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Demonstration of high-affinity folate binding activity associated with the brush border membranes of rat kidney

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ABSTRACT **Folate binding activity of high affinity was identified in the particulate fractions of rat kidney homogenates. This binding activity cofractionated with alkaline phosphatase and maltase, two brush border membranes markers. With an enriched preparation of brush border membranes, freed of endogenous folate by acid treatment, the binding of [^{3}H]folate was found to be saturable ($K_b = 4.2 \times 10^{-11}$ M) and rapid. Binding was optimal at pH 6.4–7.7. At neutral pH, competition for binding with [^{3}H]folic acid required 1.45 equivalents of pteroylheptaglutamate, 6.25 equivalents of N^5-methyltetrahydrofolate, 29 equivalents of methotrexate, and 125 equivalents of N^5-formyltetrahydrofolate. At alkaline pH, N^5-methyltetrahydrofolate was as effective a competitor as folic acid. In view of reports that renal tubular reabsorption of folate includes an initial tight binding step, the binding activity associated with the brush border membranes may participate in this process.**

Early studies by Johns *et al.* (1) and Goresky *et al.* (2) suggested that plasma folate is filtered through the glomeruli at two-thirds the rate of inulin and is reabsorbed in the tubular cells by a saturable process. Goresky *et al.* (2) also suggested that folate reabsorption involves an initial step of tight binding: 30 min after injection of [^{3}H]folic acid into the left renal artery of dogs, 6–24% of the ^{3}H was still retained in the kidney and could be flushed out into the urine by a large dose of unlabeled folic acid. Rubin *et al.* (3) showed that methotrexate, which inhibits folic acid reabsorption (1, 2), is taken up by rabbit renal cortical slices by two processes: one which is rapid and energy independent and a second which is slower and requires energy.

On the basis of these observations it has been suggested (3, 4) that this initial step of folate reabsorption in the kidney involves the binding of filtered folate to a specific protein of the proximal tubular cells. Dihydrofolate reductase has been proposed as the binding protein because this enzyme is present in relative abundance in this tissue. An alternate possibility is that this cellular factor is a high-affinity binding protein of the type that has been recently identified in milk (5), blood serum (6–9), and various other tissues including the soluble fraction of porcine kidney homogenates (10–13). Such proteins are characterized by rapid association and slow dissociation with folic acid ($K_b = 10^{-10}$–10^{-11} M), a preference for folate mono- and polyglutamates over those with one carbon substitution at the 5 position or methotrexate (7), and different effects of pH on binding of folic acid and N^5-methyltetrahydrofolate (14, 15).

The present study demonstrates that rat kidney contains a high concentration of folate-binding activity and that this activity associates with the brush border membranes of the renal proximal tubular cells.

MATERIALS AND METHODS

[3′,5′,9-^{3}H$_3$]Pteroylglutamic acid ([^{3}H]PteGlu) (34–63 Ci/mmol) was purchased from Amersham/Searle Corporation (Arlington Heights, IL). Pteroylheptaglutamic acid was prepared as described by Godwin *et al.* (16). N^5-Methyltetrahydrofolate was from Sigma Chemical Corporation (St. Louis, MO). N^5-Formyltetrahydrofolate and methotrexate were from Nutritional Biochemical Co. (Cleveland, OH) and the Lederle Laboratory Division (Pearl River, NY), respectively.

