IMPROVED CONVENTIONAL SYNTHESIS FOR ^{14}C-LABELED POLYGLUTAMATES OF FOLIC ACID

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SUMMARY

The majority of folates existing in nature are of the pteroylpolyglutamyl
form and are unable to support Lactobacillus casei growth until the ४-linked
glutamyls are digested by conjugase enzymes. Most studies involving folate
absorption have utilized monoglutamyl forms of folate, primarily folic acid.
Synthetic pteroylpolyglutamates prepared by solid phase or conventional
synthesis provided conjugated materials with which to study the absorption and
metabolism of natural derivatives; however, the synthetic hepataglutamates
support microbiological growth prior to enzymatic hydrolysis whereas the
natural conjugates do not. Incomplete purification of intermediate peptides
during the synthesis would be the most likely explanation of this growth
phenomenon. A modified solution synthesis has been developed which improves
upon intermediate peptide condensations, increases product yields, and
provides a heptapeptide which does not support microbiological growth until
after enzymatic hydrolysis.

Key words: pteroylpolyglutamic acid, folic acid, pheroylheptaglutamic acid

INTRODUCTION

Dietary folates exist primarily as reduced polyglutamic forms of folic
acid with up to seven ४-linked glutamyl residues. Humans are incapable of

synthesizing their total minimal daily requirement of folic acid, and remain

dependent upon intestinal absorption of ingested folates. Folate deficiencies

caused by gastrointestinal diseases can result from the inability of the

individual to either metabolize or transport folate across the intestinal

wall.

Difficulty in purifying small quantities of polyglutamates from yeast

extract prompted several investigators to synthesize polyglutamyl folates

possessing growth characteristics of natural "fermentation factor" (1). The

conjugated folates are not absorbed as readily as free folate but further

studies are required to determine whether gastrointestinal absorption involves

an active or passive process. Oxidized conjugates are utilized to study

enzymatic deconjugation and folate transport because of the unstable nature of

reduced folates. Although polyglutamates have been synthesized by solid-phase

(2) and solution-phase techniques (3), there has been controversy about purity

of final products. Tamura et al. (4) using the solid-phase method reported a

definite growth response to Lactobacillus casei on increased chain lengths of

polyglutamates prior to treatment with enzyme conjugase (glutamate

carboxypeptidase EC 3.4.12.10) (5), and explains this observation as a

phenomenon called "positive drift". Godwin et al. (6) also questioned the

purity of solid-phase products which led to the second method for preparation

of the poly-γ-glutamates by solution-phase synthesis. Similar conjugates with

more than three glutamic acid residues have been produced by conventional

synthesis in our laboratory and show the same phenomenon of Lactobacillus

casei growth with up to 20% total folate activity for higher conjugates prior

to enzymatic hydrolysis.

Through a modified solution synthesis method we have identified that

glutamates prepared by solution-phase synthesis contain impurities which arise

from an accumulation of contaminating intermediates produced by incomplete

purification methods.

The purpose of this paper was: 1) to identify the problem sites in the

solution synthesis of γ-linked glutamyl peptides and in the preparation of

CF_3CO-Pte, 2) to describe the modified synthesis and improved purification

techniques that eliminate and circumvent the problems, and 3) to provide complete data of all purified intermediates.

MATERIALS

The following chemicals were obtained from the sources indicated: L-[U-^{14}C] Glutamic acid (specific radioactivity 240 mC/mmol) was from ICN Pharmaceuticals, Inc. (Irvine, California, U.S.A.); Sephadex LH-20 was purchased from Pharmacia (Uppsala, Sweden); Whatman cellulose power CF-11 was from H. Reeve Angel Inc. (Clifton, New Jersey, U.S.A.); Z-Glu(DCHA)-OBu^t was from BACHEM Inc. (Marina Del Rey, California, U.S.A.); GluOBu$^t_2$, HCl was purchased from Sigma Chemical Inc. (St. Louis, Missouri, U.S.A.) and crystallized twice from ether/hexane before use; N,N-dimethylformamide and ethyl acetate were from Fisher Chemical (Pittsburgh, Pennsylvania, U.S.A.) and both were distilled before use; N-methylmorpholine and isobutylchloroformate were from Pierce (Rockford, Illinois, U.S.A.). Lactobacillus <u>casei</u> (A.T.C.C. 7469), <u>Flavobacterium</u> strain HY (A.T.C.C. 25012) and <u>Pseudomonas</u> (A.T.C.C. 25301) were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). All other chemicals were of analytical grade.

