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Differential Binding of Folates by Rat Renal Cortex Brush Border and Basolateral Membrane Preparations (41986)

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Abstract. The binding of radioactive 5-methyltetrahydrofolate and folic acid was found to be greater in brush border than in basolateral membrane preparations of rat renal cortex. This appeared to be due to an increased amount of a specific folate binding protein in the brush border membrane preparations as compared to those of the basolateral membrane. The binding was saturable and inhibited by nonradioactive folic acid and, therefore, a specific, rather than nonspecific process. The K_m's for folic acid binding in brush border and basolateral membrane preparations were similar and involved a single high-affinity binding site. In contrast, methotrexate was found to bind equally well to both brush border and basolateral membrane preparations. Moreover, folic acid binding was not inhibited by an equimolar amount of methotrexate. A folate binding protein could be extracted from either membrane preparation with 1% Triton X-100 and, to a lesser extent, with 0.6 *M* NaCl. These different extraction procedures resulted in different apparent molecular weights for folate binding protein (>160,000 for Triton X-100-extracted samples and 40,000 for NaCl-extracted samples). The membrane preparation pellets remaining after NaCl extraction were able to rebind tritiated folic acid and also the 40,000-Da folate binding protein. On the other hand, membrane preparations extracted with Triton X-100 lost the ability to bind folic acid or the 40,000-Da folate binding protein. These differences in molecular weight and rebinding capacity may be explained by the existence of a receptor for folate binding protein which was extracted by Triton X-100, but not by NaCl. The greater concentration of folate binding protein in the renal tubule cell brush border membrane preparations as compared to those from basolateral membranes ascribes, for the first time, a functional role for folate binding protein in the renal reabsorption of folates which is required to prevent loss of folate in the urine and perhaps in the membrane transport of folates in general.

Studies in animals indicate that the cell membranes of several tissues possess a high-affinity folate binding protein (FBP) which may be involved in transmembrane transport of folate (1–9). Since the kidney is particularly enriched in FBP, it has been suggested that FBP in renal tubular cell membranes may play an important role in preventing urinary loss of folate by participation in folate reabsorption from the fluid in the tubular lumen (6, 10). The physiologic necessity for renal reabsorption of folate is based on the observation that while plasma folate (10–35 n*M*) is loosely bound to albumin, it is almost completely dialyzable and, therefore, filterable at the glomerulus. Failure to reabsorb filtered folate would result in urinary losses of approximately 1 mg/day and the subsequent rapid depletion of body stores. Despite its apparent physiologic importance, the renal processing of folate has not been extensively characterized. In man, dog, and monkey, folate undergoes net reabsorption, apparently by a saturable process (11–13). Recent stop-flow studies in monkey, however, demonstrated that folate also undergoes secretion, and that bidirectional flux of folate appeared to occur via active transport processes within the proximal portion of the nephron (13).

Since net folate reabsorption is evident under physiologic conditions, it seemed likely that the capacity for folate uptake by luminal (brush border, BB) membranes would exceed

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that of contraluminal (basolateral, BL) membranes in the proximal tubule. The present studies were undertaken to directly investigate a functional role for membrane FBP by characterizing folate binding in BB and BL membrane preparations isolated from the cortex of rat kidney. A portion of these studies was published in abstract form (14).

Materials and Methods. *Reagents.* [^{3}H]-Folic acid ([^{3}H]PteGlu) (26 Ci/mmole), [^{14}C]5-methyltetrahydrofolate ([^{14}C]CH_3H_4-PteGlu) (58 mCi/mmole), and [^{3}H]methotrexate ([^{3}H]MTX) (150 mCi/mmole) were purchased from Amersham, Arlington Heights, Illinois. Mannitol, sucrose, *N*-2-hydroxyethylpiperazine-*N*^{1}-2-ethanesulfonic acid (Hepes), triethanolamine, Tris, phosphate, and sodium chloride were chemical grade I reagents. Dextran Blue was from Sigma Chemical Company, St. Louis, Missouri. Sephadex G-200 was supplied by Pharmacia (Piscataway, N.J.), DEAE (DE52-Whatman), by Fisher Scientific, Pittsburgh, Pennsylvania; and Aquasol-2, by New England Nuclear, Boston, Massachusetts. Unlabeled folic acid (Folvite) and methotrexate (MTX) were purchased from Lederle, Pearl River, New York.

Enzyme assays. Alkaline phosphatase and Na^+,K^+-dependent ATPase activities were utilized to characterize each membrane preparation. Alkaline phosphatase was determined with the Sigma test kit and Na^+,K^+-ATPase with a modification of the method of Ismail-Beigi and Edelman (15) in which the samples are pretreated with deoxycholate (16). Liberated phosphate was measured by a modification of the method of Fiske and SubbaRow (17). In addition, in some experiments, 5′-nucleotidase activity was measured in the membrane preparations obtained from intact vesicles (18).