Preparation of Tissue Homogenate and Subcellular Fractionation. Kidneys from unfasted Sprague–Dawley rats (200–300 g) were homogenized at 4° in a Waring blender with 40 vol (wt/vol) 0.05 M mannitol. After filtration through cheese cloth, the homogenate (H) was fractionated as described by Schmitz *et al.* (17). In this procedure, $CaCl_2$ was added to the homogenate to a final concentration of 10 mM. The mixture was stirred for 10 min and centrifuged at 2000 × *g* for 10 min, producing a "cell debris" precipitate (fraction P_1). Centrifugation of the supernatant at 20,000 × *g* for 15 min resulted in the separation of soluble proteins (fraction S) from a second pellet (fraction P_2). By electron microscopy, P_2 appeared to be a highly enriched preparation of membrane vesicles. All fractions were assayed for protein (18), their capacity to bind radioactive folate (see below), and markers activities for brush border membranes (alkaline phosphatase and maltase) (19, 20), mitochondria (succinate cytochrome *c* reductase) (21), basolateral membranes (Na^+,K^+-ATPase) (22), microsomes (glucose-6-phosphatase), and lysosomes (acid phosphatase) (23). For these assays the pellet fractions (P_1 and P_2) were resuspended in 0.05 M mannitol.

Determination of Folate Binding Activity. The homogenate and the various fractions were assayed for determination of unsaturated folate binding activity without further treatment. Total folate binding activity was determined after the removal of endogenous folate by acidification and charcoal treatment. The fraction was made 5 mM in acetic acid and then centrifuged for 15 min at 30,000 × *g*. The supernatant fraction was treated with albumin-coated charcoal (24) and then recombined with the 30,000 × *g* pellet after the charcoal was removed by centrifugation or assayed separately. A sample (20–100 μg of protein) from each of these preparations was incubated with excess [^{3}H]PteGlu (0.2–2 pmol) in 0.05 M potassium phosphate buffer (pH 7.0) for 30 min at 37°. The final volume was 1 ml. The mixture was passed through a single 0.45-μm cellulose nitrate filter (Schleicher and Schüll, Dassel, Germany) followed by washing with 10 ml of 0.025 M potassium phosphate buffer (pH 7.0). The filter was then air dried and assayed for radioactivity in 10 ml of scintillation fluid (50

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Abbreviations: [^{3}H]PteGlu, [3′,5′,9-^{3}H$_3$]pteroylglutamic acid.

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Table 1. Folate binding and marker enzyme activities of rat kidney homogenates before and after fractionation

Activity assayed	Activity/mg protein*			
	Homogenate	P_1	Supernate	P_2
Folate binding (unsaturated)	2.8	3.8(58)	0.6(10)	23(31)
Folate binding (total)	5.4	7.5(60)	0.8(7)	45(31)
Alkaline phosphatase	0.15	0.19(54)	0.02(6)	1.17(29)
Maltase	0.12	0.15(53)	0.02(8)	1.10(35)
Glucose-6-phosphatase	0.089	0.171(85)	0.005(3)	0.015(1)
Acid phosphatase	0.016	0.009(24)	0.030(80)	0.011(3)
Succinate cytochrome *c* reductase	0.152	0.316(87)	0.013(3.7)	0.07(2)
Na^+, K^+-ATPase	0.072	0.113(76)	0.017(9)	0.144(9)
Protein (total), mg	140	60(43)	68(48)	5.3(6.8)

* Activities are expressed in pmol of bound [^{3}H]PteGlu for folate binding and μmol of product formed per min for marker enzymes. Numbers in parentheses represent the percentage of total activities of the homogenate. The data shown are the means from three separate preparations.

mg of 2,5-diphenyloxazole and 3 g of 1,4-bis[2(5-phenyloxazolyl)]benzene in 1 liter of toluene). Activity was expressed as pmol of folate bound per assay tube. In preparations of soluble fractions, in addition to the above assay, folate binding activity was determined with albumin-coated charcoal to remove unbound [^{3}H]folate (11). Because both methods gave the same results, only the data from the filter assay are presented.

