Folate Pentaglutamate and Folate Hexaglutamate Mediated One-Carbon Metabolism†

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ABSTRACT: The proposal that folate polyglutamate cofactors of different chain lengths function differently in metabolism was investigated. We identified the one-carbon units present in rat liver folates within each of the liver folate polyglutamate groups, the folate penta-, hexa-, and heptaglutamates. This identification revealed that at the pentaglutamate level, 5-methyl-H_4folate was the major form, accounting for 18% of the total liver folates, with small amounts of H_4folate. At the hexaglutamate level, 5-methyl-H_4folate and H_4folate were major forms, accounting for 17% and 22% of the liver folates, respectively. At the heptaglutamate level, 5-methyl-H_4folate occurred in small amounts while H_4folate predominated. The rats used here had been fed a diet low in methionine and were exposed to N_2O gas. These results are qualitatively similar to those from rat brain [Brody, T., Shin, Y. S., & Stokstad, E. L. R. (1976) *J. Neurochem. 27*, 409-413] where 5-methyl-H_4folate was a major fraction of the folate pentaglutamates but a minor, if detectable, part of the hexa- and heptaglutamates. The folates 5-methyl-H_4PteGlu$_{5-7}$ were metabolically active in the liver, as illustrated by the severe contraction in the amounts of these folates following an injection of methionine. This indicates that "folate-binding proteins" do not prevent 5-methyl-H_4folates from entering into one-carbon metabolism.

Folates are used as cofactors in the enzymatic transfer of one-carbon units. For example, folates mediate the transfer of methyl groups in the biosynthesis of methionine and of formyl groups in the biosynthesis of purines. In addition to containing these transferable methyl and formyl groups, natural folates are modified by the presence of an oligo(γ-glutamyl) chain. These oligo- or polyglutamate chains are from one to nine or more glutamyl residues long, with different chain lengths predominating in different organisms (Rabinowitz & Himes, 1960; Krumdieck & Baugh, 1969; Buehring et al., 1974; Brody et al., 1976).

The functions of the polyglutamate chain have not been completely defined. The polyglutamate chain has been found to enhance the substrate properties of the folate cofactor with purified enzymes (Curthoys & Rabinowitz, 1972; MacKenzie & Baugh, 1980; Baggot & Krumdieck, 1979). In one case, there appears to be an absolute requirement for the folate polyglutamate substrate (Sakami & Ukstins, 1961). However, there has not yet been a report of an absolute substrate requirement for one folate polyglutamate over another, i.e., folate pentaglutamate over folate hexaglutamate.

The activities of certain folate-requiring enzymes are inhibited by folates not acting as the immediate cofactor. The inhibitory properties of the noncofactor folates vary with the specific polyglutamate chain length (Kisliuk et al., 1974; Friedkin et al., 1975; Matthews & Haywood, 1979). For example, dihydro-PteGlu$_n$[1] inhibits thymidylate synthetase with K_i values of 300, 20, 3, and 3 μM where n = 1, 2, 3, and 6, respectively (Kisliuk et al., 1974). A similar inhibitory series was found with 5-formyl-H_4folates (Friedkin et al., 1975).

In vivo studies with mutant Chinese hamster ovary cells (McBurney & Whitmore, 1974) and with lactic acid bacteria (Shane & Stokstad, 1975) showed that the polyglutamate chain is required for the cellular retention of folates whereas the folate monoglutamate is the preferred transport form of the vitamin. Furthermore, Shane & Stokstad (1976) have shown that once transported into the cell, folate polyglutamates are more active in bacterial metabolism than are the folate monoglutamates. The preference for folate polyglutamate substrates by enzymes rate limiting for bacterial growth is a likely source of this effect. It is not known, however, if there is an absolute requirement in metabolism for one specific folate polyglutamate over another, i.e., folate pentaglutamate over the hexaglutamate form.

We have observed differences between one-carbon metabolism as mediated by folate pentaglutamates and by folate hexaglutamates. This is based on data presented here as well as on that previously reported (Brody et al., 1976). Almost all the data appearing here were presented at a meeting of the Americal Society of Biological Chemists (Brody et al., 1980).

Materials and Methods

Animals. Male weanling Sprague-Dawley rats (16) (57.5 ± 4.5 g) from Simonson Labs, Gilroy, CA, were individually housed in stainless-steel screen-bottomed cages at 21 °C with lighting between 7:00 and 19:00. The animals were fed a soy protein based diet low in methionine and supplemented with vitamin B_{12} (100 μg/kg diet) and with water for a period of 30 days (Thenen & Stokstad, 1973). The rats gained about 4.5 g/day during the 4-week period, with weekly weighings. The final weights were 155 ± 19 g.

Nitrous oxide treatment was used as a tool to study folate metabolism. Exposure to this gas results in decreases in two activities which require folate, that of methionine synthetase (Koblin et al., 1981) and that of the histidine catabolic pathway. N_2O treatment consisted of exposure to nitrous oxide gas for 4 h, starting at 8:00 a.m. The rats were placed, two per chamber, in 2-L chambers. The chambers were flushed at atmospheric pressure with an equimolar mixture of N_2O and O_2, with a flow rate of 400 mL/min for each gas. The disruption of histidine catabolism was followed by measuring the oxidation of a tracer dose of [^{14}C]histidine.

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[1] Abbreviations: PteGlu, pteroylglutamic acid (folic acid); PteGlu$_n$, pteroylmono- to -heptaglutamic acid (n indicates the number of glutamic acid residues); H_4PteGlu$_n$, 5,6,7,8-tetrahydropteroylmono- to -polyglutamic acid.

