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PROPERTIES OF A FOLATE BINDING PROTEIN (FBP) ISOLATED FROM PORCINE KIDNEY

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Abstract—A specific high-affinity folate binding protein (FBP) that binds folic acid and folic acid derivatives and that was previously identified in porcine kidney has been purified 50,000-fold using the technique of affinity chromatography. The FBP had a molecular weight of 38,500 daltons and did not appear to aggregate in solution, as has been reported to be the case with folate binding protein from milk. At pH 7.6, the K_a was at least $5 \times 10^{12}\,M^{-1}$. At pH values greater than 9.5 or less than 5, the binding dramatically decreased. The specificity was determined by an isotopic dilution technique using [^{3}H]folic acid and folic acid analogs and derivatives. The FBP reacted more rapidly with unsubstituted folates, and the number of glutamic acid moieties ($N \geq 1$) did not influence binding. Binding of folic acid to the FBP was unaffected by a variety of anions and cations, and 8 M urea, but was disrupted by 6 M guanidine hydrochloride. Proteolytic enzymes irreversibly destroyed binding affinity, but RNase, DNase, phospholipase and neuraminidase had no effect.

Specific, high-affinity binders of folic acid (pteroylmonoglutamic acid, PteGlu) and some of its derivatives, first identified in milk, have now been found and partially purified from a variety of tissues and plasma [1]. The folate binding proteins (FBPs) studied thus far are immunologically heterogeneous and have different molecular sizes and various affinities and specificities for selected folic acid analogues and metabolites [1–3]. Although the descriptive literature on plasma and serum FBP is plentiful, the small quantities of binding protein(s) available from most sources have prevented large scale purification and analysis [4–9]. The present work describes some of the biochemical properties of a folate binding protein purified approximately 50,000-fold to apparent homogeneity from porcine kidney acetone powder. The properties of this factor are compared to other binders found in plasma, milk, cell lysate from a patient with chronic granulocytic leukemia (CGL) and, most recently, rat kidney [10–15].

MATERIALS AND METHODS

Affinity chromatography gel matrix was prepared by covalently coupling methotrexate (MTX) to AH-Sepharose 4B (Pharmacia, Piscataway, NJ) using 1-ethyl-3-(dimethylaminopropyl) carbodiimide as described elsewhere [16]. Conjugase treatment of the Sepharose beads was empirically performed because it was noted that the beads lost color and that the effluent material had increased amounts of methotrexate when incubated with plasma or a hog kidney conjugase preparation, suggesting that the enzyme released MTX γ-glutamyl linked to the activated Sepharose. Folic acid, coupled in a similar manner, has also proved successful in purifying FBPs, but in our laboratory the use of MTX has resulted in higher yields and in a faster elution of the FBP from the column. This is likely due to its markedly decreased affinity for MTX.

Assays to measure the specificity and affinity of the FBP were performed by incubating [^{3}H]PteGlu (sp. act. 20–40 Ci/mmole; Amersham Searle, Radiochemical Centre, England) with a number of folate analogs as indicated in each particular experiment, followed by separation of the bound and free [^{3}H]PteGlu using charcoal coated with dextran (T10 or T20 [5:1 (w/w) charcoal/dextran]) as described in detail previously [16, 17]. Use of Sephadex gel filtration, ethanol precipitation or sucrose density gradient centrifugation to separate PteGlu from PteGlu–binder complex gave results comparable to the charcoal method; therefore, the charcoal method was used because of its speed and economy.

Isoelectric focusing was performed with a model LKB 110 jacketed column using ampholytes to form a gradient from pH 3 to 10. The average run took 12–16 hr. Gel electrophoresis was performed according to the method of Davis [18]. The recovery and the identification of [^{3}H]PteGlu were demonstrated using DEAE A-25 [19]. Treatment of the purified binding protein with proteolytic enzymes was accomplished using assays and standards as described in the Worthington Catalog (1972).