RESULTS

Binding of Folic Acid by the Rat Kidney Homogenate before and after Fractionation. The rat kidney homogenate contained >5 pmol of folate binding activity per mg of protein; 60% was unsaturated (Table 1). This activity was distributed mostly in the particulate fractions (P_1 and P_2) with the highest enrichment in fraction P_2. The amount and degree of enrichment of folate binding in the P_2 fraction relative to the original homogenate closely resembled the pattern of alkaline phosphatase and maltase, two markers for the brush border membranes of the proximal renal tubules. The folate binding activity in the two other fractions, P_1 and S, is probably derived from the brush border membranes because the relative enrichment and distribution of this activity closely resembled those of the two brush border membrane enzyme markers. Marker enzymes for mitochondria, basolateral membranes, microsomes, and lysosomes fractionated differently than the folate binding activity. Fig. 1 shows that the enrichment of activity in both the acid- and non-acid-treated membrane preparations (fraction P_2) is due exclusively to macromolecular binding rather than to transport of free vitamin into vesicular spaces. After solubilization of the binding factor with Triton X-100, the radioactivity released from the cellulose nitrate filters was eluted quantitatively in the void volume of Bio-Gel P-10 columns.

Characteristics of Folate Binding by the P_2 Fraction. Experiments were conducted with fraction P_2 that was freed of its endogenous folate by acid treatment. The binding of folic acid was a rapid process and was faster and slightly higher at 37° than at 0° but negligible at 80° (Fig. 2). At 80° the binding activity was rapidly destroyed; cooling and further incubation at 37° yielded no significant restoration of the binding.

The binding of folic acid by the acid-treated membranes was negligible at pH 5.0, maximal at pH 6.4–7.7, and decreased at higher pH (Fig. 3).

At neutral pH, the binding of folic acid was concentration dependent and saturable (Fig. 4). The apparent K_b, as determined from a Scatchard plot, was 4.2×10^{-11} M.

The competitive effect of unlabeled folic acid and three folate analogues on the binding of tritiated folic acid is shown in Table 2. Folic acid was slightly more effective as an inhibitor of this binding than was pteroylheptaglutamate and considerably more effective than N^5-methyltetrahydrofolate, methotrexate, or N^5-formyltetrahydrofolate. Incubation of the

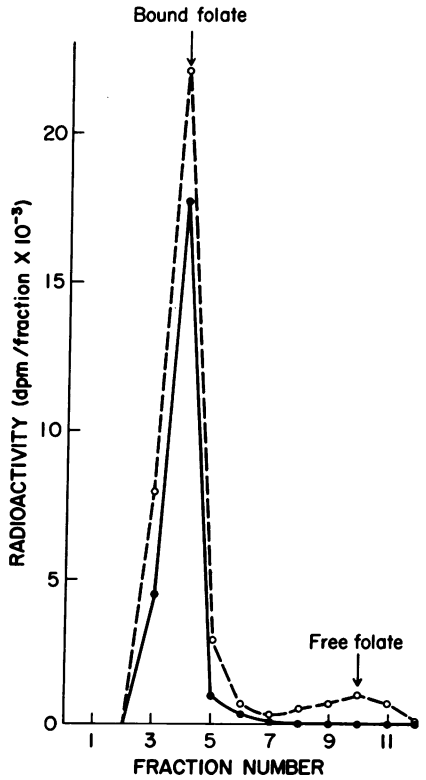

FIG. 1. Macromolecular binding of radioactive folate by the brush border membrane preparations. Incubations containing P_2 fraction before (solid line) and after (broken line) acid treatment and [^{3}H]-PteGlu were each passed through cellulose nitrate filter and the filters were washed. The filters were then shaken for 14 hr at room temperature with 5% Triton X-100/0.05 M potassium phosphate, pH 7.0. The radioactivity released into the Triton X-100 solution was applied to columns of Bio-Gel P-10 (10 ml bed vol) and eluted with the same buffer. Fractions (1 ml) were collected and assayed for radioactivity with 10 ml of Scintisol (Isolab Inc., Akron, OH).