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Of the 16 rats, 8 were then injected intraperitoneally with 300 μmol of L-methionine (neutralized with NaOH, in a volume of 1 mL). The remaining animals were injected with 1 mL of 0.85% NaCl. All animals were immediately replaced in the nitrous oxide chambers for an additional 0.5 h.

Folate metabolism was then assessed in both groups of rats by measuring excreted $^{14}CO_2$ after an injection of L-[2-^{14}C]-histidine, intraperitoneally. Each rat was injected with 150 μmol, 0.3 μCi (528 000 cpm), of [^{14}C]histidine in a volume of 0.5 mL. The rats were then placed in air-flushed chambers for $^{14}CO_2$ collection for a final 2 h (Chiao & Stokstad, 1977).

All rats were then decapitated, and livers were immediately weighed and minced, and heated at 95 °C in 10 volumes of 1.0% sodium ascorbate, pH 6.0, and 100 mM 2-mercaptoethanol was to minimize oxidative destruction of the tissue folates (Bird et al., 1969). The heated tissues were then cooled to 4 °C, homogenized with a Polytron homogenizer (Brinkmann Instruments) to extract folates, and centrifuged at 30000*g* for 30 min. The supernatants from the plus methionine (+met) and minus methionine (−met) groups were pooled separately and stored in liquid nitrogen for later analysis of the extracted folates.

A dose–response curve of $^{14}CO_2$ expiration vs. amount of methionine injected was constructed as follows. In an independent experiment, 28 rats were raised for 30 days and exposed to nitrous oxide as described above. Seven groups of four rats were then injected with graded levels of L-methionine (270, 67.5, 33.8, 16.9, 4.2, or 1.05 μmol of L-methionine, or 0.85% NaCl). Histidine catabolism was then measured in all rats as described above. The final step in histidine catabolism is dependent on folate-dependent reactions (MacKenzie & Baugh, 1980; Kutzbach & Stokstad, 1971a). Thus, histidine oxidation is used to assess folate metabolism.

Diets. Powdered rat diet was exactly as described by Thenen & Stokstad (1973) except it contained retinyl palmitate instead of retinyl acetate, vitamin B_{12} was included at 100 μg/kg diet, and there was no supplementary methionine. The soy protein contained 1% methionine by weight according to Skidmore Enterprises, Cincinnati, OH. Thus, the diet contained about 0.2% L-methionine. According to Thenen (1971), weanling Sprague-Dawley rats fed this low-methionine diet grow at a rate of 0.77 times that of rats fed this diet supplemented with either 1.0% or 0.5% DL-methionine. Nitrous oxide treatment does not result in defective [^{14}C]histidine oxidation unless the diet is low in methionine (J. E. Watson and E. L. R. Stokstad, unpublished observations).

Identification of Extracted Tissue Folates. Folates were initially fractionated by chromatography on a column of diethylaminoethylcellulose (DEAE-cellulose) (Thenen & Stokstad, 1973) into half a dozen folate activity peaks. Liver extract was loaded on the column of freshly packed DEAE-cellulose (0.9 × 28 cm) (DE-52 from Whatman) and developed with an exponential salt gradient at 4 °C in the dark. The closed mixing chamber contained 375 mL of 10 mM sodium phosphate, pH 7.0, 10 mM sodium ascorbate, and 100 mM 2-mercaptoethanol. The reservoir contained 10 mM sodium phosphate, 0.4 M KCl, pH 7.0, 10 mM sodium ascorbate, and 100 mM 2-mercaptoethanol.

Aliquots of each fraction were treated with partially purified γ-glutamyl hydrolase to convert the eluted folate polyglutamate to the microbiologically available monoglutamate and then assayed with *Lactobacillus casei* (ATCC 7469) and with *Streptococcus faecalis* (ATCC 8043) (Bird et al., 1969). These initial microbiological assays established whether the γ-glutamyl hydrolase treated fraction contained mainly 5-methyl-H_4PteGlu or some different folate monoglutamate, since *L. casei* will but *S. faecalis* will not grow on 5-methyl-H_4PteGlu.

The DEAE-cellulose fractions were stored in dry ice during the microbiological analysis. After analysis, the fractions in major and minor peaks were thawed, pooled, and stored in liquid N_2 until further analysis.

The folate oligo(γ-L-glutamate) chain length, as well as the one-carbon unit present on the folate in question, was determined by chromatography on a column of Sephadex G-25 (Pharmacia Fine Chemicals) according to Shin et al. (1972). The column was equilibrated and eluted with 0.1 M sodium phosphate, pH 7.0, and 200 mM 2-mercaptoethanol at 23 °C. Aliquots from the DEAE-cellulose peak pools were chromatographed directly on the column of Sephadex G-25 (0.75 × 190 cm) along with $PteGlu_n$ standards in order to determine the polyglutamate chain length of the folate. Fractions were assayed with *Pediococcus cerevisiae* (ATCC 8081) in order to avoid any response to the $PteGlu_n$ standards. *P. cerevisiae* does not grow on nonreduced folates. Synthetic *dl*-H_4PteGlu was used to construct the microbiological standard curves. When it was clear that the major folate in the peak pool was a form of 5-methyl-H_4 folate, $PteGlu_n$ standard markers were not used. In this case, folates in the Sephadex G-25 fractions were assayed with both *L. casei* and *S. faecalis* in order to establish unambiguously the elution position of the 5-methyl-H_4 folate polyglutamate. PteGlu was used here to construct the growth curves.

Aliquots from the DEAE-cellulose peak pools were also briefly incubated with partially purified γ-glutamyl hydrolase in order to convert the folate polyglutamate in question to the corresponding folate monoglutamate. This folate monoglutamate product was then chromatographed on the Sephadex G-25 column in order to determine if the folate was mainly 10-formyl-, 5-formyl-, 5-methyl-, or H_4PteGlu. Each fraction was assayed, with no further exposure to γ-glutamyl hydrolase, with *L. casei* and *S. faecalis*.