PteGlu and 5-methyltetrahydrofolic acid ($5CH_3H_4PteGlu$) were purchased from the Sigma Chemical Co. (St. Louis, MO) and standardized according to published spectral data [20]. Dihydrofolic acid was prepared by dithionite reduction of PteGlu [21]. MTX and 5-formyltetrahydrofolic acid ($5CHOH_4PteGlu$) were purchased from

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Lederle, Pearl River, NY. Folylhepatglutamate was a gift from Dr. Charles Butterworth and 5-methyltetrahydrotriglutamate was a gift from Dr. Warick Sakami. Other pteridines, purines, nucleotides and nucleosides used were purchased from the Sigma Chemical Co.

Liquid scintillation samples were counted at an efficiency of 25–35% in a Packard Tricarb counter in a solution of Triton X-100 and toluene (1:1, v/v) containing 5 g Preblend 2a70 (Research Product, Inc., Mt. Prospect, IL)/liter solution.

Aliquots of [^{3}H]PteGlu less than 90% pure (as determined by binding in the presence of an excess of the purified binder) were purified by chromatography on DEAE-cellulose [22].

Preparation of folate binding protein. The crude preparation of FBP was made by suspending porcine kidney acetone powder (Sigma Chemical, K7250) in 0.05 M phosphate buffer, pH 7.5 (100 g/l). The suspension was stirred for 15–20 min at room temperature, and then the pH was adjusted to 3–3.5 with 1 N HCl. After acidification, 50 g of activated charcoal (Norit A; Matheson, Coleman & Bell, Norwood, OH) was added per liter of solution and the suspension was stirred overnight at 4°. The charcoal was removed by centrifugation for 30 min at 6000 *g*. The solution was mixed with an equal volume of ethanol and held at −20° for 4–6 hr. After centrifugation at 6000 *g* for 15 min, the precipitate was discarded and the supernatant fraction was mixed with ethanol to a final concentration of 75%. This solution was held at −20° overnight. The salmon-colored precipitate was collected by centrifugation as above and dried for 3 hr at room temperature. The pellet was suspended in a minimal volume of 0.05 M phosphate buffer (pH 7.5) and centrifuged for 10 min at 10,000 *g* to remove any residual precipitate. The supernatant fraction containing the FBP was further purified by affinity chromatography as described elsewhere [16]. Briefly, the FBP was passed through the column, the column was washed with 0.5 M NaCl until no A_{280} was detected, and the FBP was eluted with 0.2 M acetic acid (1- to 2-ml aliquots). The FBP was generally found in fractions 2–6. The peak fractions were pooled, neutralized with 1 M ammonium bicarbonate (1:5, v/v) and then either frozen at −80° or lyophilized to dryness. The sample was reconstituted by dissolving in H_2O.

RESULTS

The FBP was purified approximately 50,000-fold from the initial extract with an overall yield of 30–40% (Table 1). The final specific activity of different preparations ranged from 16.0 to 22.0 nmoles PteGlu bound/mg protein. Assuming a univalent, homogeneous binding site and a molecular weight of 38,500 (see below) for the protein, the specific activity of the FBP reported here was 0.85 moles PteGlu bound/mole protein. The specific activity was not increased by further purification using standard procedures, such as DEAE chromatography or by passing the preparation through the MTX-Sepharose again. Passage of the purified binder over