The scintillation solution for radioactive determinations contained 24g of Fluoralloy TLA and 380 ml of Bio-Solv (BBS-3) from Beckman Instruments Inc. (Fullerton, California, U.S.A.) and sufficient toluene to make a total volume of 3.8 litres.

Microelemental analyses were determined by Galbraith Laboratories (Knoxville, Tennessee, U.S.A.). Abbreviations for lesser-known substituents: Z, benzyloxycarbonyl; OBu^t, tu-butyl ester; CF$_3$CO, trifluoroacetyl; Pte, Pteroic acid; DCHA, dicyclohexylammonium salt; N-MM, N-methylmorpholine; isoBuOCOCl, isobutylchloroformate; ETO$_2$, ether. DMF, N,N-dimethylformamide; TEA, triethylamine. Abbreviated forms of amino acid derivatives conform to the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature (1972). The glutamic acid residues were of the L-configuration.

METHODS AND RESULTS

Preparation of the Starting Esters

Z-Glu(OH)-OBu^t (I)   The dicyclohexylammonium salt of

benzyloxycarbonylglutamic acid-γ-t-butyl ester was removed by

suspending the salt [2.50 g, 2.80 mmol] in ethyl acetate and

extracting with 48 ml of 0.05 M-H$_2$SO$_4$ until the pH of the aqueous

solution remained acidic and the salt was totally solubilized. The

organic layer was extracted with water and the aqueous layer was

back extracted with ethyl acetate. After drying over MgSO$_4$, the

ethyl acetate layer was removed under reduced pressure and the oil

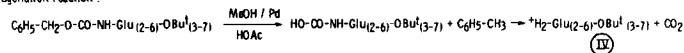crystallized one time from ether/light petroleum (b.p. 30-60°C).
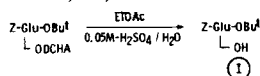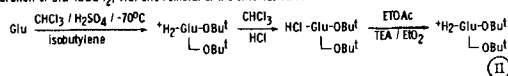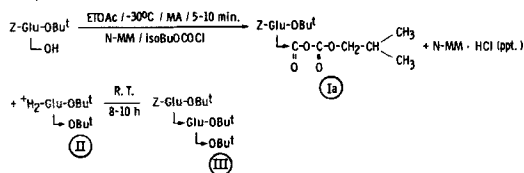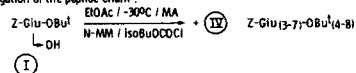
Scheme 1. Step-wise preparation of γ-linked heptaglutamic acid.

The crystalline material was chromotographed over a Sephadex LH-

20/ethanol column (5.5 cm x 200 cm) and monitored at 280 nm.

Benzyloxycarbonyl-glutamic acid-γ-t-butyl ester (I); [1.50 g, 4.40 mmol] was crystallized from ether/light petroleum (b.p. 30-60°C); m.p. 81-82°C; $[\alpha]_D^{21}$-15.4 (c1, methanol (Table 2)

$^+$H$_2$-GluOBu$^t_2$ (II)   The glutamic acid-di-t-butyl ester hydrochloride salt was crystallized from ether/hexane to remove impurities; m.p. 110-112°C (Lit. 116-117°C); $[\alpha]_D^{21}$+19.6 (c2, methanol); or was prepared from the free amine by reaction of glutamic acid with isobutylene in a mixture of chloroform/sulfuric acid following the procedure of Kramber (7). Glutamic acid [0.44 g, 3 mmol] (L-[U-^{14}C] glutamic acid, 1 mC, specific activity 240 mC/mmol) was suspended in 15 ml of chloroform in a pressure vessel; 0.3 ml of concentrated sulfuric acid was added as a catalyst and the suspension was cooled to -70°C in a solid CO$_2$-acetone bath. Condensed isobutylene (15 ml) was added and the mixture was allowed to react at room temperature with periodic vortexing until the glutamic acid had totally solubilized (about 3 days). The vessel was opened at -70°C and excess isobutylene was air evaporated in the hood. The acid was neutralized to pH 7.2 with KHCO$_3$(22 g/50 ml). The chloroform layer was separated, washed with water, dried over MgSO$_4$ and concentrated under vacuum to an oil. Treatment of the di-t-butyl ester in chloroform with excess HCl gas produced the hydrochloride salt. The residual oil was scratched under hexane at -30°C to yield an amorphous solid which was immediately washed with cold hexane and dried for 24 h over NaOH pellets. The semi-crystalline material was recrystallized from ether/hexane. Yield 67%, [0.59 g, 2 mmol], m.p. 103-106°C.