Isolation and preparation of membrane vesicles. Membrane vesicles were prepared by techniques previously described in detail (19). In brief, renal cortical slices were obtained from 8–10 male rats (Charles River Breeding Laboratory, Wilmington, Mass.), minced, homogenized, and subjected to differential centrifugation. The membrane vesicles isolated with this technique were separated into BB and BL vesicles by free-flow electrophoresis. Protein was determined on the day of each experiment from the optical density at 280 nm of each collected fraction and subsequently further quantified with the method of Lowry *et al.* (20) using bovine serum albumin as the standard. The electrophoresis samples were subsequently pooled into BB and BL vesicles on the basis of the activity of marker enzymes: BB vesicles were identified by alkaline phosphatase activity and BL vesicles by Na^+,K^+-ATPase activity. Enrichment of these enzyme activities in the BB and BL vesicles, respectively, were comparable to those previously reported (19).

In all experiments, the pooled electrophoresis fractions enriched in BB and BL vesicles were diluted in equal volumes of 0.1 m*M* mannitol in Tris–Hepes buffer (1 m*M* Hepes buffered to pH 7.4 with Tris), homogenized, and centrifuged at 32,000*g*. The pelleted materials from these centrifuged samples were each suspended in the same solution in a volume estimated to yield a protein concentration of 5–10 mg/ml.

Preparation of membranes from vesicles. BB and BL membrane preparations were prepared from the corresponding vesicles by repeated freezing (at −40°C) and thawing. To ensure that this procedure resulted in disruption of the vesicles, suspensions of the frozen and thawed vesicles were centrifuged at 32,000*g* for 20 min and portions of the resulting pellets were prepared for electron microscope examination.

Each pellet was fixed in 4% phosphate-buffered paraformaldehyde for 2 hr, postfixed with 10% phosphate-buffered osmic acid, dehydrated in graded alcohols, passed through propylene oxide, and embedded in epoxy resin. Thin sections were stained with lead citrate and uranyl acetate and examined by electron microscopy.

Folate binding to membrane preparations. The binding of [^{3}H]PteGlu to a membrane preparation was determined by adding 20 μl (50 μg protein) of membrane preparation suspension to 200 μl of phosphate in "mannitol" buffer (0.01 *M* potassium phosphate buffer, pH 7.4, 0.3 *M* mannitol) supplemented with 400 n*M* [^{3}H]PteGlu. Binding of [^{3}H]PteGlu at 25°C was found to be approximately the same as that at 37°C; therefore, binding was routinely determined at 25°C. Binding assays for [^{14}C]CH_3H_4-PteGlu and [^{3}H]MTX were determined as

for [^{3}H]PteGlu except that in place of [^{3}H]PteGlu the corresponding radioactive compound was added to yield a concentration of 40 μM in a total volume of 100 μl. In studies in which the effect of NaCl on substrate binding to membranes was evaluated, the media was prepared with 0.1 M sodium chloride and made isoosmotic with mannitol buffered to pH 7.4. After several time intervals the "insoluble" membrane material was separated from the incubation medium by a rapid dilution and filtration technique. Aliquots (20 μl) were removed from the media, pipeted into 4 ml of ice-cold "stop" solution (154 mM NaCl, Tris–Hepes buffer, pH 7.4), and simultaneously filtered through Millipore filters (Bedford, Mass.) (HATF 07500, 0.45-μm pore size) using a Millipore No. 1225 sampling manifold. The filters were immediately rinsed with an additional 16 ml of the same cold solution. Filtration and rinsing were performed in less than 10 sec. The filters were dried and dissolved in 12 ml of Aquasol-2. The level of radioactivity of each filter was determined in a Beckman Model LS-250 liquid spectrometer, to a counting error of 0.2% or less. Uptake as picomoles per milligram protein was calculated from the cpm of the filters, the specific activity of the isotope, and the protein concentration of the membrane preparation suspension.

To assess the possibility that folate was metabolized during the course of the experiments, media containing [^{3}H]PteGlu was incubated for 60 min at room temperature with and without a membrane preparation and then analyzed using DEAE column chromatography. The column was developed with a phosphate gradient in the presence of 2-mercaptoethanol (21). When [^{3}H]PteGlu was incubated with either a BB or BL membrane preparation there were no additional radiolabeled folates or other compounds noted (data not shown). Therefore, there was no evidence for metabolism of [^{3}H]PteGlu by a membrane preparation under these conditions.

The total capacity of the membrane preparations to bind [^{3}H]PteGlu was determined after removing endogenous folate (7). Endogenous folate was removed by addition of 0.3 ml of 0.17 mM acetic acid to 1.0 ml of membrane suspension (final pH 4.0). Immediately thereafter, the solubilized folate was separated from the membrane preparation by centrifugation at 30,000g for 20 min as suggested by *Selhub et al.* (22). The membrane preparation pellet was then resuspended in a volume of mannitol buffer estimated to achieve a protein concentration comparable to that used in studies with untreated membranes. The resuspended, folate-depleted membranes were vigorously vortexed and [^{3}H]PteGlu binding was determined by the rapid filtration technique described above. Kinetic studies were performed with folate-depleted and untreated BB and BL membrane preparations. Binding was assayed 30 min after the addition of 50 μg of protein from BB or BL membrane preparations to a media containing 100 μl of mannitol buffer and varying concentrations of [^{3}H]PteGlu (15 nM–4 μM). The results were analyzed using Scatchard and double reciprocal plots (23).

