group to inorganic phosphate (P_i) during catalysis. The reaction products were separated as described under Experimental Procedures, and purified P_i was converted to TMP by reaction with CH_2N_2. The presence of ^{17}O or ^{18}O in TMP can be established by NMR and/or mass spectral techniques as described below. The general experimental approach is shown in eq 1 and 2. Scheme I illustrates the specific design of the experiments employing both isotopes of oxygen and shows the maximum percentage of isotope transfer possible from each substrate.

Incorporation of oxygen isotope into pteroic acid or glutamic acid was accomplished by enzyme-catalyzed hydrolysis of folic acid in $H_2{}^{18}O$ or by acid-catalyzed exchange of $H_2{}^{17}O$ or $H_2{}^{18}O$ and the glutamate carboxyl oxygen atoms, respectively. Quantitative mass spectral analysis of *p*-aminobenzoic acid (pABA) derived from [^{18}O]pteroic acid revealed that 93.96% of this fragment was ^{18}O-enriched. Since only one of the two carboxyl oxygens can be transferred via an acyl phosphate intermediate, the maximum ^{18}O transfer is 50% of the ^{18}O enrichment, or 46.98%. Quantitative mass spectral analysis of ^{18}O-enriched glutamate di-*tert*-butyl ester precursors of

Scheme II



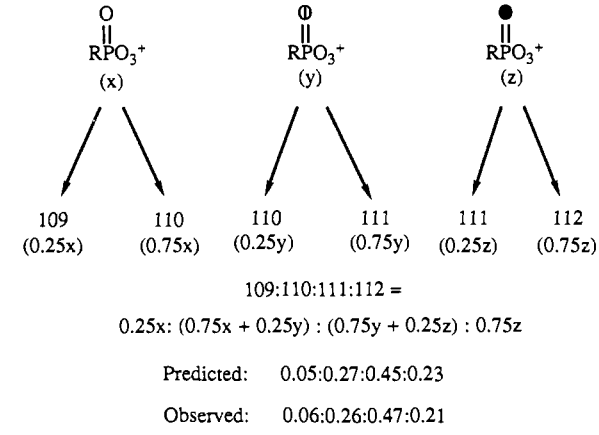
[^{18}O]folic acid (**1**) and [^{18}O]methotrexate (**2**) was more complex. Since $H_2{}^{18}O$ exchange leads to nonspecific incorporation of ^{18}O at both the α- and γ-carboxyl groups, as confirmed by the observed ^{18}O perturbations of both α- and γ-C=O ^{13}C NMR resonances, a detailed analysis of the mass spectral data was required. The probability of a single ^{18}O being located at one of the four carboxyl oxygen atoms is given by the fourth root of the relative abundance (RA) of the $^{18}O_4$ species. Thus, the probability of transferring a single ^{18}O from the γ-COOH is also (RA $^{18}O_4)^{1/4}$, or 0.6483 for **1** and 0.6622 for **2**. These data predict the maximum ^{18}O transfer for **1** and **2** (Scheme I).

Due to the isotopic composition of $H_2{}^{17}O$ (56.4% ^{17}O, 28.7% ^{18}O, 14.9% ^{16}O) used in the synthesis of [^{17}O]glutamate, the mass spectrum of the ^{17}O-enriched diester was considerably more complex. The observed nine molecular ions span the range m/z 260–268, and each ion peak represents a composite of all isotopomers with that value of m/z. In the case of m/z 260 ($^{16}O_4$) and 268 ($^{18}O_4$), the observed RA gives the frequency of finding four ^{16}O or four ^{18}O atoms in a single molecule. Thus, as described above, the probability of transferring a single ^{16}O or ^{18}O atom was calculated to be 0.4729 and 0.1899, respectively. The RA of the $^{17}O_4$ population cannot be measured directly because the mass of its parent ion coincides with that of two isotopomers, namely, $^{16}O^{17}O_2{}^{18}O$ and $^{16}O_2{}^{18}O_2$. However, the probability of transferring a single ^{17}O can be easily deduced by subtraction, i.e., 1 − (0.4729 + 0.1899) = 0.3372.