The di-t-butyl glutamic acid was liberated from its hydrochloride salt prior to condensation: 1) by treatment with an aqueous alkali and extraction into ethyl acetate or 2) by treatment with a base such as triethylamine or N-methylmorpholine in ethyl acetate. The addition of dry ether completely precipitated the HCl

salt which was removed by filtration and the free amine was dried
and concentrated in vacuo to a semi-crystalline oil (II) and used
directly in the condensation reaction. See Table 1 for
chromotography.

Mixed Anhydride Condensation

To a stirred solution of (I) [2.0 g, 5.9 mmol] in distilled ethyl acetate
at −30°C was added a slight excess of N-methylmorpholine [0.67 ml, 6 mmol] and
an equivalent amount of isobutylchloroformate [0.78 ml, 5.9 mmol]. Immediate
salt precipitation indicated formation of the mixed anhydride (Ia), and the
free amine (II) [1.52 g, 5.87 mmol] was added in ethyl acetate. Thin layer
chromatography on silica gel in ETOAc indicated that the majority of the free
amine condensed within 5 min. and the solution was allowed to stir 8–10 h at
room temperature until no free amine was detected on chromatography with
fluorescamine (8). The reaction was filtered to remove N-methylmorpholine HCl
and the organic layer was washed with saturated Na_2CO_3 and water. After
backwashing with ethyl acetate and drying over $MgSO_4$ the solvent was reduced
to a semi-crystalline oil and the condensed product was chromatographed on a
Sephadex LH–20/ethanol column (5.5 cm x 200 cm) with a flow rate of 60 ml/h
and 15 ml collections monitored at 280 nm. After a volume of approximately
1500 ml, the main peak containing product (III) was identified as a single
spot on silica gel at R_F of 0.90 (Table 1) and the fractions were combined and
evaporated. The $Z-Glu_2OBu^t_3$ (III) was crystallized (twice) from
ether/hexane. Yield 89%, [3.2 g, 5.2 mmol] m.p. 85–86°C; [$]_D^{21}$−26.6 (c1,
methanol) (Table 2).

Removal of the Benzyloxycarbonyl Group

The Benzyloxycarbonyl group was chosen as the protecting group for
glutamic acid because of its selective removal during catalytic hydrogenation
in the presence of the $-OBu^t$ groups. The peptide to be hydrogenated was
vigorously stirred in a solution of methanol, two drops of HOAc, and
approximately 100 mg of freshly prepared palladium catalyst (Pd on charcoal