To determine if [^{3}H]PteGlu binding could be competitively inhibited by nonradioactive folate, aliquots of the BB and BL membrane suspensions were preincubated in 100 μl with mannitol buffer and nonradioactive PteGlu (5 nM–1μM). After 30 min [^{3}H]PteGlu was added (to 400 nM) to each incubation and binding was determined. In additional experiments, membrane suspensions were preincubated with nonradioactive MTX (0.4–400 μM) for 5 min. Then, [^{3}H]PteGlu was added (to 400 nM) and binding was assessed. The shorter preincubation period used in studies with MTX was based on the observation that [^{3}H]MTX bound rapidly to the membrane preparations, achieving maximal binding at 1 min of incubation (see Fig. 5).

Chromatographic characterization of membrane preparation—FBP. Radiolabeled folate compounds were analyzed by DEAE ion-exchange column chromatography (22). By this technique commercially available [^{3}H]PteGlu contained a radioactive impurity which amounted to 5–10% of the total radioactivity. Membrane preparation binding of purified [^{3}H]PteGlu was similar to that of a nonpurified commercial preparation of [^{3}H]PteGlu.

The amount of [^{3}H]PteGlu bound to membrane preparations was determined with a chromatographic technique and compared to that obtained with the Millipore filtration

technique. Comparable amounts of diluted BB and BL membrane preparations, previously incubated with [^{3}H]PteGlu, were passed through a Sephadex G-200 column (1 × 15 cm). The column was equilibrated with 0.1 *M* phosphate buffer pH 7.4, and calibrated with Dextran Blue (>200,000 Da), hemoglobin (68,000 Da), human FBP (40,000 Da), and [^{3}H]PteGlu (440 Da).

In additional studies FBP was extracted from 1-mg portions of membrane preparations with 1% Triton X-100. Membranes were incubated in the Triton X-100 solution for 2 hr at room temperature, and subsequently centrifuged at 30,000*g* for 20 min. An 0.5-ml aliquot of the clear Triton X-100-extracted supernatant fluid was then incubated with 40 pmole of [^{3}H]PteGlu at room temperature. After 30 min, the entire solution was passed through a Sephadex G-200 column previously equilibrated with buffer containing 1% Triton X-100. Sequential 0.5-ml fractions were collected and the radioactivity determined in an aliquot of each fraction. FBP also was extracted from membrane preparations with 0.6 *M* NaCl after incubation for 60–78 hr at room temperature. The same techniques were employed as described above for the Triton X-100-treated samples. Simultaneously an aliquot of each supernatant fluid extract was assayed for [^{3}H]PteGlu binding capacity using charcoal adsorption as an additional method (24, 25). The [^{3}H]PteGlu binding capacity of the membrane preparation remaining after extraction with either Triton X-100 or NaCl also was determined. For this purpose, these two membrane preparations were each diluted 1:10 with mannitol buffer and then incubated for 60 min with [^{3}H]PteGlu or with partially purified endogenous FBP, which had been previously labeled with [^{3}H]PteGlu, and analyzed by Sephadex G-200 column chromatography.

Results. *Electron microscopic examination of intact vesicles and membrane preparations.* The vesicle fractions prior to free-flow electrophoresis separation of BB and BL vesicles consisted of various sized single-membrane vesicles measuring less than 486 nm in diameter. These were morphologically indistinguishable from those found in vesicle preparations separated into BB and BL fractions by free-flow electrophoresis. This suggested that the separation process did not structurally alter the vesicles. In all samples, minute vesicles with fine light particles were attached to the outer aspect of some of the vesicles. Fine granules and amorphous pale material often lined the inner surface and sometimes completely filled the central space. A few vesicles contained coarse granules or smaller vesicles. Scattered vesicles had elongated protuberances containing moderately electron-dense material. Mitochondria were not observed by electron microscopy in either the BB or BL vesicle fractions although free mitochondria were observed in the vesicle preparations prior to the separation by electrophoresis.

In the BB vesicle fraction, there were small vesicles with strongly electron-dense protuberances. Many of these projections were visualized as isolated structures often cut in cross section. The combination of fuzzy-coated vesicles and electron-dense vesicle protuberances resembled the apical portion of the proximal convoluted tubule cytoplasm. These structures were only rarely encountered in the BL vesicle fraction. Although some of the elongated structures may represent microvilli, unequivocal microvilli were not noted in either the BB or BL vesicle fractions.

In contrast to the vesicle preparations, the freeze–thawed membrane preparations prepared from the separated BB and BL vesicles were composed of smooth membrane sheets and elongated vesicles.