A quantitative analysis of ^{18}O transfer was required in order to establish that each glutamate added required intervention of an acyl phosphate intermediate. For this purpose, and also to develop a more sensitive assay of [^{18}O]TMP for use with less abundant mammalian FPGSs, mass spectroscopy was our method of choice. The mass spectrum of TMP conforms to the fragmentation pattern described by Bafus et al. (1966), i.e., molecular ion at m/z 140, daughter ions at m/z 110, 109, and 95, and granddaughter ions at m/z 80 and 79. Synthetic [^{18}O]TMP, prepared so that only the P=O is ^{18}O-labeled (see Experimental Procedures), gave a mass spectrum in which the only daughter ions observed (m/z 112, 111, 97) indicated no loss of ^{18}O via methyl migration in the fragmentation of the molecular ion, as previously observed by Abbott et al. (1979). Similarly, subsequent fragmentation of the daughter ions led to no loss of ^{18}O as indicated by the formation of ^{18}O-enriched granddaughter ions at m/z 82 and 81. Significant methyl migration and resultant loss of ^{18}O would lead to a pattern of peaks representing fragments from ^{16}O daughter ions (m/z 95, 109, 110) and/or ^{16}O granddaughter ions (m/z 79, 80). This was not observed.

With synthetic [^{17}O]TMP a similar but more complex analysis is possible. Fragmentation of the molecular ions m/z 140, 141, and 142 to daughter and granddaughter ions can be schematized as shown in Scheme II. It is immediately clear

Scheme III



that a given ion can arise from a variety of precursors; e.g., m/z 111 from 141 (^{17}O) and 142 (^{18}O). The only set of ions arising from a unique set of parent ions is the 95, 96, 97 group. In this case, the observed ratio of 140:141:142 equal to 0.17:0.53:0.30 is in good agreement with that of 95:96:97 equal to 0.20:0.53:0.27, again indicating that the doubly bonded ^{17}O and ^{18}O are not lost in the initial fragmentation. As indicated in Scheme II, the 109–112 set of fragments is composed of two subsets: 109, 110, 111 (corresponding to the 140 → 109 transition in unenriched TMP) and 110, 111, and 112 (corresponding to the 140 → 110 transition in unenriched TMP). The ratio of relative intensities of the two subsets was determined from the mass spectrum of commercial TMP and was found to be 0.25:0.75 for m/z 109–110. This is in good agreement with the values reported by Bafus et al. (1966) and Santoro (1973). Knowing this ratio and the relative abundance of the molecular ion permits the calculation of a predicted ratio of the m/z 109–112 group as shown in Scheme III. In this scheme, the focus is on the fate of the oxygen atoms. The symbol R represents the remainder of the molecule and has no structural meaning.

A similar calculation can be made with the m/z 79–82 group based on a 0.6:0.4 ratio of m/z 79–80 observed in unenriched TMP. The data (not shown) from this analysis are also consistent with the retention of the double-bonded ^{17}O and ^{18}O during fragmentation. Thus, 79:80:81:82 = $0.6x$:$(0.4x + 0.6y)$:$(0.4y + 0.6z)$:$0.4z$, where x:y:z is the ratio ^{16}O:^{17}O:^{18}O in the parent ion.