Table 1. Thin layer chromotography of polyglutamyl peptides

Layers*	S_1	S_2	S_1	S_1	S_2	S_2
Solvent**	A_1	A_2	A_2	A_3	A_4	A_5
Solvent front in cm.	13	13	13	8	10	10
GluOBu$_2^t$, HCl					0.75	0.98
Z-Glu(OH)-OBu^t	0.85	0.50	0.85	0.50	0.79	
Z-Glu(OBu^t)-OH	0.85	0.50	0.85	0.56	0.74	
$^+$H$_2$-GluOBu$_2^t$ ***	0.78		0.69		0.77	0.98
Z-Glu$_2$OBu$_3^t$	0.90	0.64	0.94	0.83	0.95	
$^+$H$_2$-Glu$_2$OBu$_2^t$	0.36					
Z-Glu$_3$OBu$_4^t$	0.91	0.38	0.75	0.85	0.95	
$^+$H$_2$-Glu$_3$OBu$_4^t$	0.25					
Z-Glu$_4$OBu$_5^t$	0.91	0.15	0.56	0.89	0.95	
Z-Glu$_5$OBu$_6^t$	0.89	0.13	0.39	0.89	0.95	
$^+$H$_2$-Glu$_5$OBu$_6^t$	0.17					
Z-Glu$_6$OBu$_7^t$	0.88	0.11	0.36	0.89	0.96	
$^+$H$_2$-Glu$_6$OBu$_7^t$	0.15		0.04			
Z-Glu$_7$OBu$_8^t$	0.89	0.08		0.89	0.96	
$^+$H$_2$-Glu$_7$OBu$_8^t$	0.15					
CF$_3$CO-Pte						0.73
CF$_3$CO-PteGlu$_3$OBu$_4^t$						0.86
CF$_3$CO-PteGlu$_7$OBu$_8^t$						0.92

* = 0.25 mm plates 5 cm x 20 cm. Layers: S_1 = Silica gel-glass-Brinkman activated 80°, S_2 = Silica gel-glass-brinkman air dried

** = Spotted 5ug/ul. Solvent: A_1 = Ethyl acetate, A_2 = Ethyl acetate/hexane (35:25), A_3 = 15% (v/v) ammonium hydroxide/sec-butanol (3:17), A_4 = Methanol/chloroform (1:9), A_5 = Methanol

*** = Free amines were detected with Fluorescamine (8)

Table 2. Analytical data of Benzyloxycarbonyl-oligo-γ-l-Glutamic acid-t-Butyl esters. All melting points are uncorrected. For abbreviations used see materials section. All condensations were done using the mixed anhydride procedure (MA).

Compound	Product Yield (%)	M.p. (°C)	Optical Rotations $[\alpha]_D^{21}$ (c1, methanol)*	Elemental Analyses (%) Calculated	Found
L-Glutamic acid			+10.8(c1.4,1MONaOH)[21]		
GluOBu$_2^t$, HCl	67	103–106	+19.6(c2, methanol)[21]		
Z-Glu(OH)-OBu^t	91	81–82	-15.4	C=60.53 H=6.82 N=4.15	C=60.51 H=6.90 N=4.07
Z-Glu$_2$OBu$_3^t$	89	85–86	-26.6	C=62.27 H=8.01 N=4.84	C=62.34 H=7.61 N=4.77
Z-Glu$_3$OBu$_4^t$	94	77–80	-27.4	C=61.34 H=7.99 N=5.51	C=61.29 H=8.05 N=5.34
Z-Glu$_4$OBu$_5^t$	80	80–82	-33.1	C=60.76 H=8.01 N=5.91	C=60.75 H=8.10 N=5.99
Z-Glu$_5$OBu$_6^t$	83	68–72	-36.2	C=60.37 H=8.03 N=6.18	C=60.53 H=8.09 N=6.14
Z-Glu$_6$OBu$_7^t$	87	72–74	-38.1	C=60.09 H=8.04 N=6.37	C=60.02 H=8.14 N=6.21
Z-Glu$_7$OBu$_8^t$	79	86–88	-42.3	C=59.88 H=8.05 N=6.52	C=59.61 H=8.17 N=6.39
CF$_3$CO-Pte	75	300(decomp)		C=43.25 H=3.35 N=18.91 F=12.83	C=43.06 H=3.25 N=18.80 F=12.68

* = All optical rotations were done on a Perkin Elmer 241 in methanol at 21°C unless otherwise stated.

was not as effective) in the presence of a nitrogen barrier. Hydrogen gas was bubbled through the solution and the evolution of CO_2 was monitored by $BaCO_3$ precipitation in saturated $Ba(OH)_2$. See Table 1 for R_Fs of hydrogenated peptides. The catalyst was filtered, washed with methanol, and the methanol evaporated to a semi-crystalline oil. The free amine was dried over P_2O_5 and added directly to the mixed anhydride condensation.