Binding of [^{14}C]CH_3H_4PteGlu and [^{3}H]PteGlu to BB and BL membrane preparations. The time course and amount of [^{14}C]CH_3H_4PteGlu and [^{3}H]PteGlu bound to BB and BL membrane preparations are shown in Fig. 1. Each point represents the mean of at least five triplicate experiments. The range was ±5 pmole for BB and ±2 pmole for BL. In both BB and BL membrane preparations, binding of [^{14}C]CH_3H_4PteGlu increased rapidly during the first minute. Thereafter, binding increased further, but at a much reduced rate. Throughout the 60 min of observation the binding capacity of the BB membrane preparation was significantly greater than that of BL ($P < 0.001$). The time course and pattern of binding of [^{3}H]PteGlu was very similar to that of [^{14}C]CH_3H_4PteGlu and, as evident with the

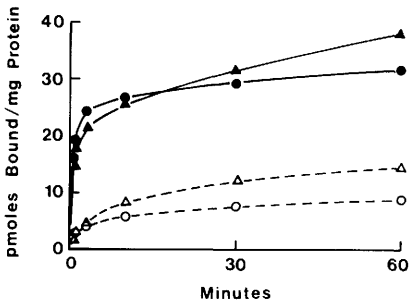

FIG. 1. Time course of binding of [^{3}H]PteGlu and [^{14}C]CH_3H_4PteGlu to BB (● [^{3}H]PteGlu, ▲ [^{14}C]-CH_3H_4PteGlu) and BL (○ [^{3}H]PteGlu, △ [^{14}C]CH_3-H_4PteGlu) membrane preparations as determined by the millipore filter assay described under Materials and Methods.

naturally occurring folate, binding of [^{3}H]-PteGlu by BB membrane preparations was severalfold greater than that by BL membrane preparations ($P < 0.001$) (Fig. 1). The presence of sodium chloride (0.1 M) in the incubation media reduced the binding of [^{3}H]PteGlu to both BB and BL samples by 30% within the first minute of observation and it remained reduced over a 30-min period (data not shown).

To determine total folate binding capacity, the endogenous folate was removed by acid treatment of the membrane preparations. This resulted in increased binding of [^{3}H]PteGlu in both BB and BL membrane preparations (Fig. 2). The increment in binding was greater in BB than BL membrane preparations so that the difference in total binding capacity was even greater after endogenous folate was removed from the membrane preparations (Fig. 2). This finding indicates that the greater [^{3}H]PteGlu binding in untreated BB membrane preparations was not due to either a difference in saturation of the BB and BL membrane preparations with endogenous folate or to displacement of folate from the membrane preparations during the preparative procedures. The latter conclusion was supported by the finding that the vesicle preparations (before separation by electrophoresis) had a [^{3}H]PteGlu binding capacity which was 18.2 pmole/mg and was intermediate between that of the BB (26.3 pmole/mg) and BL membrane preparations (5.9 pmole/mg). Similarly the difference in binding did not appear to be due to a difference in the amount of membrane material in the BB and BL preparations, since 5'-nucleotidase activity was remarkably similar in both types of preparations (2.07 nmole/hr/mg protein for BB and 2.1 for BL preparations).

Kinetics of [^{3}H]PteGlu binding. In order to determine if the binding sites for [^{3}H]PteGlu were of high or low affinity, kinetic studies were performed. The free concentration of substrate was obtained by subtracting the bound substrate from the total amount of substrate added to the reaction mixture. The binding affinity of folate binding protein for [^{3}H]PteGlu is extremely high (K_a 1.0 nm^{-1}). Therefore, there is little dissociation during the course of this study. In addition, the complex or the individual components are stable over a 24-hr period under these conditions. These studies revealed that [^{3}H]PteGlu binding was a saturable process (Fig. 3). Estimation of the V_{max} for [^{3}H]PteGlu binding to the membrane preparations, using a Lineweaver–Burke plot, indicated that it was approximately 2.5-fold greater in BB than in BL membrane preparations. Estimates of the K_m's for [^{3}H]PteGlu using this technique revealed quite similar K_m's for BB and BL membrane preparations (approximately 3.12 and 3.33 nM, respectively).

Further analysis of the kinetic data using

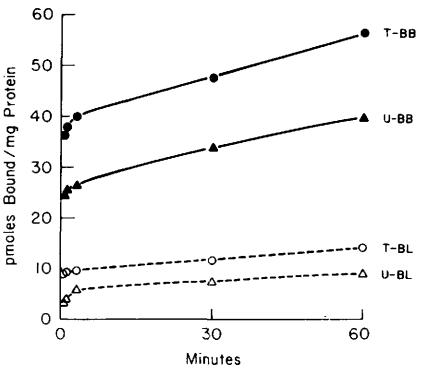

FIG. 2. Binding of [^{3}H]PteGlu by BB and BL membrane preparations after removing endogenous folate by acid treatment (total folate binding capacity—T) compared with that of untreated membrane preparations (unsaturated folate binding capacity—U) determined as described in Fig. 1 legend.

