Adequacy of Enzymatic Deconjugation in Quantification of Folate in Foods†

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A critical aspect of many assay methods for folate in foods and other biological materials is the enzymatic deconjugation of polyglutamyl forms of the vitamin. This research was conducted to evaluate the properties of pteroylpolyglutamate hydrolase (conjugase) from hog kidney and the efficacy of this enzyme when used to deconjugate sample extracts for folate analysis. Extracts of a variety of foods caused detectable inhibition of the enzyme, although appropriate combinations of incubation time and enzyme concentration yielded complete or nearly complete hydrolysis of synthetic polyglutamyl folates. These results support the efficacy of the enzyme from hog kidney and indicate the need for care in establishing proper conditions for deconjugation in folate assays.

Naturally occurring folates in most materials of plant or animal origin exist in long-chain polyglutamyl form, with chain lengths primarily from five to seven γ-linked glutamyl residues. The determination of folate in foods and other biological materials may be performed by microbiological assay (generally with *Lactobacillus casei*), high-performance liquid chromatography (HPLC), or, to a lesser extent, ligand-binding methods. In each procedure, the enzymatic deconjugation of polyglutamyl folates is an important factor affecting the accuracy of the analysis.

The assay organism *L. casei* has been shown to yield full response to folates having monoglutamyl, diglutamyl, or triglutamyl chain lengths, while longer chain polyglutamyl folates elicit markedly less growth (Tamura et al., 1972a). Various HPLC methods have been reported for the determination of folates as their monoglutamyl forms in extracts of foods and other biological materials (McMartin et al., 1981; Duch et al., 1983; Gregory et al., 1984; Wilson and Horne, 1984, 1986; Rebello, 1987; Holt et al., 1988). Because polyglutamyl chain length is a major determinant of chromatographic retention in ion-exchange and reversed-phase HPLC, complete deconjugation of folates to their mono-glutamyl forms is an absolute requirement in sample preparation for these HPLC procedures. Competitive ligand-binding assay methods have been evaluated for the analysis of foods and animal tissues (Tigner and Roe, 1979; Graham et al., 1980; Klein and Kuo, 1981; Gregory et al., 1982). The influence of polyglutamyl chain length on the response of these assays is unclear at present, although several of the folate-binding proteins employed exhibit varying folate-binding affinities according to the chain length of the ligand (Shane et al., 1980).

Pteroylpolyglutamate hydrolase (conjugase; EC 3.4.12.10) from several sources has been used for deconjugation in folate analysis (Keagy, 1985). The conjugase enzyme from chicken pancreas yields primarily a diglutamyl folate product (Leichter et al., 1977), while forms of the enzyme from kidney, certain other tissues, and serum yield a monoglutamyl folate as the terminal product (Lakshmaiah and Ramasastri, 1975; Silink et al., 1975; Wilson and Horne, 1984; Day and Gregory, 1985). Very little information exists concerning the relative merits of these conjugase preparations for deconjugation in folate analysis.

Of the various conjugases suitable for use in conjunction with HPLC methods of folate determination, those from hog kidney and rat serum have received the greatest application (McMartin et al., 1981; Gregory et al., 1984; Wilson and Horne, 1984, 1986; Rebello, 1987; Holt et al., 1988). In the development of methods for folate analysis in this laboratory, hog kidney conjugase was adopted because of the generally greater availability of hog kidneys than rat serum. The present study was conducted to provide a kinetic description of the deconjugation of polyglutamyl folates by hog kidney conjugase as employed in folate analysis and to determine the susceptibility of this enzyme to inhibition by the components of food extracts.

MATERIALS AND METHODS

Note: All experimental procedures were conducted in a laboratory that was illuminated by gold fluorescent lights to protect labile folates from photochemical degradation.

Materials. Synthetic pteroyltriglutamate ($PteGlu_3$) was prepared by solid-phase synthesis as described by Krumdieck and Baugh (1980). Folic acid monoglutamate was obtained from Sigma Chemical Co. (St. Louis, MO). The concentration of these folates in standard solutions was determined spectrophotometrically from molar absorptivity values reported by Blakley (1969).