Elongation of the Peptide Chain

Fully protected peptides containing from two to six glutamic acid residues, $Z-Glu_{(2-6)}OBu^t_{(3-7)}$ were hydrogenated as needed by the above procedure. The removal of the benzyloxycarbonyl group was usually completed within two hours, and the dried material was added to the mixed anydride preparation of compound (I) as previously discussed. After condensation each intermediate was purified by chromatography over a Sephadex LH-20/ethanol column (5.5 cm x 200 cm). The free γ-acid (I) and $Z-Glu_{(2-4)}OBu^t_{(3-5)}$ were monitored at 280 nm or 272 nm. As the peptide chain increased to $Z-Glu_{(5-7)}OBu^t_{(6-8)}$, the absorption shifted downscale, and the longer peptides were monitored at 230 nm and 280 nm (to detect any unreacted $Z-Glu(OH)-OBu^t$). Peaks were identified by t.l.c. (Table 1), dried over $MgSO_4$, and evaporated to dryness. The residues were crystallized from either ether/hexane or ETOAc/hexane. Yields averaged 80%. See Table 2 for elemental data.

Preparation of N^{10}-trifluoroacetylpteroic acid ($CF_3CO-Pte$)

Pteroic acid was prepared from folic acid by the method of Houlihan et al. (9) using a _Pseudomonas_ (A.T.C.C. 25301). The organism was cultured in a nutrient-glutamic acid broth and transferred to 15 litres of a folic acid – salts medium at pH 7.3. The solution was stirred for six days at 30°C and refrigerated overnight at 4°C. The clear supernate was decanted from the gelatinous pteroic acid-cell mixture which precipitated during the incubation. The mixture was centrifuged at 9000 rev/min in a refrigerated centrifuge and washed (twice) with water. The pteroic acid was dissolved in 950 ml of 2% (w/v) Na_2CO_3 containing 50 ml of 1M-NaOH and separated from the insoluble cells by filtration on a Buchner funnel. Free pteroic acid was

precipitated by careful adjustment of the supernate to pH 3 with 2M–HCl so as
to avoid formation of a red gelatinous complex which forms at pH 8 if the
material is rapidly precipitated with acid. The product was washed (twice)
with water and lyophilized to an orange powder. The sodium salt was formed by
dissolving the dried powder in a minimal amount of 2.5 M–NaOH with warming to
40°C to effect solution. Cooling overnight at 4°C precipitated sodium
pteroate. The salt was extracted with minimal amounts of warm water and free
pteroic acid was carefully precipitated using 2M–HCl. The precipitate was
washed with water and lyophilized to an orange powder. Preparation of pteroic
acid using the <u>Flavobacterium</u> strain HY organism (A.T.C.C. 25012) and the
method of Pratt et al. (10) produced a dark orange product having similar R_Fs
to the pteroic acid prepared by the <u>Pseudomonas.</u> After elimination of heavy
fluorescent impurities, yields averaged 10–20% and the precipitation of the
sodium salt was often impossible even when very high concentrations of pteroic
acid were added to the warm NaOH. The compounds showed an ultraviolet
quenched spot with R_F value of 0.92 and strongly fluorescent impurities when
chromatographed on silica gel in 10% (v/v) NH_4OH. The pteroic acid was
dissolved in 0.1 M–glycine/NaOH (15% w/v ascorbic acid) buffer, purged with
nitrogen, and chromatographed on a cellulose CF–11 column (100 cm x 2.5 cm).
Five milliliter fractions were collected and the major ultraviolet absorbent
spot was isolated and precipitated with 2M–HCl. After lyophilization, the
purified pteroic acid was identified as a single ultraviolet absorbent spot
having the ultraviolet spectrum and paper chromatographic behavior reported by
Houlihan et al. (9). The modified procedure for the preparation of pteroic
acid using the <u>Pseudomonas</u> averaged yields of 85%.