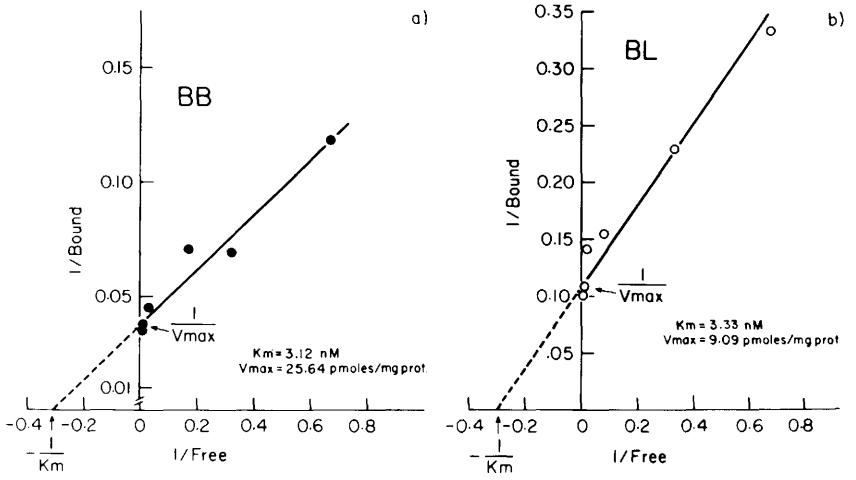

FIG. 3. Determination of K_m and V_{max} for BB (a) and for BL (b) membrane preparations determined as described in Fig. 1. legend.

a Scatchard plot (23) revealed plateauing of specific binding. This suggests two classes of binding sites in BB membrane preparations, one with a high and one with a lower affinity for [^{3}H]PteGlu (Fig. 4). After removal of endogenous folate by acid treatment of membrane preparations, however, only one binding site was observed since the bound:free ratio of [^{3}H]PteGlu was linearly related to the specific activity of the bound [^{3}H]PteGlu (Fig. 4). Similar findings were obtained with BL membrane preparations (data not shown).

In both BB and BL membrane preparations, [^{3}H]PteGlu binding was progressively inhibited in the presence of increasing concentrations of nonradioactive PteGlu. Maximal inhibition of binding to BL membrane preparations was achieved at a lower concentration of nonradioactive PteGlu than was required to maximally inhibit binding to BB membrane preparations (data not shown). The effect of 0.4–400 μM MTX on the binding of [^{3}H]PteGlu to the membrane preparations also was determined (Table I). The preparations of MTX tested inhibited [^{3}H]PteGlu binding to BB and BL membranes (Table I). When MTX was added in equimolar amounts to [^{3}H]PteGlu (400 nM), no inhibition was evident. At higher levels,

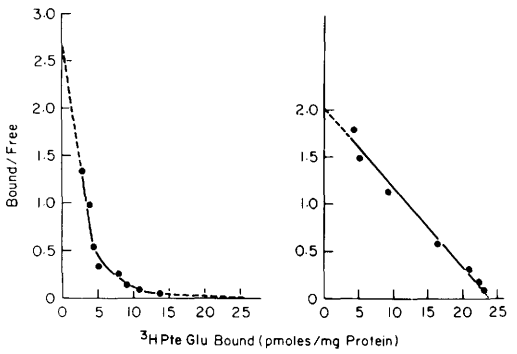

FIG. 4. Scatchard plots of data obtained with folate-containing (untreated) (left) and folate-depleted (acid treated) (right) BB membrane preparations.

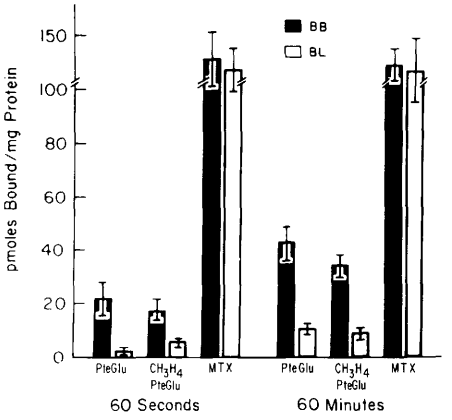

FIG. 5. Binding capacity of BB (closed bars) and BL (open bars) membrane preparations for [^{3}H]PteGlu, [^{14}C]CH_3H_4PteGlu, and [^{3}H]MTX were determined after incubations of 60 sec and 60 min.

TABLE I. EFFECT OF MTX ON THE BINDING OF [^{3}H]PteGlu TO MEMBRANE PREPARATIONS[a]

MTX (μM)	[^{3}H]PteGlu binding by BB membrane preparation (%)		[^{3}H]PteGlu binding by BL membrane preparation (%)	
	1 min	60 min	1 min	60 min
0.4	106.4	105.5	107.1	100.9
4	41.5	78.5	59.9	84.5
40	15.2	63.6	26.1	57.6
400	5.8	11.2	12.7	19.4

[a] The level of binding of [^{3}H]PteGlu (at a concentration of 0.4 μM) to membrane preparations was determined in 0.1 ml in the presence of the concentrations of MTX and for the time intervals indicated as described under Materials and Methods.