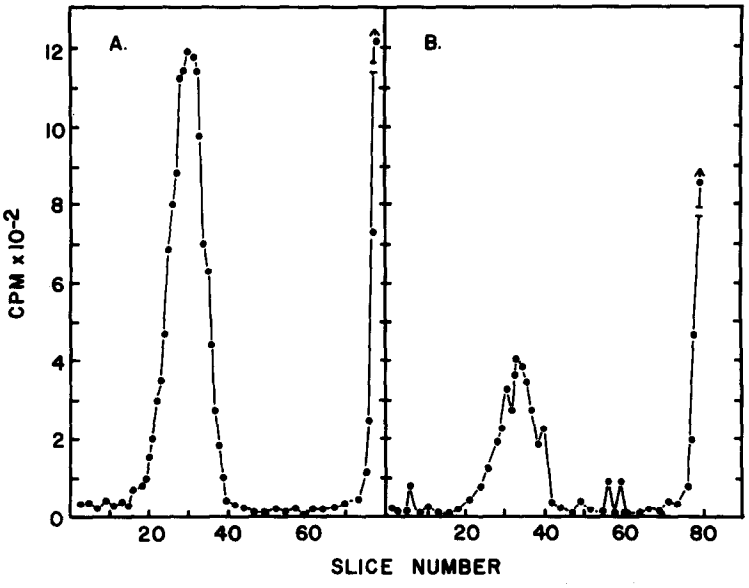

Fig. 1. Polyacrylamide disc gel electrophoresis of folate–binder complex. Affinity chromatography purified hog kidney binder (16 μg) having a 0.24 nmole PteGlu binding capacity was incubated for 20 min at room temperature with 2.4 nmoles [^{3}H]PteGlu (sp. act. 2.6 Ci/mmole) in a volume of 0.2 ml; then 0.1 ml was heated for 10 min at 95° and each sample was electrophoresed on separate 6 × 80 mm 7.5% polyacrylamide gels. Electrophoresis was done at pH 8.6 and at 0.5 mA/gel until the tracker dye, bromphenol blue (on a third gel), was 0.5 cm from the end of the gel. This generally took 8–10 hr. The gels were sliced into 1-mm sections, placed in a scintillation vial, and treated with 0.2 ml NH_4OH overnight; then the radioactivity was determined by adding 5 ml of scintillation fluid and counting. (A) unheated sample; and (B) heated sample. The top of the gel is fraction one. The tracker dye correlated with the peak radioactivity at fractions 75–80.

Table 1. Purification of folate binder from hog kidney acetone powder

	Total volume (ml)	Total protein (mg)	Total binding (dpm)	Specific activity (dpm [^{3}H]PteGlu bound/mg protein)
Crude extract	5,000	100,000	1.7×10^9	1.7×10^4
75% Ethanol ppt	350	8,050	1.0×10^9	1.25×10^5
Affinity column	7	0.8	6.8×10^8	8.5×10^8

Increase in specific activity = 5×10^4. Recovery of initial activity = 40%. Protein was determined as detailed [23].

a Sephadex G-100 molecular sieve column increased the specific activity about 3–5%, but resulted in a 10% loss of total activity. Chromatography of the FBP on Sephadex G-200 yielded a molecular weight of 40,000. Sodium dodecyl sulfate (SDS) gel electrophoresis of 1–10 μg FBP yielded a single band (Coomassie blue stain) at 38,500 daltons (data not shown). Analysis of the [^{3}H]PteGlu–binder complex by gel electrophoresis is shown in Fig. 1. Similar gels, except for substitution of [^{1}H]PteGlu for [^{3}H]PteGlu, stained for protein, showed a single band correlating to fractions 25–40. The radioactivity in fractions 25–40 was not adsorbed by treatment with coated charcoal, whereas 95% of the radioactivity in fractions 70–80 was, implying that this latter fraction was non-bound PteGlu. Sucrose density gradient sedimentation experiments demonstrated that the FBP did not appear to self-aggregate or shift its apparent molecular weight over a 1–80 μg/ml range (Fig. 2).