Unfractionated liver extracts were also incubated with γ-glutamyl hydrolase and analyzed on Sephadex G-25, as above, to determine the one-carbon units present overall in cellular folates. Preliminary analysis of liver extracts by DEAE-cellulose chromatography following treatment with γ-glutamyl hydrolase revealed that no H_2 folates or nonreduced folates were present.

γ-Glutamyl Hydrolase. Hydrolysis of folate polyglutamates to the corresponding monoglutamate forms was necessary before microbiological assay of folates as well as prior to analysis of overall one-carbon units by chromatography on Sephadex G-25. γ-Glutamyl hydrolase from hog kidney was purified about 200-fold as follows. Kidney (800 g) was homogenized in 3 volumes of 10 mM 2-mercaptoethanol at 4 °C, brought to pH 5.0 with 1 M acetic acid to precipitate proteins, followed by warming at 50 °C for 20 min for protein denaturation. The solution was brought to 50% saturation with ammonium sulfate at 4 °C. Inactive protein was removed by centrifugation at 4 °C after each of the above four steps. Active protein was then precipitated in 70% ammonium sulfate, dialyzed, and loaded (218 mL, 4.3 g of protein) on a column of carboxymethylcellulose (3.6 × 23 cm). The column was washed with 220 mL of mixing chamber buffer to remove most of the inactive protein. The column was then developed at 4 °C with a linear gradient of 0–0.3 M NaCl in 50 mM sodium acetate and 10 mM 2-mercaptoethanol at pH 5.0. The mixing chamber and reservoir volumes were each 650 mL. Active protein eluted near the end of the gradient and was

washed out completely with an additional 150 mL of reservoir buffer. Active protein (275 mL, 143 mg of protein) was stored in liquid N_2.

Enzyme activity was measured by incubating the protein in a volume of 1.0 mL with 2.5 ng of synthetic $PteGlu_7$ in 50 mM sodium acetate at pH 4.7 (Bird et al., 1946). The reaction was terminated by boiling and product measured by assay of the mixture with *S. faecalis*.

The partially purified enzyme (50 μL, 22 μg of protein, derived from the equivalent of 70 mg of kidney) catalyzed the apparent maximal hydrolysis of $PteGlu_7$ substrate in 50 min and in 7 min in two separate determinations. Accurate measurement of enzyme activity during purification was difficult because of the nonlinearity of activity vs. incubation time. Although about 20% of enzyme activity was lost per week with storage at 4 or −20 °C, the activity was stable to repeated freezing in liquid N_2.

Chemicals. L-[*ring*-2-^{14}C]Histidine (50 mCi/mmol) was from Amersham. L-Methionine was from Sigma. $PteGlu_{3-7}$ chromatographic markers were synthesized by Dr. Barry Shane using the Merrifield solid-phase method according to Baugh et al. (1970). $H_4PteGlu$ was used for constructing standard growth curves in microbiological assays from *P. cerevisiae* and was synthesized according to Blakley (1957). PteGlu was converted to 10-formyl-PteGlu in formic acid (Roth et al., 1951), purified on a column of Sephadex G-25 in the absence of 2-mercaptoethanol, and reduced to 10-formyl-H_4PteGlu (Blakley, 1957) for use as a chromatographic marker. Nitrous oxide was from Ohio Medical Products, a division of Airco, Inc., Madison, WI. Industrial-grade oxygen was from Liquid Carbonic, Chicago, IL.

Results

Changes in [^{14}C]Histidine Oxidation with Nitrous Oxide Treatment and Various Doses of Methionine. We wished to determine whether one-carbon metabolism at the folate pentaglutamate level is, under any condition, different from that at the folate hexaglutamate level. One-carbon metabolism was assessed by determining the prevalence of methyl-, formyl-, and tetrahydrofolates at the various folate polyglutamate levels. Folates were examined after exposing animals to one of two conditions to increase the likelihood of finding differences in one-carbon metabolism. These were exposure to nitrous oxide gas followed by injections of methionine or saline. The condition of folate metabolism was assessed by measuring the oxidation of injected [^{14}C]histidine to $^{14}CO_2$. Histidine oxidation was found to be impaired in rats after a brief exposure to N_2O. About 10% of the [^{14}C]histidine was oxidized in rats raised on a low-methionine diet without exposure to nitrous oxide (J. E. Watson and E. L. R. Stokstad, unpublished experiments). But with exposure to N_2O, only 0.5% of the [^{14}C]histidine was oxidized, as shown in Figure 1. No depression of histidine oxidation with exposure to N_2O was detected with rats raised on a methionine-sufficient diet.

Various amounts of L-methionine were injected in different animals to determine a dose for a maximal and consistent increase in [^{14}C]histidine oxidation. Oxidation was consistently low, though definite, with doses of 0–16.9 μmol of L-methionine (Figure 1). Variable results were obtained with 67.5 μmol of methionine. Doses of 270 μmol of methionine resulted in consistently high oxidations of about 20% (Figure 1). Thus, doses of 270 μmol, or 2 μmol/g of body weight, were used in further experiments.