Hog kidney conjugase was partially purified according to the method of Brody et al. (1982) with minor modifications described by Gregory et al. (1984). The final fraction used in the present research was that precipitated between 50% and 75% saturation with ammonium sulfate following acidification to pH 5.0 and heat treatment (50 °C for 2 h). This fraction was dialyzed versus 0.05 M potassium acetate, pH 5.0, containing 10 mM 2-mercaptoethanol and then stored at −20 °C until used. The preparation contained 4.3 mg of protein/mL and no folate was detected when assayed by HPLC (Gregory et al., 1984). Little or no loss of activity occurred over 6-month storage.

Determination of K_m and V_{max}. The kinetic parameters K_m and V_{max} for the hog kidney conjugase preparation were determined at pH 4.9 with $PteGlu_3$ as substrate with the extraction buffer 50 mM sodium acetate, pH 4.9, containing 50.5 mM sodium ascorbate as the reaction medium. Incubation mixtures, in duplicate, contained 120 μL of 0.5–20.0 μM $PteGlu_3$ and 10 μL of the 10-fold-diluted enzyme preparation. The reactions were terminated by addition of 10 μL of 100% (w/v) trichloroacetic acid after 20-min incubation at 37 min. By allowing no more than 11% of the original amount of substrate to be deconjugated, the measurement of initial rates was assured. In this and all other studies, the relative concentration of residual

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substrate ($PteGlu_3$) and the products $PteGlu_2$ and folic acid were determined by HPLC as described below.

Determination of pH-Activity and pH-Stability Profiles. For the pH-activity profile, 37 μM folic acid (over $10K_m$) was used. Enzyme activity was determined at pH 3.0, 3.6, 4.5, 4.9, 5.3, 5.6, 6.4, and 6.9. The reaction mixtures contained 50 mM potassium acetate, 20 mM sodium phosphate, and 50.5 mM sodium ascorbate, which provided sufficient buffer capacity in the range pH 2.5–8.0. Freshly thawed enzyme preparation was diluted with 4 volumes of the above buffers containing 10 mM 2-mercaptoethanol. Fifty-microliter aliquots of diluted enzyme at the pH values listed above were mixed with 150 μL of 50 mM $PteGlu_3$ of the same pH (i.e., pH 3.0–6.9). Reaction mixtures were incubated for 5–30 min, depending on the enzyme activity at the given pH.

Modifications of these procedures were used to determine the pH-stability profile. The diluted enzyme preparations, of the same pH values listed above, were preincubated in the absence of substrate at 37 °C for 30–40 min. The pH was then adjusted to pH 4.5, the optimum pH, by addition of 1.0 M NaOH or HCl as needed. Fifty-microliter aliquots of diluted, preincubated enzyme solutions at pH 4.5 were mixed with 150 μL of 50 mM $PteGlu_3$ in the pH 4.5 buffer. Reaction mixtures were incubated for 5–30 min, depending on the enzyme activity remaining following preincubation.

Preparation of Food Extracts and Evaluation of Enzymatic Reactions. Selected foods were experimentally fortified with $PteGlu_3$, subjected to thermal extraction as previously described (Gregory et al., 1984), and then used for evaluation of the hydrolytic action of hog kidney conjugase. For these studies, 5 g of single-strength orange juice (from reconstituted Minute Maid concentrate), whole milk (Flav-O-Rich), and fresh chicken liver, or 25 g of fresh cabbage and canned kidney beans (Bush's Light), were homogenized in 4 volumes of extraction buffer (50 mM sodium acetate, pH 4.9, containing 50.5 mM sodium ascorbate). Whole wheat flour (Pillsbury), 5 g, was similarly homogenized except with 9 volumes of the buffer. Homogenization was performed with a Waring Blendor or a Polytron Type P100/35 (Brinkmann Instruments, Westbury, NY) operated at high speed for 1 min. For evaluation of the effect of citrate on the action of hog kidney conjugase, the extraction buffer (50 mM sodium acetate, pH 4.9, containing 50.5 mM sodium ascorbate) was prepared with the addition of 10.4 or 52.0 mM sodium citrate.