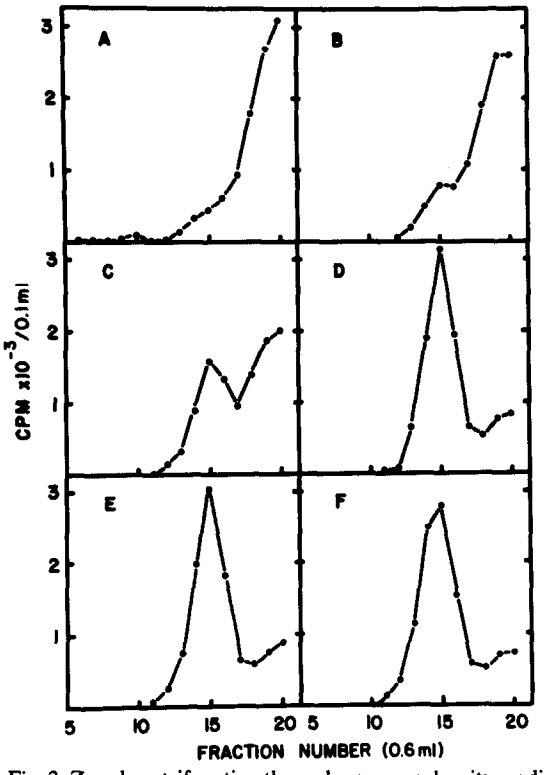

Fig. 2. Zonal centrifugation through a sucrose density gradient. Affinity chromatography purified folate binder (1.0 to 80 μg) having a 15–1200 pmole PteGlu binding capacity was incubated with 300 pmoles of [^{3}H]PteGlu (sp. act. 0.26 Ci/mmole) for 45 min at room temperature in a total volume of 1 ml. A 0.6-ml aliquot was layered on a 5–20% sucrose gradient prepared in 0.05 M potassium phosphate, pH 7.6, on top of a 0.5 ml 30% sucrose cushion. Total volume was 10.0 ml. The samples were centrifuged for 24 hr at 40,000 rpm in a Beckman SW 41 rotor. Samples of 0.6 ml were collected from the bottom. Radioactivity was determined in a 0.1-ml aliquot. Radioactivity in fractions 14–16 from samples D, E and F was not removed by charcoal-coated dextran. A_{280nm} determinations of samples E and F showed a single symmetric band with a peak at fraction 15 of 0.177 and 0.328 A_{280} units respectively. Key: (A) 1.0 μg; (B) 5.0 μg; (C) 10 μg; (D) 20 μg; (E) 40 μg; and (F) 80 μg.

Isoelectric focusing of the saturated or unsaturated FBP resulted in three fractions, with mobilities at pH 5.4, 6.0, and 6.6 for FBP saturated with folate and three fractions at pH 5.7, 6.4, and 6.8 for unsaturated FBP.

The effect of pH on the binding of PteGlu is shown in Fig. 3. Below pH 5.5, binding of PteGlu rapidly diminished. This loss of binding was immediately and completely recovered upon neutralization. At pH 7.5, Scatchard analysis yielded an approximate binding constant because the [^{3}H]PteGlu was not of high enough specific activity to measure an equilibrium between bound and free PteGlu. That is, within the limits of the experimental design, when the FBP ≥ the PteGlu (initial concentration of 10^{-10} M PteGlu), all the tracer was bound. Using an alternative method for calculating association constants, estimates of the binding constant at neutral pH were $1\text{–}5 \times 10^{12}\,M^{-1}$ (Fig. 4) [24]. Scatchard analysis of [^{3}H]PteGlu binding at pH 5.4, 9.8 and 10.15 yielded K_a values of $2.2 \times 10^{10}\,M^{-1}$, $7.5 \times 10^9\,M^{-1}$, and $3.0 \times 10^9\,M^{-1}$, respectively, but a number of binding sites equal to that measured at neutral pH (Fig. 5). At pH 9.8, binding of $5CH_3H_4$PteGlu and folic acid were equivalent, based upon a competitive binding technique using [^{3}H]folic acid as detailed in Table 2 (data not shown).

The specificity of the FBP, at neutral pH, was analyzed by incubating a number of PteGlu derivatives and analogues in a competitive ligand binding system with [^{3}H]PteGlu. The degree of inhibition of tracer binding was compared with a standard solution of PteGlu. The results are presented in Table 2. Binding was most selective for oxidized or reduced (but non-substituted) folates, independent of the number of glutamyl residues, and required at least the pteroic acid moiety of the PteGlu molecule for any significant competition to be detected.