The rapid changes in folate metabolism following exposure to nitrous oxide and following the methionine injection were desirable in order to minimize the effects of breakdown and

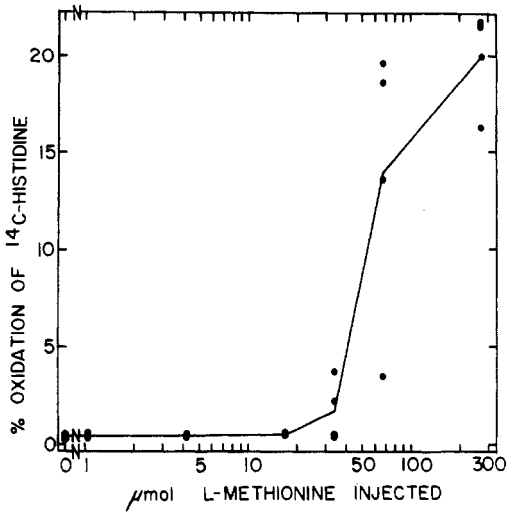

FIGURE 1: Respiratory excretion of $^{14}CO_2$ following injection of [*ring*-2-^{14}C]histidine. All rats were exposed to N_2O for 4 h, then injected with 0–270 μmol of L-methionine (0–2 μmol/g of body weight), and replaced in the N_2O chambers for an additional 30 min. Each rat was then injected with 0.3 μCi of [^{14}C]histidine (150 μmol) and respiratory $^{14}CO_2$ collected over 2 h. The excreted $^{14}CO_2$ was measured in order to assess stimulations of folate metabolism. Each point represents the percentage excretion of ^{14}C by one rat.

excretion of liver folates and replacement by dietary folates (Thenen et al., 1973).

Liver Folate Identification. Liver folates from rats receiving nitrous oxide but no methionine (−met rats) and from rats receiving nitrous oxide and methionine (+met rats) were fractionated initially on DEAE-cellulose. Microbiological assay of the −met fractions revealed four discrete major peaks, as shown in Figure 2. The peak centered at fractions 210–215 and labeled III (−met) contained mainly a form of 5-methyl-H_4 folate, because it supported significant growth of *L. casei* but not of *S. faecalis*. Similarly, peak V (−met) and the region labeled VII (−met) contained 5-methyl-H_4 folate, as well as nonmethylated forms. Major peaks containing nonmethylfolates were labeled VI (−met) and VIII (−met). Folates with longer polyglutamate chains and thus more negative charges elute later from DEAE-cellulose columns. Thus, the methylfolate in peak V (−met) had a longer chain length than that in peak III (−met).

Microbiological assay of the +met fractions revealed seven peaks (Figure 3). The peaks labeled V (+met) and VI (+met) were the largest and contained mainly nonmethylfolates. Folates in the region labeled IV (+met) supported a shoulder of *L. casei* growth but not of *S. faecalis* and thus contained methylfolate. There was also some indication of methylfolate in fractions 247–250.

In summary, the −met liver extract contained quantitatively more 5-methyl-H_4 folate than did the +met extract. None of the major folates in the +met extract were methylfolates. The +met extract did contain some unique early eluting nonmethylfolates. Fractions from the various activity peaks were pooled for further analysis.

The one-carbon unit present in the folate in question was identified by γ-glutamyl hydrolase treatment followed by analysis on a column of Sephadex G-25. With the −met column, rechromatography of the major nonmethyl peaks VI (−met), shown in Figure 4A, and VIII (−met) (data not shown) showed that these peaks contained 100% $H_4PteGlu_n$. All fractions were assayed by *S. faecalis* to avoid response to methylfolates present as contaminants from adjacent DEAE-cellulose peaks. Other regions of the DEAE-cellulose eluate such as I (−met) and IV (−met) were not analyzed. Peak I

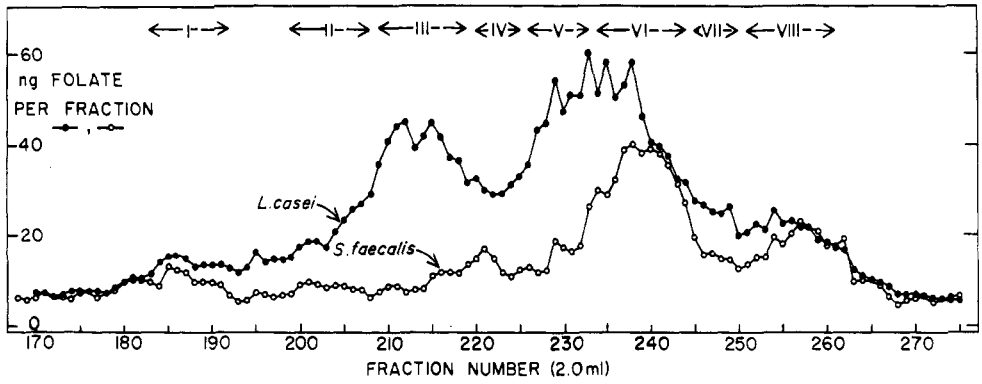

FIGURE 2: DEAE-cellulose chromatography of liver folates. Eight rats were exposed to N_2O for 4 h, injected with saline, and then exposed to N_2O for 30 min more. All rats were injected with [^{14}C]histidine as described in Figure 1. After respiratory $^{14}CO_2$ was collected, the rats were decapitated, liver folates were extracted, and all eight extracts were pooled. A 4-mL aliquot of the liver extract (2.2 μg of total folate by *L. casei*) was loaded on the column followed by development with an exponential salt gradient. The recovery of total folate in fractions 170–270 was 2.5 μg, by *L. casei*. For microbiological analysis, 100 μL of each fraction was exposed to 50 μL of partially purified γ-glutamyl hydrolase (22 μg of protein) for 5 h, 37 °C, in 1.0 mL of 50 mM sodium acetate and 0.3% sodium ascorbate at pH 4.7. All incubation mixtures were then brought to 2.9 mL with folate-free bacterial growth medium. The final concentration of fresh ascorbate was 0.4% and the final pH 6.7. All samples were autoclaved for 6 min, immediately cooled, and inoculated with *L. casei* or *S. faecalis*. Mixtures were incubated for 20 h, 37 °C, followed by measurement of growth at 650 nm. Folic acid was used to construct standard curves. (●) *L. casei*; (○) *S. faecalis*; averages of duplicate assays. Groups of fractions which appeared enriched for a specific form of folate were pooled for further analysis, as indicated by the roman numerals.