MTX progressively reduced the binding of [^{3}H]PteGlu, however, even at these high levels (400 μM) MTX did not completely inhibit binding of [^{3}H]PteGlu. Since it has been reported that MTX does not bind to FBP at pH 7.4 (34), the inhibition of [^{3}H]PteGlu binding by MTX observed here may have been due to an impurity in the MTX preparations (see Discussion). At each level of MTX used, inhibition of [^{3}H]PteGlu binding was greater at 1 min than at 60 min, suggesting a reversible, time-related, inhibitory process (Table I). The binding capacities of the two types of membrane preparations for various folates were compared. The binding capacity of the membrane preparations for [^{3}H]MTX was markedly greater than that of equimolar concentrations of [^{3}H]PteGlu or [^{14}C]CH_3H_4PteGlu. [^{3}H]MTX binding was similar in BB and BL membrane preparations. In contrast to [^{3}H]MTX binding, an increase in [^{3}H]PteGlu and [^{14}C]CH_3H_4PteGlu binding at 60 min compared to 1 min was noted. In addition as observed above, BB membrane preparations bound more of these two compounds than BL preparations.

Characterization of FBP by chromatographic studies. As depicted in Fig. 6, after incubation with [^{3}H]PteGlu, radioactive material from BB or BL membrane preparations was recovered in the void volume. The amount of [^{3}H]PteGlu bound to the rapidly eluting fraction derived from BB membrane preparations was threefold greater than that bound to the rapidly eluting fraction from BL membrane preparations as measured by Sephadex G-200 column chromatography (data not shown). This difference in binding capacity was comparable to that observed when binding was evaluated with the Millipore filtration technique. In order to determine if a receptor for PteGlu or FBP could be extracted from BB membrane preparations, they first were incubated with [^{3}H]-PteGlu and then treated with either Triton X-100 or NaCl. The material labeled with [^{3}H]PteGlu and extracted with Triton X-100 eluted from a Triton X-100-equilibrated Sephadex G-200 column at a position close to that of the void volume (160,000 Da) (Fig. 6). In contrast, the Sephadex G-200 elution profile of the [^{3}H]PteGlu-labeled extract obtained from 0.6 *M* NaCl-treated membrane preparations eluted at a position equivalent to a moiety of approximately 40,000 Da. The molecular weight of the [^{3}H]PteGlu-bound material in the NaCl extract was similar to that of the FBP prepared from human granulocytes (2) which was used as a marker for the column (data not shown).

Triton X-100 extracted virtually all of the [^{3}H]PteGlu bound to the membranes. The extracted [^{3}H]PteGlu-labeled material presumably represents [^{3}H]PteGlu bound to endogenous membrane FBP. Following centrifugation and exhaustive washing and dialysis, the resuspended Triton X-100-treated membrane preparation pellet failed to rebind either [^{3}H]PteGlu, [^{3}H]PteGlu-labeled NaCl-

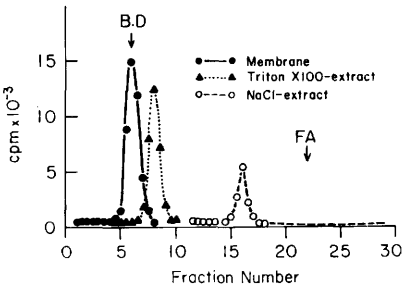

FIG. 6. Elution profile on Sephadex G-200 of [^{3}H]PteGlu-labeled untreated membrane preparation (●), Triton X-100 membrane preparation extract (△), or NaCl membrane extract (○). The radioactive material was completely recovered. The unbound radioactive material was recovered in the free folate (FA) peak (not shown). B.D. = Dextran Blue.

TABLE II. BINDING OF PteGlu AND FBP TO FBP-DEPLETED MEMBRANE PREPARATIONS[a]

Type of folate	Extraction solution	
	Triton X-100	NaCl
	[^{3}H]PteGlu binding to extracted membrane pellet (pmole/mg protein)	
(1) [^{3}H]PteGlu	0	23.1
(2) ([^{3}H]PteGlu–FBP)	0	1.9
(3) ([^{3}H]PteGlu–granulocyte FBP)	0	1.2

[a] BB membrane preparations were treated with 1% Triton X-100 or 0.6 *M* NaCl at room temperature for the times indicated under Materials and Methods. The incubation mixtures were centrifuged and the capacity of the pelleted material to bind (1) [^{3}H]PteGlu, (2) [^{3}H]PteGlu-labeled, NaCl-solubilized, membrane FBP, and (3) partially purified, [^{3}H]PteGlu-labeled, human granulocyte FBP was determined by the millipore filter assay.

extracted membrane FBP, or [^{3}H]PteGlu-labeled human granulocyte FBP (Table II, Fig. 7). In contrast, NaCl only partially extracted FBP from the membranes; the amount extracted by NaCl was one-fourth to one-third that extracted by Triton X-100 (Fig. 6). In addition, the binding capacity of the NaCl-extracted membranes, assayed by elution on Sephadex G-200 (Fig. 6) and by the coated charcoal technique (not shown), was 25–30% of the total [^{3}H]PteGlu binding capacity of "nonextracted" membrane preparations. The NaCl-extracted membrane preparation pellet was also able to bind [^{3}H]PteGlu or endogenous FBP extracted from the membrane preparation as well as that obtained from human granulocyte FBP, (Table II, Fig. 7). It was previously demonstrated that [^{3}H]PteGlu binds to FBP with high affinity (5×10^{12} liters/mole at pH 7.6) and was not dissociated by high concentrations of salt (26). Therefore, it was unlikely that the [^{3}H]PteGlu–FBP complex dissociated allowing for rebinding of free [^{3}H]PteGlu to the membrane preparations. In contrast, "nonextracted" membrane preparations showed no binding capacity for exogenous FBP suggesting the presence of a saturated receptor for FBP in the intact membrane.