The effect of temperature on the rate of PteGlu–

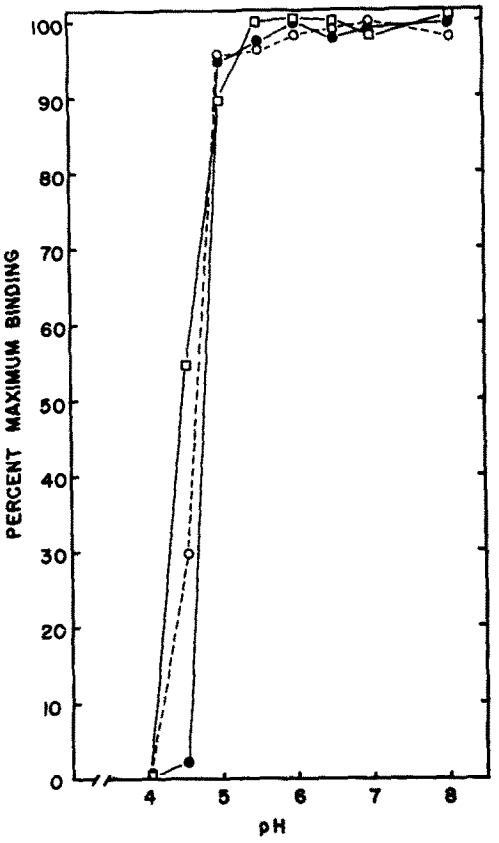

Fig. 3. Effect of pH on binding of [^{3}H]PteGlu. Hog kidney folate binder (10^{-12} pmole PteGlu binding capacity) was incubated at room temperature with 2×10^{-12} moles [^{3}H]PteGlu in a total volume of 1.0 ml. The reaction was stopped after 20 min by addition of 1 ml of ice-cold suspension charcoal coated with dextran and processed as usual. The percent of maximum binding was calculated as follows:

$$\frac{dpm\ bound\ sample - dpm\ charcoal\ control}{dpm\ bound\ at\ pH\ 7.6 - dpm\ charcoal\ control} \times 100$$

Key: (□) 0.05 M sodium citrate; (●) 0.05 M sodium or potassium acetate; and (○) 0.05 M potassium phosphate.

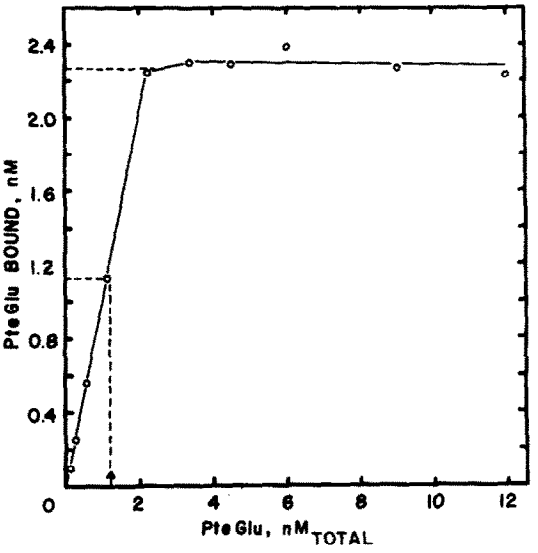

Fig. 4. Estimation of the binding constant of purified hog kidney folate binder for PteGlu. Folate binder (0.16 μg) was incubated with increasing amounts of [^{3}H]PteGlu as indicated. Incubation was for 20 min at room temperature in a total volume of 1 ml. The reaction was stopped by addition of 1 ml of charcoal coated with dextran. The binding capacity, 2.28 ± 0.04 pmoles/ml (mean ± S.D.) was determined by measuring the binding in the presence of up to a 50-fold excess of [^{3}H]PteGlu. At a concentration of 1.14×10^{-9} PteGlu, one-half maximum binding of 1.14×10^{-9} was measured, i.e. all available PteGlu was bound.

FBP complex formation was examined at three temperatures with 1.5×10^{-9} M folic acid and 1.0×10^{-9} M active FBP. The initial rates at 4°, 21°, and 37° were 0.65%/sec, 1.2%/sec, 2.5%/sec and were linear to approximately 75% of completion. Once formed, the complex was stable for at least 48 hr, even in the presence of a 10,000-fold excess of PteGlu, at 4°. Heat denaturation of the FBP, either free or complexed with PteGlu, was irreversible and took about 20 min at 99° to complete.

To partially test for the possibility that there was some enzymatic function of the purified FBP, the PteGlu–binder complex was incubated with ATP, NADPH, NAD, NADH, and dihydrofolic acid reductase in several combinations for 10 min at 37°. The samples were boiled for 20 min in the presence of 5 mg ascorbic acid/ml solution in order to separate the PteGlu from binder. [^{3}H]PteGlu was recovered and identified with a DEAE A-25 column as shown in Fig. 6. The peak of radioactivity of fraction 3 most likely as ^3H_2O. It eluted from the column in the wash and was lost upon lyophilizing. The peak at fraction 43 corresponded to *p*-aminobenzoylglutamate which may have been formed by degradation of the [^{3}H]PteGlu during the boiling. When chromatographically pure [^{3}H]PteGlu was boiled and chromatographed, there was a small peak at this position and at fraction 3. In these experiments, there was a >90% recovery of the radioactivity as [^{3}H]PteGlu