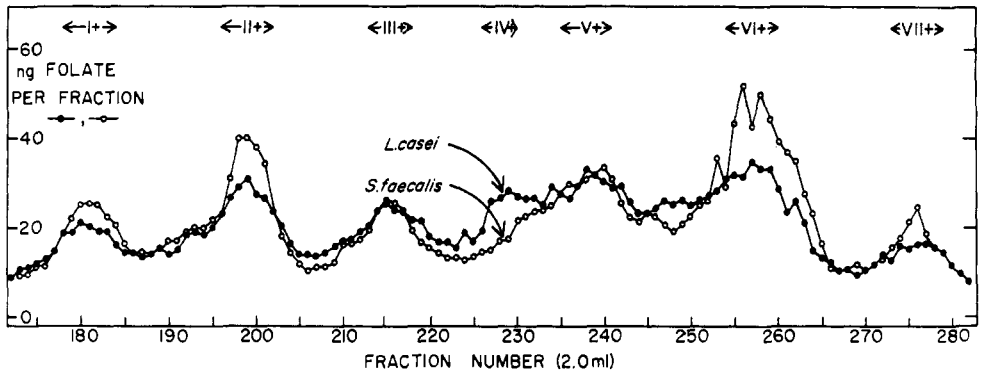

FIGURE 3: DEAE-cellulose chromatography of liver folates. Eight rats were exposed to N_2O as described in Figure 2 and injected with 300 μmol of L-methionine (2 μmol/g of body weight) instead of with saline. All rats were injected with [^{14}C]histidine as described in Figure 1 followed by killing, extraction of livers, and combination of extracts. A 4-mL sample of the pooled extract (2.4 μg of total folate by *L. casei*) was loaded on the column. The recovery of total folates in fractions 170–285 following chromatography was 2.3 μg by *L. casei*. (●) *L. casei*; (○) *S. faecalis*.

(−met) probably contained formylfolates because of identification of the corresponding fraction from the +met rat column.

The chain length of the reduced folate oligo(γ-glutamate) in each pool was determined by rechromatography of about 40 ng of this folate directly on the Sephadex G-25 column. Fractions were treated with γ-glutamyl hydrolase and assayed with *P. cerevisiae* in order to avoid response to the nonreduced $PteGlu_n$ markers (20 000 ng of folate). The only major nonmethylfolate in the fractionated −met extract was in peak VI (−met). With rechromatography, this folate eluted at the position of $PteGlu_6$, as shown in Figure 5A. Since this folate previously was determined to be in the tetrahydro form, its identity is $H_4PteGlu_6$. The folate in the pool labeled IV (−met) was a mixture of three unidentified folates eluting at the positions of the $PteGlu_5$ and $PteGlu_6$ markers and before $PteGlu_7$. The nonmethylfolates in fractions 215–227 accounted for 6.4% of the total folate from the −met liver extract. $H_4PteGlu_5$ would be expected to elute in these fractions. Since pool IV (−met) contained some material eluting at the $PteGlu_5$ marker position, only about 2% of the −met folates are $H_4PteGlu_5$.

Peak VIII (−met), determined earlier to be in the tetrahydrofolate form, eluted from Sephadex G-25 at or slightly ahead of the $PteGlu_7$ marker and thus was $H_4PteGlu_7$, as shown in Figure 5B. With rechromatography, the methylfolates in peaks III (−met) (Figure 6A), V (−met) (Figure 6B), and VII (−met) (Figure 6C) eluted one fraction ahead of the markers $PteGlu_{5-7}$ and thus were identified as 5-methyl-$H_4PteGlu_{5-7}$, respectively.

In summary, the −met extract contained large amounts of the methylfolates 5-methyl-$H_4PteGlu_{5,6}$ and the tetrahydrofolate $H_4PteGlu_6$. $H_4PteGlu_5$ was not a major form here. Folates from an independent group of four −met animals were extracted and chromatographed on DEAE-cellulose. Aside from a better resolution of peaks, there was essentially no difference from the pattern presented in Figure 2.

The identities of the +met peaks are listed in Table I. Some of the chromatographic patterns used for this identification are shown in Figure 4B for peak II (+met), Figure 6D for peak IV (+met), Figures 4C and 5C for peak V (+met), and Figure 5D for peak VI (+met). The +met extract contained major amounts of $H_4PteGlu_{5,6}$ and smaller amounts of $H_4PteGlu_7$ and the various formylated forms. 5-Methyl-$H_4PteGlu_n$ was a minor component of the +met liver extract. The various one-carbon units were distributed more consistently at the penta-, hexa-, and heptaglutamate levels among the +met folates than among the −met folates. It should be noted that folates eluting in the DEAE-cellulose fractions 1–165 were below the limit of detection by microbiological analysis. Thus,