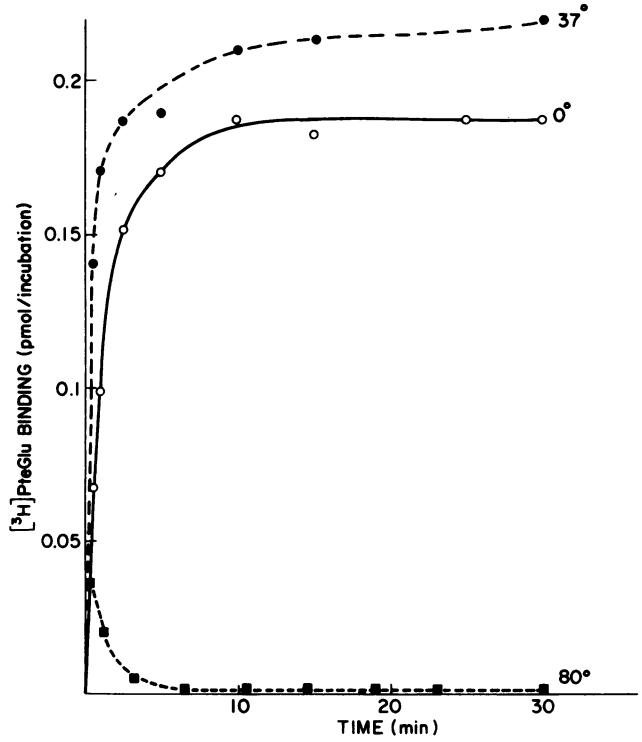

FIG. 2. Dependence of the binding of [^{3}H]PteGlu on time and temperature. Binding activities were determined after incubation, for the times and at the temperatures indicated, of the acid-treated membrane preparation (0.05 mg of protein) with 1 pmol of [^{3}H]PteGlu.

acid-treated P_2 fraction at alkaline pH (9.0) was associated with an increase in the binding activity for N^5-methyltetrahydrofolate that became nearly identical to that for folic acid.

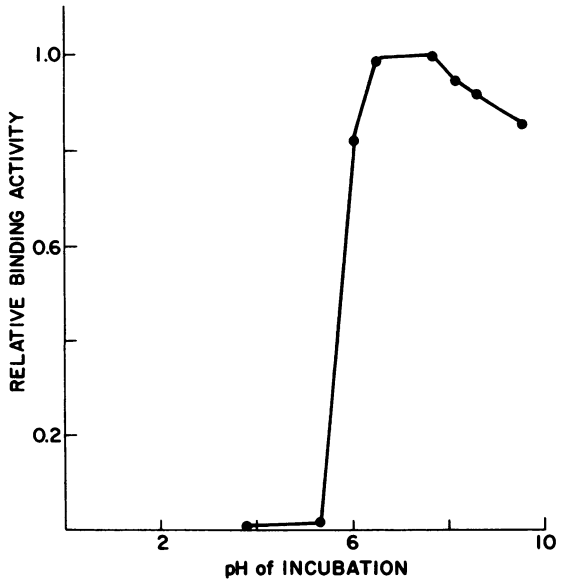

FIG. 3. Dependence of the binding of [^{3}H]PteGlu on pH. Binding of [^{3}H]PteGlu (1 pmol) by the acid-treated P_2 fraction (0.05 mg of protein) was determined at various pH values by using a universal buffer (25).