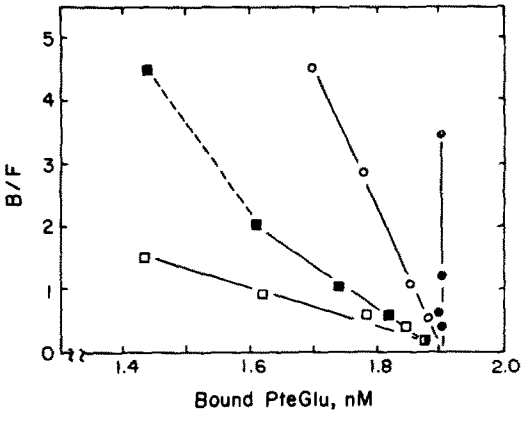

Fig. 5. Scatchard plot of [^{3}H]PteGlu binding at different pH values. Conditions were as in Fig. 4 but 1.9 pmoles of PteGlu binding activity was used. Key: (□) pH 10.15; (■) pH 9.8; (●) pH 7.6; and (○) pH 5.5. Alkaline buffers were made with 0.05 M barbital or lysine. Acid buffers were made with acetate or citrate.

Table 2. Effect of folic acid, derivatives, or analogs on [^{3}H]PteGlu binding

Compound	Percentage inhibition at amounts shown (pmoles) 1.0	2.0	10.0	100.0
Folic acid	52	66	85	98
Dihydrofolic acid	55	64	80	99
Tetrahydrofolic acid	50	60	75	95
Diopterin	53	65	85	97
Teropterin	54	67	85	98
Folylheptaglutamate	52	65	84	98
5-Methyltetrahydrofolate	20	40	68	95
5-Formyltetrahydrofolate	0	0	0	5
5-Methyltetrahydrofolyltriglutamate	20	42	65	95
10-Formyltetrahydrofolate	30	45	70	90
Aminopterin	0	0	0	0
Methotrexate	0	0	0	5
3',5'-Dichloromethotrexate	0	0	0	0
Pterin			0	0
Pterin-6-carboxylic acid			0	0
Xanthopterin			0	0
p-Aminobenzoic acid			0	0
Glutamic acid			0	0
p-Aminobenzoylglutamic acid			0	0
Pteroic acid	0	0	5	10
Thymidine			0	0
Adenosine			0	0
NADH			0	0
NADPH			0	0

Purified hog kidney folate binder (0.75×10^{-12} moles PteGlu binding capacity) was incubated with 1×10^{-12} moles [^{3}H]PteGlu and competitor as noted, in a total volume of 1 ml. Thus, 10^{-12} moles competitor was equal to the [^{3}H]PteGlu. If there was equimolar competition, then 10^{-12} moles competitor would reduce binding of [^{3}H]PteGlu by 50%. The percent inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{dpm control} - \text{dpm sample}}{\text{dpm bound in control}} \times 100$$

Control was binding in the absence of competing compound. The reaction was started by adding binder to the solution of [^{3}H]PteGlu and competitor and was stopped after 15 min using charcoal coated with dextran. The results are from duplicate experiments run in duplicate on two different days with freshly prepared compounds.

Table 3. Effect of enzymes on binding activity

Enzyme	Percent of maximum binding 2 min	30 min
Chymotrypsin (1 mg/ml)	90	0
Trypsin (1 mg/ml)	92	0
DNase I (1 μg/ml)	97	98
RNase (1 μg/ml)	98	96
Phospholipase C (1 mg/ml)	100	98
Neuraminidase (1 mg/ml)	99	100
β-Galactosidase (1 mg/ml)	97	98

Enzymes were all obtained from Worthington, and used as directed in the *Worthington Enzyme Manual*, Worthington Biochemical Corp. DNase and RNase, phospholipase C, neuraminidase and β-galactosidase were shown to be active using the assay described in the manual. The enzymes were incubated in a total volume of 1 ml with hog kidney binder (10^{-12} mole PteGlu binding cpacity) and 2×10^{-12} moles [^{3}H]PteGlu for the time indicated. The reaction was stopped by placing on ice and adding 1 ml of ice-cold charcoal-coated suspension. Because the pH optimum of neuraminidase is 5.0–5.1, incubation with this enzyme was done in 0.5 ml vol. at pH 5.0 and then adjusted to about pH 7.0 by adding 0.5 ml of 0.1 M potassium phosphate buffer, pH 8.5, and incubating for an additional 2 min.

after incubation with the FBP. Similar results were obtained when FBP purified from fresh kidney was analyzed.