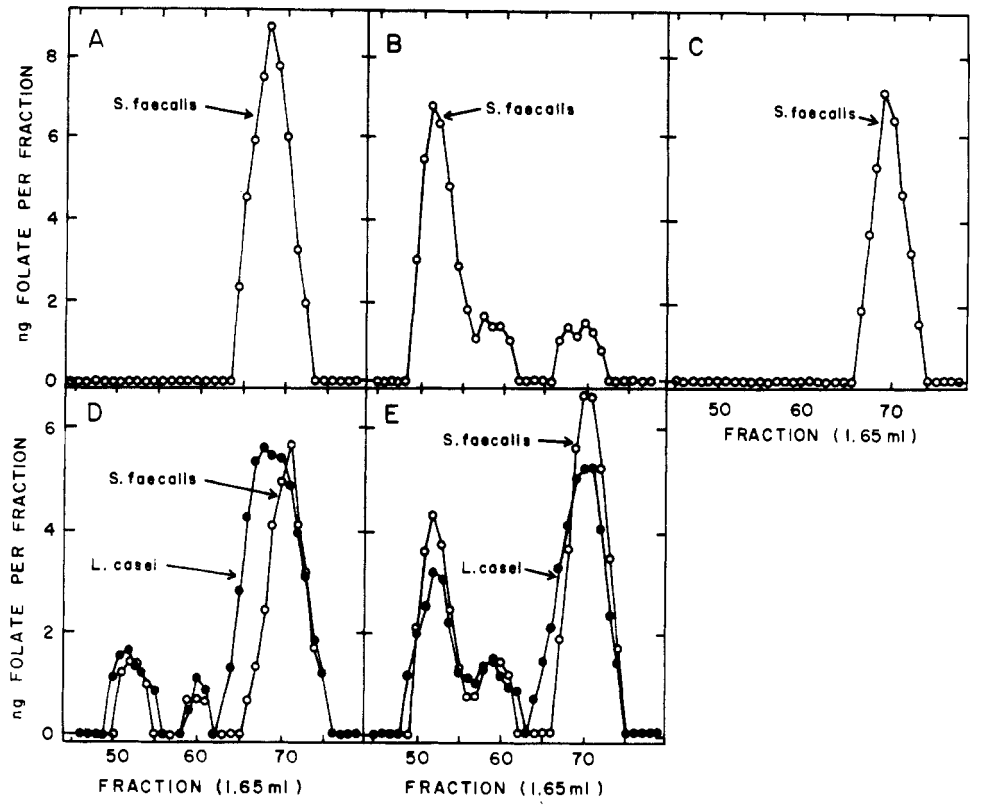

FIGURE 4: Sephadex G-25 chromatography of γ-glutamyl hydrolase treated liver folates. Folates (20–100 ng) were incubated with 1.0 mL of γ-glutamyl hydrolase (0.45 mg of protein) for 2 h under N_2 at 37 °C. The incubation mixture was 50 mM sodium acetate, 0.3% sodium ascorbate, and 50 mM 2-mercaptoethanol, pH 4.7, in a volume of about 3 mL. Samples were loaded on a column of Sephadex G-25 (0.75 × 190 cm) equilibrated and eluted with 0.1 M sodium phosphate, pH 7.0, and 200 mM 2-mercaptoethanol. The recoveries of folates after chromatography were between 109% and 126%, by *L. casei*. The marker compounds blue dextran, 10-formyl-H_4PteGlu, 5-formyl-H_4PteGlu, 5-methyl-H_4PteGlu, and H_4PteGlu eluted at fractions 23, 52, 59, 67, and 70, respectively, when run separately. DEAE-cellulose peak VI– (A), peak II^+ (B), peak V^+ (C), liver extract from –met rats (D), and liver extract from +met rats (E). (●) *L. casei*; (○) *S. faecalis*.

Table I: Identification of Liver Folates[a]

treatment				
met	N_2O	peak	major constituent	% of total folates
–	+	III	5-methyl-H_4PteGlu$_5$	18.3
–	+	IV	H_4PteGlu$_5$	2
–	+	V	5-methyl-H_4PteGlu$_6$	16.7
–	+	VI	H_4PteGlu$_6$	21.7
–	+	VII	5-methyl-H_4PteGlu$_7$	3.7
–	+	VIII	H_4PteGlu$_7$	11.0
–	+		unidentified	26.6
+	+	I	10-formyl-H_4PteGlu$_5$	10.7
+	+	II	10-formyl-H_4PteGlu$_6$	14.1
+	+	III	10-formyl-H_4PteGlu$_7$	8.8
+	+	IV	5-methyl-H_4PteGlu$_5$	2.2
+	+	V	H_4PteGlu$_5$	24.6
+	+	VI	H_4PteGlu$_6$	22.1
+	+	VII	H_4PteGlu$_7$	8.2
+	+		unidentified	9.3

[a] Two groups of rats were grown on a diet low in methionine, a diet which permits inhibition of histidine catabolism by subsequent nitrous oxide treatment. All animals were then exposed to nitrous oxide at 4 h. One group of eight rats (–met group) was injected with saline. The other group of eight rats (+met group) was injected with methionine. Then all animals were injected with a tracer dose of [^{14}C]histidine to confirm that histidine oxidation had been inhibited by the nitrous oxide treatment. Finally, livers from the two groups of rats were removed and extracted, and the extracts of each group were combined. Folates in the two pooled extracts were separated by chromatography and the fractions assayed microbiologically.

folate mono- to tetraglutamate comprised less than 5% of the liver folate pool.

Overall One-Carbon Units. The overall one-carbon unit forms present in liver folates were determined by treatment of extracts with γ-glutamyl hydrolase followed by chromatography on Sephadex G-25. The results appear in Table II. The one-carbon unit forms, as calculated from the data in Table I, are also listed here. The –met livers contained a high proportion of folates in the 5-methyl form and a low proportion in the formyl forms (Figure 4D), as compared with the folates from the +met livers (Figure 4E). About 70% of the +met formylfolates and 75% of the –met formylfolates were in the 10-formyl form, rather than in the 5-formyl form.