Twenty-five milliliters of each homogenate or buffer alone was placed in 50-mL screw-cap polypropylene centrifuge tubes and a concentrated solution of $PteGlu_3$ added to provide 5 μM added $PteGlu_3$ in each tube. The homogenates were blended with the Polytron for 1 min at high speed, and then all samples (homogenates and appropriate buffer solutions) were flushed with nitrogen gas, sealed, and incubated in a boiling water bath for 60 min. After cooling on ice, the mixtures were centrifuged at 10000*g* for 20 min at 2 °C, followed by removal of the supernatants and adjustment of the pH, if needed, to 4.9 by addition of HCl or NaOH. Each was then filtered on a syringe filtration device (0.45-μm membrane, Gelman, Ann Arbor, MI). Enzymatic reactions were conducted by mixing a 125-μL aliquot of each food extract or buffer containing 5 μM $PteGlu_3$ with 30 μL of the hog kidney conjugase preparation with incubations up to 180 min at 37 °C.

The effect of enzyme concentration on the rate of deconjugation in the kidney bean extract was also evaluated. In this study, reaction mixtures conisted of 125 μL of kidney bean extract and either 30, 45, 60, or 75 μL of conjugase preparation with enzyme dialysis buffer (50 mM potassium acetate, pH 5.0, and 10 mM 2-mercaptoethanol) to yield a total volume of 215 μL. Mixtures were incubated at 37 °C for 90 min.

Analytical Methods. Following each enzymatic reaction, activity was terminated by the addition of 10 μL of 100% (w/v) trichloroacetic acid, followed by centrifugation at 2000*g* for 10 min. The extent of conversion of the substrate, $PteGlu_3$, to the products $PteGlu_2$ and folic acid (PteGlu) was determined by reversed-phase HPLC (Gregory et al., 1987). In this procedure, separations were achieved with use of an isocratic mobile phase (0.1 M potassium acetate, pH 5.0, containing 1.2% v/v acetonitrile) at a flow rate of 1.5 mL/min and an octadecylsilyl column (3 × 3; Perkin-Elmer, Norwalk, CT). Highly sensitive and specific fluorometric detection was accomplished by post-column oxidative cleavage of the $PteGlu_3$, $PteGlu_2$, and folic acid with a hypochlorite reagent (100 μL of 5% w/v NaOCl in 50 mL of 0.1 M K_2HPO_4 containing 0.2 M NaCl) as described previously (Gregory et al., 1987). This reagent was pumped into the column effluent stream at 0.1 mL/min via a T-junction. The combined stream was passed to the fluorometric detector (FluoroMonitor, American Instrument Co., Silver Spring, MD) through a 5-m-long coil of 0.8-mm-i.d. Teflon tubing to allow for complete oxidative derivatization.

Quantification of each folate was performed by measurement of peak area. The degree of hydrolysis of the initial $PteGlu_3$ was calculated by dividing the combined area of the hydrolysis products ($PteGlu_2$ and folic acid) by the total peak area, with correction for the trace amounts of $PteGlu_2$ initially present in the $PteGlu_3$ preparation. Typical chromatograms are presented in Figure 1.

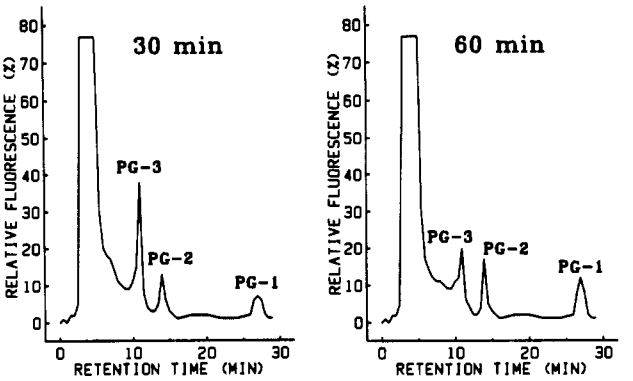

Figure 1. Chromatograms illustrating the time-dependent deconjugation of $PteGlu_3$ by hog kidney conjugase in an extract of red kidney beans. Reaction times are shown above chromatograms.

Table I. Kinetic Parameters for Hog Kidney Conjugase at pH 4.9 with $PteGlu_3$ as Substrate

method of calculn[a]	K_m, μM	V_{max}, nmol/(mg protein min)
Lineweaver–Burk	2.57 (0.27)	0.53 (0.06)
Eadie–Hofstee	3.12 (0.44)	0.61 (0.05)
Michaelis–Menten	3.17 (0.48)	0.61 (0.06)

[a] Values are from regression analysis of the same 16 data points, with standard deviations in parentheses. The Michaelis–Menten calculation was performed by the nonlinear weighted regression method of Duggleby (1981).