A second goal of this research was to develop a method for the simultaneous analysis of ^{17}O- and ^{18}O-enriched P_i derived from two reactions catalyzed by a multifunctional protein (Scheme I). The first experiment was designed to analyze for the sequential formation of [^{18}O]P_i and [^{17}O]P_i in reactions catalyzed by the DHFS and FPGS activities of the *E. coli folC* gene product (Bognar et al., 1985, 1987). The second experiment sought to use the same analytical techniques to probe mechanistic details of sequential glutamylation reactions catalyzed by FPGS. The experiments with methotrexate served to extend our studies to both the mechanism with the antifolate and with the mammalian FPGSs. Product analysis was carried out as described under Experimental Procedures, and the results are given in Table I. Allowing the reactions to proceed for 6 h resulted in the incorporation of nearly 2 mol of glutamate/mol of pteroyl substrate. The product analysis data permit the determination of the percent P_i formed as the result of each glutamate addition. With [^{18}O]H_2Pte as one of the substrates (expt 1), 48.01% of the P_i formed is the result of DHFS-catalyzed formation of dihydrofolate. Subsequent FPGS-catalyzed addition of a second and third glutamate to

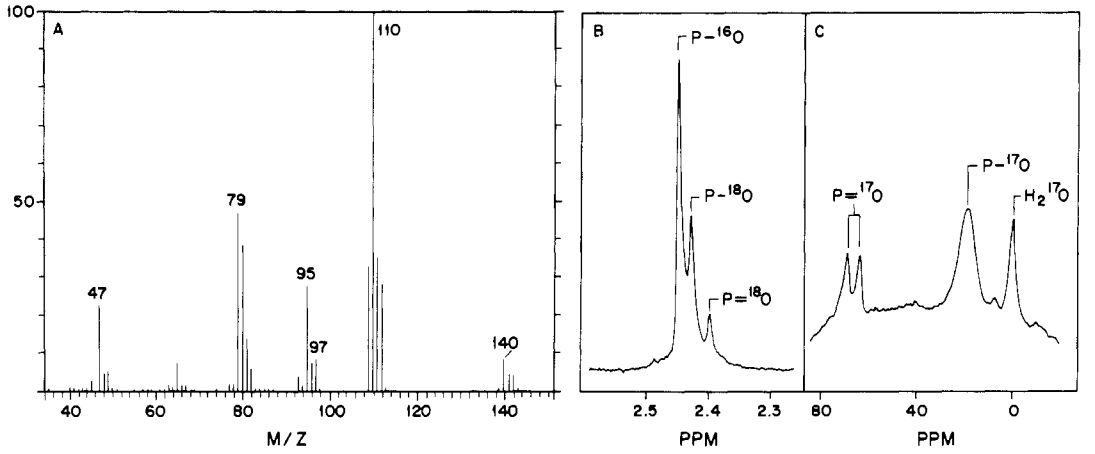


FIGURE 1: Spectra of [^{17}O, ^{18}O]TMP derived from *E. coli* DHFS/FPGS-catalyzed reaction of [^{18}O]H_2Pte and [^{17}O]glutamate (expt 1): (A) mass spectrum; (B) ^{31}P NMR; (C) ^{17}O NMR.

Table II: Quantitative Mass Spectral Analysis of TMP Derived from ^{17}O- and ^{18}O-Enriched Substrates by Using *E. coli* Bifunctional Enzyme

ion group (m/z)	expt 1[a,b]	expt 2[a,b]
140:141:142	0.50:0.25:0.25	0.45:0.20:0.35
	(0.50:0.18:0.32)	(0.41:0.16:0.43)
109:110:111:112	0.17:0.51:0.18:0.15	0.15:0.47:0.18:0.20
	(0.15:0.50:0.16:0.19)	(0.14:0.45:0.17:0.24)
95:96:97	0.64:0.17:0.19	0.60:0.15:0.26
	(0.62:0.14:0.25)	(0.56:0.12:0.32)
79:80:81:82	0.45:0.36:0.13:0.06	0.43:0.34:0.15:0.07
	(0.45:0.36:0.13:0.06)	(0.42:0.33:0.16:0.09)

[a] Refers to experiments described in text and listed in Table I. [b] Numbers in parentheses are calculated values. See text for details.