Table II: Identification of Overall One-Carbon Units of Liver Folates

treatment				
met	N_2O	folate	%[a]	%[b]
–	+	H_4PteGlu$_n$	51	33
–	+	5-methyl-H_4PteGlu$_n$	31	39
–	+	formyl-H_4PteGlu$_n$	18	9 (estimate)
+	+	H_4PteGlu$_n$	51	55
+	+	5-methyl-H_4PteGlu$_n$	10	3
+	+	formyl-H_4PteGlu$_n$	39	34

[a] Identification by γ-glutamyl hydrolase treatment followed by chromatography on Sephadex G-25. [b] Identification by chromatography on DEAE-cellulose and analysis of the folate activity peaks.

In an entirely separate experiment, one group of six rats was exposed to N_2O, and another group of six rats was not. All rats were grown on a low-methionine diet, and exposure to N_2O was for 6 h, from 9:30 to 15:30. None of the 12 rats was injected with methionine or histidine. By differential micro-

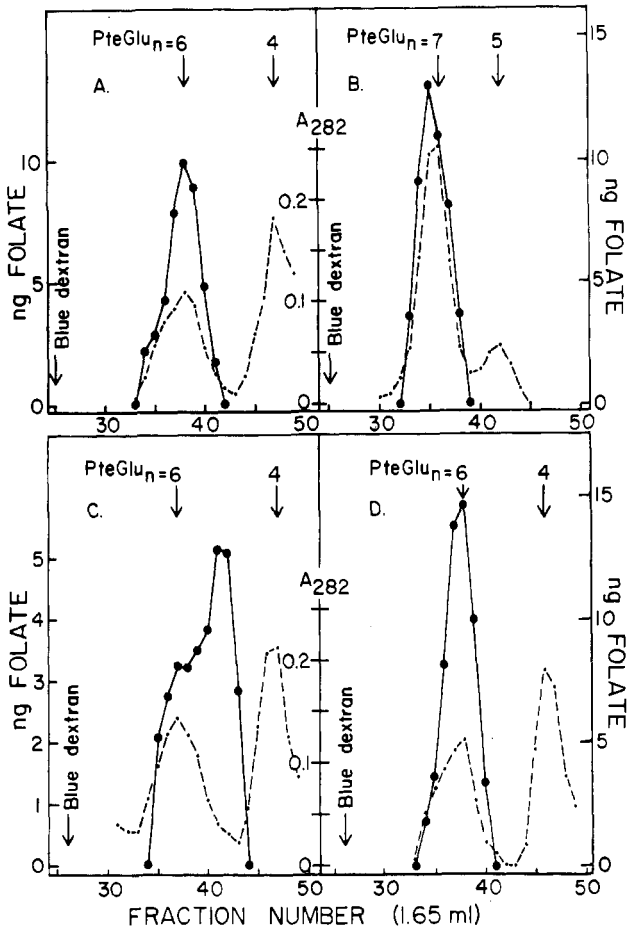

FIGURE 5: Sephadex G-25 chromatography of folates without prior treatment with γ-glutamyl hydrolase. Folates (30–50 ng) from the peaks indicated in Figures 2 and 3 were chromatographed on a column of Sephadex G-25, as described in Figure 4. Marker folate polyglutamates (about 20 000 ng) were run with the unknown folate. Aliquots (100–150 μL) of each fraction were treated in duplicate with γ-glutamyl hydrolase (22 μg of protein) in a volume of 1.2 mL for 3–4 h at 37 °C and then used for microbiological assay. The recoveries of folates as measured before chromatography with *S. faecalis* and after with *P. cerevisiae* were 103–133%. (●) Nanograms of folate per fraction by *P. cerevisiae*; (···) A_{282} of folate polyglutamate markers. DEAE-cellulose peaks VI– run on Sephadex G-25 (A), VIII– (B), V$^+$ (C), and VI$^+$ (D).

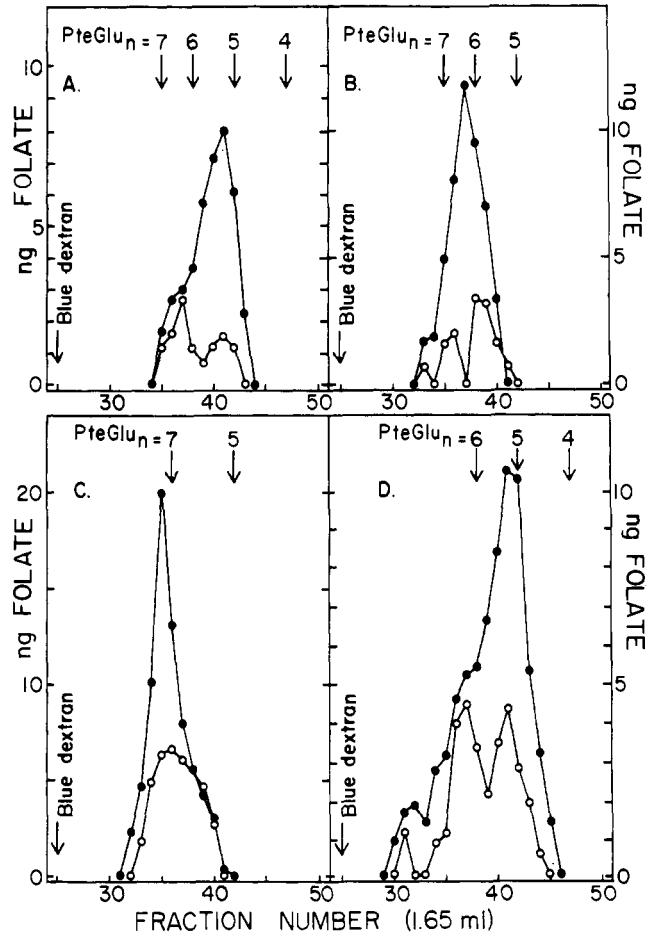

FIGURE 6: Sephadex G-25 chromatography of folates without prior treatment with γ-glutamyl hydrolyase. Folates were chromatographed and assayed as described in Figure 5 except that marker folate polyglutamates were not included with the unknown folate. The recoveries of folates were 100–140% by *L. casei*. (●) Nanograms of folate per fraction by *L. casei*; (○) nanograms of folate by *S. faecalis*. DEAE-cellulose peaks III– run on Sephadex G-25 (A), V– (B), VII– (C), and IV$^+$ (D).