Discussion. The present studies demonstrate that both CH_3H_4PteGlu, the physiological form of folate in blood and one of the folates found in the urine (27), and PteGlu bind to isolated luminal (BB) and contraluminal (BL) membrane preparations derived from rat renal cortex. Binding has been shown to be saturable and was inhibited by nonradioactive folates and, therefore, binding appears to be a specific rather than nonspecific process in both types of membrane preparations. Similar conclusions regarding folate binding were previously derived from *in vivo* studies (11–13). The present studies extend these observations insofar as they demonstrate that BB membrane preparations have a higher V_{max} and greater binding capacity than BL membrane preparations. These observations are consistent with there being a significantly greater amount of a specific FBP in the BB than in the BL membrane preparations. Both the presence of FBP and the quantitative difference in its distribution in the cell membrane suggest that FBP may play an important physiologic role in folate transport in

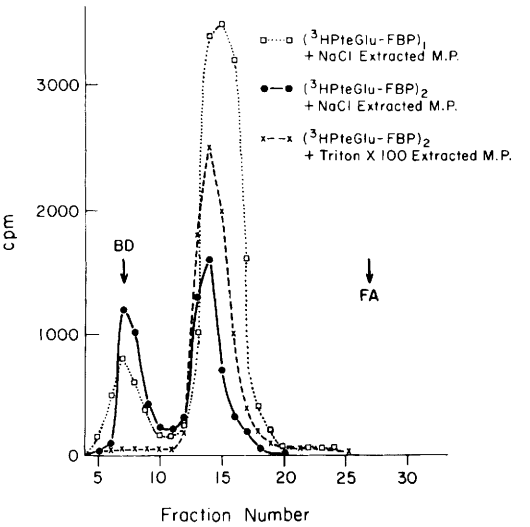

FIG. 7. Chromatographic analysis on Sephadex G-200 of the binding of [^{3}H]PteGlu-labeled FBP preparations to 1% Triton X-100- or NaCl-extracted BB membrane preparation pellets. [^{3}H]PteGlu-FBP)$_1$ = 0.6 M NaCl-extracted (solubilized) membrane FBP preparation. (^{3}HPteGlu–FBP)$_2$ = partially purified human granulocyte FBP. BD = Dextran Blue. FA = free folic acid. M.P. = membrane pellet.

the proximal tubule. Although bidirectional transport of folate occurs at this nephron site, reabsorption exceeds secretion so that net reabsorption occurs (13). The greater amount of FBP in BB membrane preparations may be responsible for the difference in the magnitude of the reabsorptive and secretory fluxes of folate.

It has been reported that FBP cofractionated with markers for the BB membrane, however only BB, but not BL membranes, were separated from the crude membrane preparations (22). In the present study, BB and BL membranes were separated by free-flow electrophoresis and it was found that FBP was present in both BB and BL membrane preparations. However, a greater FBP concentration was found in BB preparations than in those containing BL material. The pellet containing BB membrane material was enriched approximately 8-fold in alkaline phosphatase activity over that of the BL preparation and depleted of BL membrane material, since only 65% of the sp act of Na^+,K^+–ATPase activity of the crude homogenate was present. The pellet containing BL membrane material was enriched 4- to 5-fold in Na^+,K^+–ATPase activity and 2.5-fold in alkaline phosphatase activity compared to BB preparations. The latter may represent some contamination with BB membrane material. However, it has been suggested that alkaline phosphatase may not reside solely in BB membranes; some activity also may occur in BL membranes (28). Therefore, the presence of the alkaline phosphatase in the BL fractions does not necessarily indicate BB membrane contamination. It is highly unlikely that the FBP activity in the BL membrane preparations was due to contamination from BB membrane material since the FBP activity should be proportional to the degree of contamination. This was not the case since folate binding increased approximately 60% in BB membrane preparations and 40% in BL membrane preparations after acid treatment. Moreover, if folate binding in BL membrane preparations was overestimated by contamination with some BB material, then the difference in the capacity of the two membrane preparations to bind folate would even be greater than that observed. Therefore, it is the concept of differential folate binding to BB and BL membrane preparations, rather than the precise quantitative difference, that appears to be important.

The K_m of the [^{3}H]PteGlu binding by BB and BL preparations was very similar suggesting that the FBP on both sides of the cell membrane has the same affinity for folate. Since the K_m's are lower than the concentration of folate in rat serum (29), folate binding should be efficient under physiological conditions. Although the Michaelis–Menten plots suggested a single K_m, further analysis with a Scatchard plot revealed two components of [^{3}H]PteGlu binding by BB preparations and BL native (nonacid treated) membrane preparations. When endogenous folate was removed by acid, however, only one binding site was evident. These results are consistent with a previous report (22) that showed that under physiological conditions, the membrane is partially saturated with endogenous folate. In this context, the initial rapid binding of [^{3}H]PteGlu to the membrane preparations may be due to binding to unsaturated FBP, while the subsequent slower binding may reflect slow displacement of endogenous folate from saturated FBP (Fig. 4). Similar phenomena may explain the biphasic rate of [^{14}C]CH_3H_4PteGlu binding.