The effects of various hydrolytic enzymes are presented in Table 3. The FBP was irreversibly destroyed by incubation with proteolytic enzymes, but was unaffected by lipase, DNase, RNase and neuraminidase. Common anions and cations such as Ca^{2+}, Mg^{2+}, NH_4^+, Ba^{2+}, Na^+, K^+ and HPO_4^{2-}, CH_3COO^- at concentrations from 0.1 to 0.5 M did not affect binding. Preincubation with either 8 M urea or 6 M guanidine hydrochloride could prevent complex formation between folate and FBP. The effect was reversible by dialysis. The complex was stable to 8 M urea, but was disrupted by 6 M guanidine hydrochloride.

DISCUSSION

In 1969, a folate binder in cow's milk was partially purified and some of the properties described [25]. The existence of this factor had been postulated by others [26]. Since then, folate binders have been found in a number of different tissues and plasma [1–11, 27–35]. This paper presents characteristics of

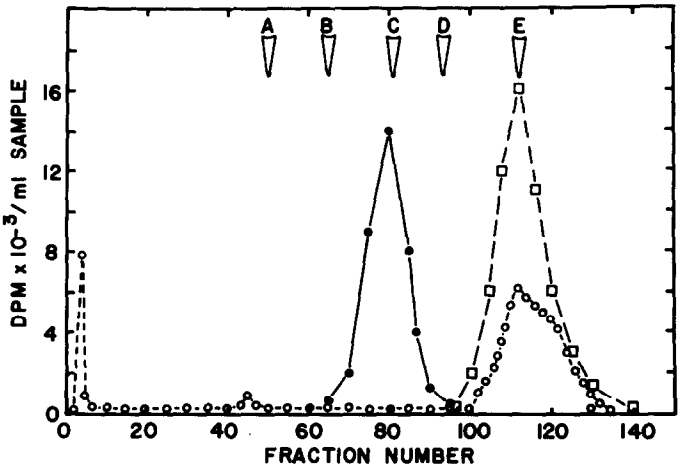

Fig. 6. DEAE A-25 chromatography of tritium released from a [^{3}H]PteGlu–binder complex. [^{3}H]PteGlu (2 pmoles; sp. act. 36 Ci/mmole) was incubated for 20 min at 37°C in the presence of excess binder at pH 7.6 in a total volume of 1 ml. The reaction was terminated by adding 5 mg of ascorbic acid and then boiling for 20 min. The sample was centrifuged for 10 min at 12,000 *g* to pellet the heat-denatured material. The clear supernatant fraction, containing more than 98% of the total radioactivity, was applied to a 1 × 10 cm DEAE A-25 column prepared according to Nixon and Bertino [19]. Elution was also carried out according to these workers. The column had been calibrated with known folates as indicated. The ability to separate [^{14}C]$5CH_3H_4$ and [^{3}H]PteGlu was also shown in a separate experiment. Key: (○) radioactivity released from PteGlu–binder complex; (●) marker [^{14}C]$5CH_3H_4$Glu; (□) marker, [^{3}H]PteGlu (A) *p*-aminobenzyolglutamate; (B) $5CHOH_4$PteGlu; (C) $5CH_3H_4$PteGlu; (D) H_2PteGlu; and (E) PteGlu.

a folate binder extracted from acetone powder of porcine kidney and purified 50,000-fold. Acetone powder was used for convenience because insufficient FBP was present to warrant processing of fresh kidney.

As with previously described factors, the porcine kidney folate binder reacted more rapidly with unsubstituted folates and required at least the pteroic acid moiety of the folate molecule. Folate binding appeared to be independent of the number of glutamyl residues (N = 1–3), and analogs of the pteridine moiety such as purines and pyrimidines did not complete for [^{3}H]PteGlu binding. The change in selectivity for PteGlu and $5CH_3H_4$PteGlu as a function of pH is discussed below.