Pteroic acid [1.5 g, 4.8 mmol] was stirred in a round bottom flask with
careful elimination of moisture and trifluoroacetic anhydride (24 ml) was
added dropwise over a period of 30 minutes using a vented separatory funnel.
After three days, the excess anhydride was evaporated under reduced pressure
and the creamy white precipitate washed on a sintered glass filter with cold
water. The crude material was crystallized from a mixture of
DMF/methanol/ethanol/water (6:10:3:60, by vol) by dissolving the powder first

in DMF/methanol/ethanol (6:10:3 by vol) with gentle warming to 40°C, filtering
through Whatman #1 filter paper, and cooling to room temperature. Warm water
was added and slow evaporation yielded off-white crystals. Chromatography on
silica gel indicated gross streaking due to contamination. Repeated
crystallizations yielded transparent crystals with a single fluorescent spot,
R_F of 0.73 with over 50% loss of product. Alternately after one
crystallization, small amounts of impure CF_3CO-Pte were chromatographed on a
Sephadex LH-20/DMF column (60 cm x 1.8 cm) with 3 ml collections. The main
peak was located by t.l.c. and after cooling to 4°C in an ice-ethanol bath,
ice water was added to precipitate crystalline CF_3CO-Pte. This procedure
effectively eliminated decomposition and raised the yield to 75%; m.p. 300°
(decomp). Analytical data appears in Table 2.

Condensation of CF_3CO-Pte with the Blocked Peptides

The CF_3CO-Pte and δ-t-butyl-γ-glutamyl peptides were condensed by the
same mixed anhydride condensation procedure used earlier for the elongation of
the peptide chain. Crystalline CF_3CO-Pte was dissolved in distilled DMF at
40°C in a closed vessel and a 10% excess of N-methylmorpholine was added
followed by an equivalent amount of isobutylchloroformate. The reaction was
allowed to stir for 7 min in an ice water bath. Hydrochloride salt
precipitation does not occur in DMF. The blocked peptide of desired chain
length was hydrogenated in methanol using freshly prepared palladium catalyst
prior to the mixed anhydride condensation, and added to the mixed anhydride in
a minimal amount of distilled DMF. The condensation reaction was followed on
t.l.c. by visually monitoring the conversion of fluorescent starting materials
with an u.v. lamp indicating formation of a new ultraviolet condensation spot
of R_F midway between the two starting products. The reaction was terminated
on disappearance of the free peptide. Incorporation of the starting materials
into the new spot was verified by condensing CF_3CO-Pte with radiolabeled $^+H_2$-
Glu_2-δ-[U-^{14}C]GluOBu$_4^t$. The fluorescent spots were scraped from the plate and
eluted in scintillation fluid. Counts indicated a shift of the free peptide
to the newly formed fluorescent spot.

The reaction was terminated and the product partially separated from unreacted CF_3CO-Pte by adding cold water. The precipitate was collected by centrifugation at 9000 rev/min for 15 min. Cooling the supernate for several hours precipitated the CF_3CO-Pte. The condensed material was chromatographed over an LH-20/ethanol column (5.5 cm x 200 cm), concentrated in vacuo and dried over P_2O_5.

Deprotection of Fully Condensed Material

The $-OBu^t$ groups were selectively removed by stirring CF_3CO-PteGlu$_7$OBu$_8^t$ [0.35 g, 0.19 mmol] in 20 ml of distilled trifluoroacetic acid for 10 min at room temperature. The acid was removed under reduced pressure and the residue washed with ether on a sintered glass filter. After drying over P_2O_5, the CF_3CO- group was removed in the dark by dissolving the partially protected material in 19 ml (10 equiv) of 0.1 M-piperidine, in an ice-ethanol bath at 4°C with a nitrogen barrier for 30 min. For removal of the CF_3CO- group the pH of the base must remain between pH 11-12 or the trifluoroacetic acid from the previous deprotection was not totally removed. The yellow solution was centrifuged at 7000 rev/min, decanted from any insoluble material, and adjusted slowly to pH 3 with 2M-HCl. The gelatinous product was centrifuged at 9000 rev/min and lyophilized to a hygroscopic orange powder which moved as a single fluorescent quenched spot of R_F 0.92 on Whatman #1 paper using ascending chromatography in 5% (w/v) NH_4HCO_3.