H_4PteGlu account for 46.00% and 5.88% of the total P_i, respectively. As shown in Scheme I, the DHFS-catalyzed reaction should lead to [^{18}O]P_i, whereas subsequent FPGS-catalyzed reactions should result in the formation of [^{17}O]P_i. The predicted ^{18}O transfer from [^{18}O]H_2Pte is 46.98%, while the predicted transfer from the γ-carboxyl of the newly formed folate is 33.72% ^{17}O (together with 18.99% ^{18}O). In enriched TMP of biosynthetic origin, the observed isotopic ratio of the parent ions is not reflected in the ratio of the fragments. This is due to the statistical capture of ^{18}O or ^{17}O single- or double-bonded to phosphorus during esterification of P_i with CH_2N_2. Thus, in the fragmentation of a molecular ion to a daughter ion there is a one in four chance that ^{18}O- or ^{17}O-TMP will lose the oxygen isotope to yield an unlabeled fragment. In a similar manner, subsequent fragmentation of the daughter ions will result in a 1:3 ratio of unlabeled:labeled granddaughter ions. On the basis of these relationships, the predicted ratios of molecular ions and various derived fragments are

molecular ions

$$m/z\ 140{:}141{:}142 = x{:}y{:}z$$

daughter ions

$$(x + 0.25y + 0.25z){:}0.75y{:}0.75z = a{:}b{:}c \qquad (3)$$

granddaughter ions

$$(a + 0.33b + 0.33c){:}0.67b{:}0.67c = m{:}n{:}o$$

As shown in Scheme II, only the 95:96:97 set of fragments is uniquely derived from a defined set of molecular ions. The other sets of daughter ions (m/z 109–112) and granddaughter ions (m/z 79–82) are derived from a variety of precursors. Application of the relationships between the ^{16}O:^{17}O:^{18}O in

Table III: Quantitative Mass Spectral Analysis of TMP Derived from [^{18}O]MTX by Using *E. coli* DHFS/FPGS[a]

m/z		m/z	
140:142	0.51:0.49 (0.49:0.51)	95:97	0.66:0.34 (0.62:0.38)
110:112	0.64:0.36 (0.62:0.38)	80:82	0.74:0.26 (0.75:0.25)
109:111	0.62:0.38 (0.62:0.38)	79:81	0.75:0.25 (0.75:0.25)

[a] Predicted values are given in parentheses. See text for details.

the m/z 109–112 (Scheme III) and m/z 79–82 sets of ions similarly leads to the predictions for biosynthetic TMP:

daughter ions

$$m/z\ 95{:}96{:}97 = a{:}b{:}c$$

$$m/z\ 109{:}110{:}111{:}112 = 0.25a{:}(0.75a + 0.25b){:}(0.75b + 0.25c){:}0.75c \qquad (4)$$

granddaughter ions

$$m/z\ 79{:}80{:}81{:}82 = 0.6m{:}(0.4m + 0.6n){:}(0.4n + 0.6o){:}0.4o$$

Given these relationships and from the product distribution data, one can calculate a predicted ratio of molecular ions in biosynthetic TMP from expt 1 as 140:141:142 = 0.50:0.18:0.32. A similar analysis for expt 2 leads to a predicted ratio of 0.41:0.16:0.43. The results from this analysis of the mass spectra (e.g., Figure 1A) for biosynthetic TMP derived from expt 1 and 2 are shown in Table II.

Use of [^{18}O]MTX led to significant amounts of triglutamate products in addition to the predominant diglutamate with the bifunctional enzyme (Table I). Incorporation of additional glutamyl residues in the products results in dilution of ^{18}O in P_i because any subsequent acyl phosphates are not enriched with ^{18}O. Simple calculations based on the product analysis and percent ^{18}O enrichment in the substrate correct for incorporation of ^{16}O into P_i in the second glutamylation reaction. This correction leads to a predicted ratio of ^{16}O:^{18}O in P_i isolated from the enzyme-catalyzed reactions and esterified to TMP for analysis. These predicted values are shown in Table III, together with the observed ratios of all fragments observed in the mass spectra derived from [^{18}O]MTX.