RESULTS AND DISCUSSION

The kinetic parameters K_m and V_{max} as well as the pH dependence of enzyme stability and activity were determined in this study. Although the K_m and optimum pH of the enzyme had been reported previously from a study using microbiological assay procedures and a heterogeneous substrate (Bird et al., 1946), we considered it important to assess the kinetic behavior of the enzyme using more direct and specific methods. The selection of pH 4.9 for the measurement of K_m and V_{max} was based on previous studies in our laboratory that showed that this pH was suitable for both effective extraction and subsequent deconjugation with hog kidney conjugase in preparation for HPLC or microbiological assay of folates in foods and other biological materials.

These kinetic studies (Table I) indicated a K_m for $PteGlu_3$ of 2.6–3.2 μM. This is in agreement with the results of Bird et al. (1946) who reported K_m 3.3 μM for polyglutamyl folates from yeast. The observed low K_m supports the use of hog kidney conjugase for deconjuga-

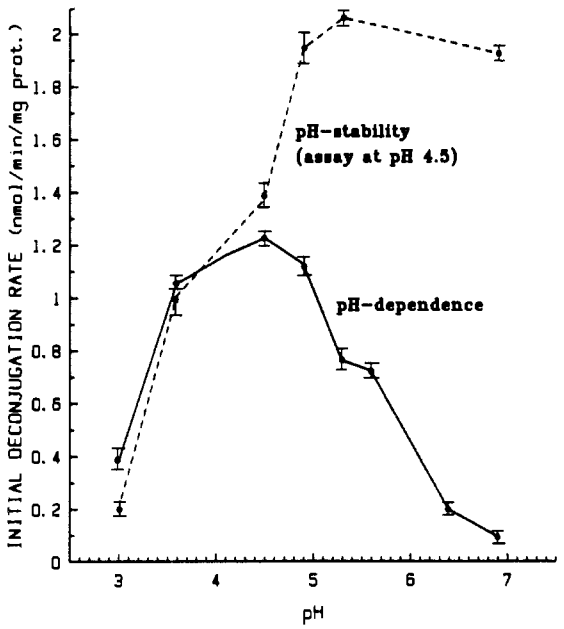

Figure 2. pH-activity and pH-stability profiles for hog kidney conjugase. Each determination of reaction rate was calculated from four time points, with each reaction performed in two or three replicates. Data points show means and standard deviations.

tion of polyglutamyl folates at the low concentration typically found in food extracts.

The pH-activity and pH-stability profiles are shown in Figure 2. In deriving the data of Figure 2, high linear correlation coefficients ($r > 0.990$) were found between the extent of deconjugation and incubation time for each pH series, with the exception of extreme pH values that yielded very low activity. The pH-activity curve exhibited a maximum at pH 4.5, which was in agreement with the results of Bird et al. (1946). However, while Bird et al. observed a narrow pH-activity profile, the present study revealed a broader range of high activity with near-maximum activity over the range pH 3.6–4.9. These results are consistent with the use of pH 4.9 for deconjugation of polyglutamyl folates with hog kidney conjugase.

The pH-stability curve indicated an apparent activation of the enzyme during preincubation at pH 4.5–6.9 before assaying at pH 4.5. To our knowledge, a pH-stability profile of this enzyme has not been reported previously. Although the presence of other enzymes (e.g., proteases) in the partially purified hog kidney conjugase could be responsible for the observed activation, the actual mechanism is unclear. Preliminary studies indicated that the activation occurred immediately after pH adjustment. This activation was not caused by the concurrent increase in sodium concentration derived from NaOH used for pH adjustment.

Studies of the hydrolysis of $PteGlu_3$ added to food extracts provided valuable information about the inhibitory effect of food components on the deconjugation of polyglutamyl folates. In addition to food extracts, we also investigated the influence of citrate, an anion known to inhibit other conjugases (Elsenhans et al., 1984; Kirsch and Chen, 1984), on the activity of hog kidney conjugase. The citrate concentrations used were 10.4 mM (0.2% w/v), which was similar to that found in extracts of orange juice, and 52.0 mM (1.0% w/v), which exceeded that found in orange juice extracts. The pH of all food extracts and citrate solutions was adjusted to 4.9 to eliminate pH as a variable. Although some competitive inhibition of the $PteGlu_3$ deconjugation would be expected