Assay of Folate Derivatives

Derivatives to be assayed for folate content were accurately weighed before each hydrolysis and u.v. spectra were determined in 0.05-M potassium phosphate buffer pH 7.0. Determinations using Lactobacillus casei (A.T.C.C. 7469) employing a modified method of Cooperman (11). Folate concentrations were calculated from a standard reference curve using serial dilutions of folic acid (25-250 x 10^{-11} g/ml) obtained from Lederle Laboratories (Standard PteGlu was kindly provided by Lederle Laboratories, Pearl River, New York, U.S.A.). The polyglutamyl folates were hydrolyzed by incubating 0.1 ml of

substrate (25-200 M) with 0.1 ml of enzyme conjugase (glutamate carboxypeptidase EC 3.4.12.10) and 0.8 ml of 0.05M-universal buffer containing 0.2% (v/v) mercaptoethanol in a metabolic shaker at 37°C for 60-90 min. Either chick pancreas (20 mg/ml) at pH 7.0 or human mucosal jejunal homogenate (20 mg/ml) at pH 4.7 was used as source of enzyme. The enzyme was deactivated by adding 2.0 ml of 0.05 M-phosphate buffer at pH 6.5 and boiling for 5 min.

The folate assay was set up in 16 mm x 100 mm glass tubes using folic acid casei medium (Difco #1822-15, Detroit, Michigan, U.S.A.). The L. casei was maintained in Difco agar #0900-15 and Difco broth #0901-15. The assay was incubated for 14-16 h in a waterbath shaker at 37°C and the growth of each tube was measured at 660 nm using a Coleman Junior Spectrophotometer.

Repeated experiments with purified pteroylheptaglutamate yielded no microbiological growth with L. casei prior to hydrolysis of the substrate whereas folates without rigorous purification (6) averaged 15% growth on the substrate blanks.

DISCUSSION

Adequate amounts of pure polyglutamyl folates were the limiting factor in continuing studies on the absorptive mechanism of natural folates until 1969 when Krumdieck and Baugh (2) reported the solid phase synthesis of folic acid polyglutamates. Later Godwin et al. (6) reported the synthesis of pteroylheptaglutamic acid by solution phase synthesis. The availability of these compounds permitted further studies involving conjugated materials. The preparation of radiolabelled polyglutamyl folates provided a tool for further insight into normal absorption as studies could be performed with physiologic amounts of the vitamin. The availability of radiolabelled material decreased the necessity for measuring folates microbiologically.

Both published procedures for preparing synthetic polyglutamyl folates, the solid-phase and solution-phase methods, produce final products containing up to seven δ-linked glutamyl residues. When condensed to the pteroyl moiety the resulting folates exhibit microbiological growth characteristics at variance with natural "fermentation factor" (1). Although Tamura et al. (4)

reported that pteroylheptaglutamic acid synthesized by solid-phase technique
supported microbiological growth, other investigators have not commented on
this issue.

The solid-phase method for preparing peptides, though rapid, has the
disadvantage of accumulating unreacted side products during preparation. The
solution-phase synthesis as published also allows for accumulation of
intermediate products unless rigorous purification techniques are incorporated
into the synthesis procedure. The preparation of the peptide was not the only
problem area discovered in the synthesis of pteroylpolyglutamates. We found
that the production of pteroic acid from folic acid using a Pseudomonas was
superior to that of Flavobacterium strain HY. Yields were higher and
crystallization of the sodium salt was easier. Most important, when this
material was condensed to the heptaglutamate, the final product failed to
support growth with Lactobacillus casei until hydrolysis with "conjugase".
The purification procedures used in earlier works were found to be unsuitable
for the complete removal of labile pterins which result from oxidative
degradation of the pteroyl compounds. Pteroic impurities allow production of
pteroyl polyglutamyl forms of degradation products. With crystalline CF_3CO-
Pte acid and δ-linked glutamyl peptides free of contaminating impurities, the
folate analogs can be easily condensed using the N-methylmorpholine mixed
anhydride coupling procedure. Stepwise purification techniques eliminate the
need for column purification of the deprotected compound thus decreasing
chances of oxidative degradation of the conjugated material. Microbiological
analysis of the heptaglutamate after enzymatic hydrolysis yielded results in
agreement with folate concentrations determined by ultraviolet absorption data
based on the molecular extinction coefficient.

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