The factor described here is similar to the FBP recently purified in a similar manner from CGL cells with respect to isoelectric points, binding specificity, affinity and molecular weight [14] although the initial reports of the properties of the partially purified CGL binder presented somewhat different molecular weight classes and binding specificities [36, 37]. These differences may be related to the degree of purity, extent of endogenous saturation with folate, or to the process of purification [14, 37]. Neutral pH extracts of porcine kidney contained unbound (free folate) and no "unsaturated" folate binder with respect to [^{3}H]folic acid; therefore, a comparison of endogenously "saturated" and "unsaturated" FBP was made.

The recently described rat kidney folate binder was localized in brush borders and released by means of Triton X-100 in the extraction buffer [15]. Rat kidney folate binder was found to be 28,500–30,000 daltons and had a K_a of only $2.5 \times 10^{10}\ M^{-1}$. It was affected by a number of anions (especially Cl^-). The reasons for these differences from the FBP presented here are not known but could be related to differences during initial purification (acetone powder vs fresh extract) and/or the presence or effect of Triton X-100. The FBPs purified from milk and placenta appear in multiple molecular weight as determined by gel electrophoresis and column chromatography; the binding affinity and specificity have not yet been further detailed [38, 39]. As demonstrated by sucrose density centrifugation, the FBP described here did not appear to aggregate in solution (Fig. 2).

The effect of acidic pH on the binding of PteGlu by porcine kidney binder was similar to that described for other FBPs. The acid treatment forms the basis for removal of endogenously bound folate, thereby rendering the binding protein unsaturated and capable of reacting with [^{3}H]PteGlu. This was first noted by others [38], and used in our laboratory to identify FBP in extracts of fresh porcine kidney [40]. The same procedure has been modified to analyze and measure unsaturated and saturated FBPs in serum, milk and placenta. The effects of pH on porcine kidney FBP are shown in Figs. 3 and 5. The apparently equal specificity of the binding protein for folic acid and methyltetrahydrofolic acid at alkaline pH is similar to that observed when the FBP in milk was studied [41].

The recovery of PteGlu from the PteGlu–FBP complex confirms that under the limited conditions tested the FBP did not alter folic acid (Fig. 6). Reduced folates have not been tested. This is important because at the present time, despite the descriptive literature, there is no known physiologic role

(e.g. transport or intracellular regulation of folate) for the FBP. some preliminary work has permitted the suggestion that the FBP can retard folate uptake *in vitro* or inhibit thymidylate synthetase activity by limiting the availability of the folate substrate [42]. Others have demonstrated that FBP from human plasma (obtained from pregnant women) facilitated uptake of radiolabeled folate by liver of pregnant animals [43]. However, transport studies with biologically available folate ($5CH_3H_4PteGlu$) at physiological concentrations may be needed before an appraisal of this putative function can be evaluated seriously.

Lower affinity FBPs ($K_d \geq 10^{-6}$ M) have been reported previously. Rat liver contains several different molecular weight folate binding proteins [5]; most recently, a tetrahydrofolate–protein complex in mitochondria co-purified with dimethylglycine dehydrogenase [44]. A folate–protein complex in bovine liver associated with cytochrome *c* reductase has also been described [45]. Whether this type of FBP is the same as the high-affinity type described here has not yet been fully ascertained.

Further analysis and comparison of secretory (milk), plasma, and intracellular FBPs may give clues as to their biological function and overall heterogeneity. It has already been suggested that the goat milk FBP can retard the availability of the folate for microorganisms, thus saving it for the kid [46], but whether bound folate is absorbed in the intestine is not yet known. One could speculate that the plasma or milk FBP could facilitate folate uptake in a carrier-mediated process, analogous to transcobalamin or transferrin with cobalamin or iron respectively. Alternatively, since the FBP is also a membrane factor (placenta, kidney), it could regulate the cellular accumulation of folate via a receptor-mediated process. Cellular folate transport, done at physiological concentration (10^{-9}–10^{-8} M) of folate in the presence and absence of FBP, should be done *in vitro* in order to study these two possibilities.

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