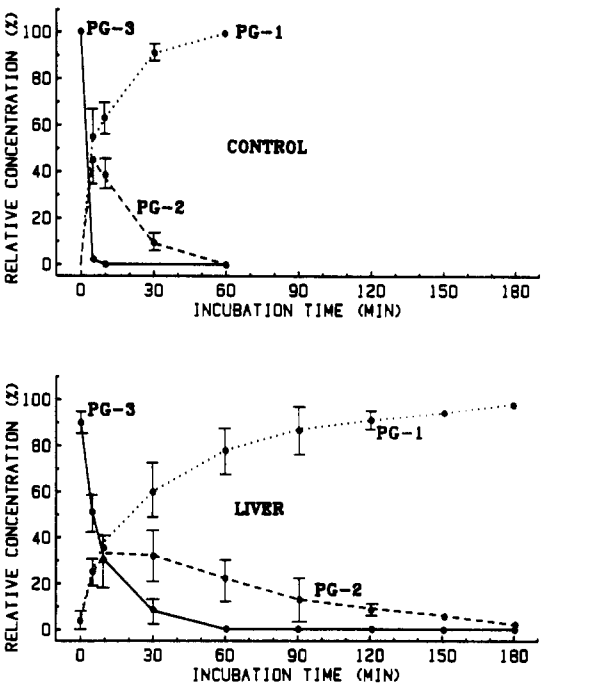

Figure 3. Relative distribution of $PteGlu_3$, $PteGlu_2$, and folic acid (PteGlu) versus incubation time in control (buffer only) and chicken liver extract. Data points show means and standard deviations of triplicate analyses.

Table II. Relative Distribution of $PteGlu_3$, $PteGlu_2$, and Folic Acid (PteGlu) following 60- and 180-min Reaction of Hog Kidney Conjugase with Food Extracts or Buffer Solutions Fortified with 5 μM $PteGlu_3$

	relative distribution,[a] %					
	60 min			180 min		
test matl in incubn med	Pte-Glu_3	Pte-Glu_2	Pte-Glu	Pte-Glu_3	Pte-Glu_2	Pte-Glu
control buffer	0	2	98	0	0	100
10.4 mM citrate	0	24	76	0	4	96
52.0 mM citrate	2	32	66	ND		
orange juice	8	31	61	0	2	98
milk	3	36	61	0	5	95
raw cabbage	0	8	92	0	0	100
boiled cabbage	0	32	68	0	0	100
chicken liver	0	22	78	0	2	98
red kidney beans	30	39	31	1	27	72
whole wheat flour	43	*b*	57	5	*b*	95

[a] Liver data are means of triplicate analyses. All other values are based on single analyses. ND = not determined. [b] $PteGlu_2$ in reaction medium containing wheat flour extract could not be determined due to the presence of an interfering non-folate peak.

in view of the presence of naturally occurring polyglutamyl folates, the 5 μM $PteGlu_3$ concentration used in this study, relative to the lower concentrations of other polyglutamyl folates, would minimize such an effect.

A typical time course study for the deconjugation of $PteGlu_3$ is presented in Figure 3. As shown in Table II, all food extracts and citrate caused some degree of inhibition relative to the control (i.e., extraction buffer alone). $PteGlu_3$ in controls underwent essentially complete conversion to monoglutamyl folic acid within 60 min under the conditions of this study, while $PteGlu_3$ in most food extracts and citrate buffers required 180 min. Increasing the concentration of citrate over the range 0–52 mM yielded proportional decreases in conjugase activity. Since the orange juice extract caused greater inhibition than 10 mM citrate, orange juice appears to contain compounds other than citric acid that inhibit conjugase activity. Similarly, the citrate concentration of milk is about