^{31}P NMR also was employed for an assessment of the transfer of ^{18}O to P_i in all three experiments with the bacterial FPGS. A representative spectrum from expt 1 is shown here (Figure 1B). The perturbation of the phosphorus resonance by both the single- and double-bonded ^{18}O are clearly seen.

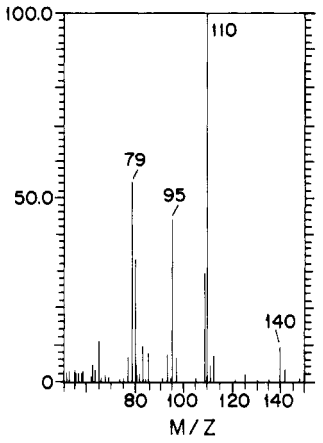


FIGURE 2: Mass spectrum of [^{18}O]TMP derived from the hog liver FPGS-catalyzed reaction of [^{18}O]MTX and L-glutamate.

Although quantitative integration of the overlapping peaks is not possible, estimates of areas under the peaks in Figure 1B indicate that the resonances due to P-^{18}O and P=^{18}O together represent ca. 33% of the total ^{31}P resonances, and the area due to P-^{18}O is ca. 3 times as great as that due to P=^{18}O. Thus, the ^{31}P NMR data of Figure 1B suggest that ^{18}O transfer occurred approximately to the extent predicted and that during methylation by CH_2N_2 a statistical capture (3:1) of ^{18}O in the single-bonded and double-bonded positions resulted. Neither the line broadening effect of ^{17}O nor ^{31}P–^{17}O coupling was observed in TMP obtained from expt 1 and 2 using a variety of plotting parameters. This was probably due to the low ^{17}O enrichment of the product P_i (17% and 18% in expt 1 and 2, respectively), together with the low concentration of the samples. This is consistent with results reported by Tsai (1979). A direct means of monitoring the ^{17}O in the same samples is by ^{17}O NMR (Figure 1C). The two peaks correspond to the resonances from the single- and double-bonded ^{17}O atoms. The observed magnitudes of J_{P-O} = 148 and 151 Hz (expt 1 and 2, respectively) are in close agreement with the value of 153.8 ± 2.4 Hz reported by Sammons et al. (1983).

Finally, the mass spectra of TMP derived from [^{18}O]MTX using FPGS from two mammalian sources, hog liver (Figure 2) and rat liver (not shown), also reveal the presence of ^{18}O in the TMP isolated from these experiments. The hog liver enzyme catalyzed a ca. 5% conversion to polyglutamate products (ca. 280 nmol), whereas the rat liver enzyme catalyzed a ca. 10% conversion to polyglutamate products (ca. 25 nmol). Purification and esterification of P_i isolated from these two reactions were accomplished in ca. 30–50% overall yield, to give ca. 140 nmol (hog liver) and 7 nmol (rat liver) of TMP available for spectral analysis. Unfortunately, the small amounts of mammalian enzyme available precluded isolation of sufficient quantities of TMP for NMR analysis. However, the mass spectral evidence of ^{18}O in the TMP isolated from the reactions catalyzed by mammalian FPGS clearly indicates that ^{18}O transfer occurs from the γ-CO_2H of MTX to P_i during biosynthesis of MTX polyglutamates.