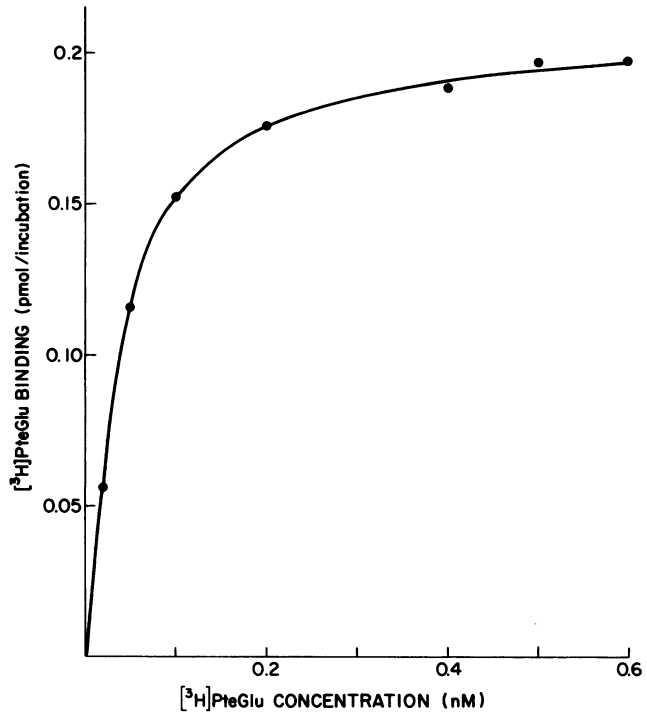

FIG. 4. Dependence of the binding on [^{3}H]PteGlu concentration. Conditions for incubations and assays were as described except that the final volume of incubation was 5 ml instead of 1 ml.

DISCUSSION

The folate binding activity identified here in rat kidney homogenates resembles in many of its properties the soluble folate binders previously reported in milk (5, 14, 15), plasma (6–9), and cell cytoplasm from leukocytes (26) and hog kidney (11). These properties are clearly not those of dihydrofolate reductase: the observed affinity for folic acid is much too high (Fig. 4) and methotrexate, a strong inhibitor of the enzyme, is a weak inhibitor of folate binding (Table 2).

The unique observation of the present study is the high concentration and localization of this binding activity in the P_2 fraction and the correlation of this binding with activities of brush border membrane markers, which indicate that the

Table 2. Inhibition of [^{3}H]PteGlu binding by unlabeled folic acid and its derivatives

Binding inhibitor	Relative concentration for 50% inhibition	
	At pH 7.2	At pH 9.0
Pteroylglutamic acid	1	1
N^5-Methyltetrahydrofolate	6.25	1.25
Pteroylheptaglutamic acid	1.45	
Methotrexate	29	
N^5-Formyltetrahydrofolate	125	

Incubation mixtures contained, in a final volume of 1 ml, 0.2 pmol of [^{3}H]PteGlu with or without increasing concentrations of the inhibitor in 0.05 M of either phosphate buffer (pH 7.2) or lysine·HCl (pH 9.0); aliquots of fraction P_2 sufficient to bind 0.1 pmol of the [^{3}H]folate were added and the mixtures were incubated at 37° for 30 min; then the binding of [^{3}H]PteGlu was determined. The values shown were obtained by dividing the concentration of the inhibitor that produced 50% inhibition by the concentration of unlabeled folic acid that produced the same degree of inhibition (i.e., 0.2 pmol/ml).

binder is in this membrane. This observation is of potential physiological interest in light of the fact that the kidney plays an important role in folate conservation. To achieve the high degree of renal folate conservation in the face of a rapid rate of glomerular filtration of plasma folate (1, 2), an efficient reabsorption by the tubular cells is required. The binder in the brush border membranes could contribute to the necessary mechanism for the rapid trapping of filtered folate. The full process of reabsorption across the tubular cells would require release from such binding and transport into the returning circulation.

There have been other reports of membrane-associated folate binders that were suggested to participate in folate transport. Of these, the binder in the membrane of *Lactobacillus casei* appears to have such function (27). The rat intestinal epithelium (28) and liver plasma membranes (29) were reported to bind folic acid and its derivatives. In both tissues the activity found was much lower than that in the kidney, making it difficult to evaluate their function experimentally. Rabbit choroid plexus does possess a high concentration of folate binding activity associated with a particulate fraction (13), but the localization of this activity to the cell membranes was not demonstrated. The present study thus provides a framework for exploring the possible involvement of specific folate binders in transport of folate into mammalian cells and across epithelia.

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