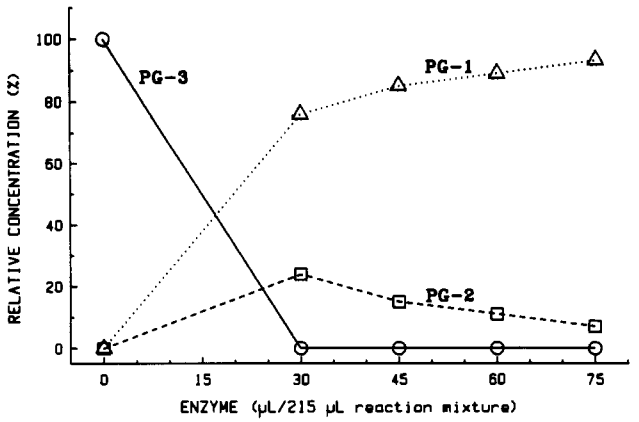

Figure 4. Relative distribution of $PteGlu_3$, $PteGlu_2$, and folic acid (PteGlu) versus concentration of hog kidney conjugase. All reaction mixtures were incubated for 90 min at 37 °C. Standard deviations of triplicate analyses are not indicated as they fell within the range designated by the data point symbols.

one-sixth that of orange juice (Jenness and Patton, 1959). The observed inhibition of conjugase action by milk extracts suggests that other inhibitory compounds are present in milk.

A complicating factor in studies of this type is the action of endogenous conjugases in raw plant and animal tissues. For example, the greater extent of $PteGlu_3$ hydrolysis in raw cabbage was presumably due to the action of endogenous conjugase activity (Tamura et al., 1972b) prior to heat treatment. Liver samples were heated to inactivate endogenous conjugase activity prior to the addition of $PteGlu_3$. The components responsible for the observed inhibitory effects of liver extracts on hog kidney conjugase are unclear. A variety of anionic compounds, such as citrate and nucleic acids, is present in mammalian cells. Nucleic acids have been reported to inhibit hog kidney conjugase (Mims et al., 1947; Olson et al., 1948). The inhibition caused by raw and boiled cabbage, whole wheat flour, and kidney beans may have been due to the presence of anionic polysaccharides, which can be present in concentrations up to several percent of dry matter (Englyst et al., 1982).

The most potent inhibition was observed in the red kidney bean extract (Table II). Strong inhibition of several conjugases by extracts of red kidney beans was previously observed by Butterworth et al. (1974) who found most of the inhibitory activity in the skin of the beans. Another study was conducted to determine whether deconjugation of $PteGlu_3$ in the bean extract could be accomplished by increasing the enzyme concentration in the incubation mixture. The results (Figure 4) indicate that increasing enzyme concentration effectively enhanced the rate and extent of $PteGlu_3$ deconjugation.

Relatively few studies have been reported concerning the inhibition of conjugase enzymes used for folate analysis. It has been reported that conjugase from chicken pancreas is less susceptible to inhibition than that from hog kidney (Eigen and Shockman, 1963), although no substantiating data were provided. Kirsch and Chen (1984) found that optimal conditions for deconjugation of polyglutamyl folates in spinach extracts could be determined (e.g., enzyme concentration, buffer, pH, and incubation time) for either hog kidney or chicken pancreas. Citrate was found to be a more potent inhibitor of the conjugase from chicken pancreas than that from hog kidney in their study. Phillips and Wright (1985) reported data showing generally higher values for apparent folate in several vegetables when treated with hog kidney conjugase compared treatment with chicken pancreas conjugase. These findings suggest that reaction conditions involving the chicken pancreas enzyme were not optimal. Pedersen (1988) found that the deconjugation of folates in extracts of peas and potatoes could be maximized by increasing the amount of conjugase from either hog kidney or chicken pancreas. Under optimal conditions, equivalent microbiological assay results were obtained when using either type of enzyme. As indicated previously, HPLC methods for the determination of folate are restricted to the use of conjugases that yield a monoglutamyl folate as the terminal product. Thus, conjugase from hog kidney would be compatible with both microbiological and HPLC methods while the enzyme from chicken pancreas is only suitable for use in preparation of samples for microbiological assay.

SUMMARY AND CONCLUSION

The results of this research indicate that partially purified hog kidney conjugase is appropriate for the deconjugation of polyglutamyl folates in extracts of foods and other biological materials in conjunction with various assay methods. Complete deconjugation of extracted polyglutamyl folates with this enzyme is feasible. The proper combination of enzyme concentration and incubation time should be determined for each type of sample to be analyzed. In addition, this information should be determined for each batch of hog kidney conjugase prepared before use in folate analysis.

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Registry No. Folic acid, 59-30-3; pteroyltriglutamate, 21919-06-2; pteroyldiglutamate, 6807-82-5; pteroylglutamate hydrolase, 9074-87-7.

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