DISCUSSION

We have used mass spectroscopy and NMR spectroscopy to assess, in a single analysis, the possibility of oxygen transfer in two distinct enzyme-catalyzed reactions. The results reported here demonstate the intermediacy of acyl phosphates in both reactions catalyzed by the *E. coli* DHFS/FPGS bifunctional protein and by the mammalian FPGSs isolated from hog liver and rat liver. We have used oxygen transfer from an ^{18}O-labeled carboxylic acid to inorganic phosphate as the method to establish this mechanism. The NMR method was used to analyze TMP derived from P_i isolated from reactions catalyzed by the *E. coli* bifunctional protein. Limited amounts of TMP derived from the reactions catalyzed by the mammalian enzymes, hog liver FPGS and rat liver FPGS, precluded the use of NMR in those cases. The ^{18}O isotope effect in ^{31}P NMR spectroscopy was first reported by Cohn and Hu (1978) and by Lowe and Sproat (1978). The magnitude of the ^{18}O shift of the ^{31}P resonance of phosphate derivatives ranges from 0.01 to 0.04 ppm and correlates well with the double-bond character of the P–O bond (Cohn & Hu, 1980). The ^{31}P NMR spectrum shown in Figure 1B demonstrates clearly the presence of ^{18}O in the TMP samples derived from biosynthetic P_i. Incorporation of ^{17}O (expt 1 and 2) was observed directly by ^{17}O NMR of the TMP derivatives (Figure 1C). These data clearly show that the overall approach, outlined in Scheme I, for the simultaneous investigation of two reactions catalyzed by a multifunctional enzyme is effective.

A more precise and sensitive assessment of ^{18}O transfer may be obtained via mass spectral analysis. The expected fragmentation pattern and isotopic enrichment of the molecular ion and all the fragments were observed in TMP derived from each experiment. A detailed analysis of the ^{16}O:^{17}O:^{18}O or ^{16}O:^{18}O ratio in the molecular ion and major fragments (Scheme III) for TMP derived from H_2Pte, H_2PteGlu, and MTX is summarized in Tables II and III. In expt 1 and 2 (Table I), the agreement between the predicted and observed values is best in the granddaughter ions (m/z 79–82) and becomes less satisfactory as the number of oxygen atoms increases in the daughter ions and molecular ions. We have observed self-protonation of the molecular ion to the extent of ca. 2–12% (after correction for natural abundance ^{2}H and ^{13}C spillover) in numerous control experiments. It is probable that a similar degree of self-protonation occurs with the various fragments observed. Since the presumed site of protonation is on oxygen, it is reasonable to expect a greater extent of protonation, and hence divergence from the predicted ratios, in the more oxygenated daughter ions and molecular ions. The self-protonation process complicates the interpretation of the data of Table II but does not alter the conclusion that oxygen transfer occurs in both reactions studied simultaneously by using ^{17}O- and ^{18}O-enriched substrates. These complications are not a concern when only ^{18}O transfer is monitored as in expt 3, where quantitative agreement is observed.

The mechanism of ATP-dependent amide or peptide formation has been studied extensively (Walsh, 1979), and examples of carboxylate attack at the γ-phosphorus of ATP to produce an acyl phosphate and ADP (e.g., glutamine synthetase) or at the α-phosphorus of ATP to produce an acyl adenylate and pyrophosphate (e.g., asparagine synthetase) are known. In the case of DHFS (Shane, 1980) and also FPGS (Shane, 1980b; Cichowicz & Shane, 1987) it has been shown previously that ADP is the only nucleotide product formed. These results have been confirmed and extended in the present work, where no AMP was detected in any of the experiments described. The use of ^{18}O-perturbed ^{31}P NMR together with mass spectroscopy has allowed us to demonstrate rigorously the involvement of an acyl phosphate intermediate, as previously postulated (Coward, 1979). The availability of large amounts of the *E. coli* bifunctional protein has allowed us to extend the quantitative analysis beyond that previously reported in related systems. It provides a firm basis for the qualitative analysis carried out with TMP derived from mammalian FPGS-catalyzed reactions and allows us to draw the conclusion that all FPGS-catalyzed reactions investigated

in this research proceed via a γ-glutamyl phosphate intermediate.

The growing number of multifunctional proteins being reported in the literature suggests that a significant amount of cellular metabolism may be mediated by either multifunctional enzymes and/or multienzyme complexes (Benkovic, 1984). In addition, the enzyme-mediated polymerization of monomers occurring in the absence of a template [e.g., glycogen synthase (EC 2.4.1.11)] is a well-known and important aspect of cellular biochemistry. With the judicious choice of appropriately labeled substrates, experiments analogous to those described in this paper should provide useful mechanistic data for a variety of enzymes.

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