In contrast to the natural folates, MTX, a widely used antifolate which can cause nephrotoxicity (30), binds equally well to BB and BL membrane preparations and in higher amounts than equimolar concentrations of CH_3H_4PteGlu (Fig. 5). These results suggest that the membrane binding sites for MTX are different than those for folates. The competition studies support this hypothesis (Table I). At equimolar concentrations, preincubation of BB or BL membrane preparations with MTX did not affect binding of [^{3}H]PteGlu and, even at a MTX concentration 1000 times that of [^{3}H]PteGlu, a significant amount of [^{3}H]PteGlu was bound to the membrane preparations. Nevertheless, a progressive reduction of [^{3}H]PteGlu binding was evident with increasing amounts of MTX in both BB and BL membrane preparations (Table I). This inhibitory effect was greater at 1 min that at 60 min. These data may be

due to the presence of N^{10}-methylPteGlu as an impurity in the commercially available MTX used in these studies (31). This PteGlu analog may have an affinity for the membrane FBP similar to that of PteGlu. In addition, this analog may partially saturate this protein in the kidney and other tissues when large doses of MTX are used *in vivo*. In contrast, these studies suggest that the renal membrane FBP has a very low affinity for MTX. This conclusion is consistent with those made previously regarding the affinity of FBP, derived from various sources (32), for MTX. Thus although MTX binds extensively to some portion of the renal membranes, it appears that it binds to sites that differ from those that bind folate.

To further characterize the membrane protein that binds folate, FBP was extracted from BB membrane preparations with Triton X-100 or a strong salt solution. The difference in the apparent size of the [^{3}H]PteGlu-labeled proteins extracted from the membrane by the two methods may have several explanations. Triton X-100 apparently binds to the proteins it extracts from the lipid bilayer; once bound, it may change the shape of the protein, making it appear larger (7). However, partially purified FBP extracted from human granulocytes did not change its chromatographic position on Sephadex G-200 from 40,000 Da despite a comparable incubation with Triton X-100 (not shown). Alternatively the lower MW of the FBP extracted with NaCl may represent a portion of a FBP complex or a subunit of a 160,000 Da polymer. The membrane may contain a FBP complex composed of two different proteins, a receptor for FBP having a mol wt of 120,000 and FBP itself with a mol wt of 40,000. Extraction of the proposed FBP receptor with Triton X-100 would render the residual membrane incapable of rebinding FBP, while extraction of the FBP, but not the receptor, with NaCl would permit the residual membrane to rebind FBP. Although the data obtained in the rebinding studies performed with Triton X-100- and NaCl-extracted membrane preparations are consistent with this hypothesis, it is possible that Triton X-100 produced a greater "derangement" of the membrane material or remained bound to the membrane preparation pellet (despite washing and exhaustive dialysis) and thereby prevented rebinding of FBP. Nevertheless, the hypothesis that the membrane preparation contains a FBP receptor is supported by the findings that [^{3}H]PteGlu and [^{14}C]CH_3H_4PteGlu are taken up by the liver (*in vivo*) more rapidly when they are bound to FBP (33). A high molecular weight complex which binds folate has been described in human milk (34) and in the plasma of patients with acute hepatitis and liver diseases (35) which may represent either a FBP polymer or the postulated receptor bound to FBP.

In order to further evaluate the physiologic role for FBP in the kidney, preliminary studies were done with intact vesicles (data not shown). There was greater [^{3}H]PteGlu uptake in BB than there was in BL vesicles (36), however, the major portion (95%) of this uptake was due to binding of [^{3}H]PteGlu to membrane vesicles. In view of the large amount of [^{3}H]PteGlu binding, it has been difficult to assess the contribution of energy-requiring transport to total uptake of [^{3}H]PteGlu. However, the level of uptake of [^{3}H]PteGlu, determined at different osmolalities, suggests that 3 to 4 fmole/mg of protein are transported across BB membranes into the intravesicular space. Transport of PteGlu by these vesicles is further supported by preliminary experiments which suggest that [^{3}H]PteGlu uptake can be accelerated by preincubating vesicles with nonradiolabeled PteGlu (homeoexchange diffusion). The present finding of a greater capacity for PteGlu uptake (binding plus transport) in the BB membrane preparations is consistent with *in vivo* data that net PteGlu reabsorption is evident under physiologic conditions in the proximal tubule of the kidney (13). FBP may play a role in the renal reabsorption and reutilization of folates. Since membrane FBP may be partially extracted with a strong salt solution, it appears that it is in part bound to the polar head of the lipid layer. Proteins bound to the polar head of the lipid membrane, may exchange with plasma and/or cytoplasmic proteins (37) and would be readily accessible to bind folate. Such a process may represent the initial step in trans-

cellular transport of folate in the kidney and in other cell types (7).

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