biological assay of unfractionated extracts treated with γ-glutamyl hydrolase, the percent of methylfolate in each group was determined. The air- and N_2O-exposed rats contained 15% and 17% 5-methyl-H_4folates in the liver, respectively. These percentages are similar and were confirmed by fractionating the extract, pretreated with γ-glutamyl hydrolase, on a column of Sephadex G-25. Here, the air and N_2O rats contained 18% and 14% of their liver folates in the 5-methyl-H_4PteGlu$_n$ form. Although all these percentages are lower than that of the eight-rat –met group, it does appear that N_2O treatment did not result in increases or changes in the methylfolate pool. Similarly, Lumb et al. (1980) reported little change in the methylfolate pool with N_2O treatment.

Discussion

The folate identification data presented here and earlier (Brody et al., 1976) demonstrate that there are differences in the one-carbon metabolism mediated by folate pentaglutamates and folate hexaglutamates. These data show that 5-methyl-H_4folate is a major component of the folate pentaglutamate pool but comprises a smaller proportion of the folate hexaglutamate pool. The extreme case found in the brain is the near absence of methylfolates at the hexa- and heptaglutamate levels. The extreme case in liver from rats deficient in methionine is the near absence of tetrahydrofolate at the pentaglutamate level. The above situations could mean that 5-methyl-H_4PteGlu$_6$ formation is a more rate-limiting step in methionine biosynthesis than is 5-methyl-H_4PteGlu$_5$ formation. Alternatively, 5-methyl-H_4PteGlu$_5$ may be forced to accumulate by binding to proteins which prevent it from participating in one-carbon metabolism.

It was expected that the identification of rat liver folates in the present study would reveal differences in folate penta- and hexaglutamate metabolism, and to make such a discovery more likely, we studied rat liver folates under two metabolic conditions rather than only one. In the first condition, folate-dependent [^{14}C]histidine catabolism was made defective by nitrous oxide treatment, and it was here that large differences were found. In the second condition, this defect was relieved rapidly by an injection of methionine, and here only small differences were found.

It was expected that with nitrous oxide treatment, cellular folates would be forced to accumulate as 5-methyl-H_4PteGlu$_n$ at the expense of other forms of folate through the following mechanism. Nitrous oxide treatment apparently disturbs folate metabolism in rats, as indicated by the 20-fold decrease in [^{14}C]histidine oxidation. This decrease is thought to result from the assumed accumulation of folates in the 5-methyltetrahydro form. This assumed accumulation is thought to result from the inactivation of methionine synthetase (Deacon

et al., 1980) arising through oxidation of the cobalamin cofactor. Reduction of the nitrous oxide molecule has been shown to proceed at the terminal nitrogen atom (Dawson & Nibbering, 1978). Oxidation of the superreduced cobalamin cofactor (Taylor & Hanna, 1970; Fujii & Huennekens, 1979) may occur with a one-electron oxidation and production of a highly reactive hydroxyl radical (Blackburn et al., 1974) or with a two-electron oxidation, the exact mechanism not yet being known. However, in the experiment reported here comparing one group of rats exposed to nitrous oxide and another exposed to air, net accumulation of methylfolates did not occur with the nitrous oxide.

In addition, it was expected that with the subsequent injection of methionine the cellular methylfolate pool would contract, along with an expansion of the tetrahydrofolate and formyltetrahydrofolate pools and an increase in the tetrahydrofolate-dependent [^{14}C]histidine oxidation. A mechanism for this contraction, involving feedback inhibition of 5,10-methylenetetrahydrofolate reductase by *S*-adenosylmethionine, has been described (Kutzbach & Stokstad, 1971b). All these expectations were fulfilled.

With the contraction of the methylfolate pool, the amount of $H_4PteGlu_5$ increased from less than 5% up to 25% of the cellular folates. However, there were only negligible changes in the amounts of $H_4PteGlu_{6,7}$. This indicates the possibility that efficient histidine catabolism has an absolute requirement in vivo for $H_4PteGlu_5$ or 5,10-methylene-$H_4PteGlu_5$. Although a variety of $H_4PteGlu_n$ can accept the ring-2 carbon of histidine in the reaction catalyzed by formiminotransferase, the pentaglutamate chain length is especially favorable for the channeling effect, as shown by MacKenzie & Baugh (1980) for pig liver formiminotransferase. The correction of histidine oxidation occurring with the increased amount of $H_4PteGlu_5$ may also mean that $H_4PteGlu_5$ or the methylene derivative is an activator of an enzyme in the oxidative pathway.

When the methionine-deficient rats were injected with methionine, the formylfolate pool also increased. However, it is not clear from the present data if this increase occurred disproportionately at any one polyglutamate level.

It has been speculated that methylfolate polyglutamates bind tightly to proteins and are stored in the liver (Suzuki & Wagner, 1980). However, it is unlikely that 5-methyl-$H_4PteGlu_n$ is forced to accumulate in liver through tight binding to proteins which sequester the folate from one-carbon metabolism. This is because rapid conversion of 5-methyl-$H_4PteGlu_n$ to nonmethylated forms occurs with an injection of the methionine-deficient rats with methionine.

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