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EFFECT OF ANIONS ON FOLATE BINDING BY ISOLATED BRUSH BORDER MEMBRANES FROM RAT KIDNEY

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Summary

The characteristics of folate binding by brush border membranes from rat kidney homogenates were investigated. At pH 7.4, binding of [3′,5′,9-^{3}H]-pteroylglutamic acid to membranes containing endogenous folate is inhibited by anions, with chloride being most effective followed by bromide, thiocyanate, iodide, phosphate and sulfate. A maximum inhibition of 70—75% is attained at a concentration of 0.1 M chloride and an incubation time of 30 min. The inhibition diminishes with increased incubation time and at 24 h is negligible. The binding of [3′,5′,9-^{3}H]pteroylglutamic acid to brush border membranes stripped of endogenous folate by acid treatment is not inhibited by anions. Anion sensitivity can be restored to these treated membranes by reconstitution with membrane-derived folate, particularly 5-methyltetrahydropteroylglutamic acid, or by preincubation with synthetic 5-methyltetrahydropteroylglutamic acid. Inhibition of [3′,5′,9-^{3}H]pteroylglutamic acid binding by anions in membranes with endogenous folate is best explained by an anion-induced stabilization of endogenous folate-binding protein complex resulting in a decreased rate of exchange with exogenous [3′,5′,9-^{3}H]pteroylglutamic acid.

Introduction

Recent studies from this laboratory demonstrated the presence of high-affinity (K_b = 0.035—0.04 nM) folate-binding proteins in brush border membranes from rat kidney and intestine [1,2]. These membrane-associated binding proteins exhibit many of the binding characteristics of soluble folate-binding

Abbreviations: [^{3}H]PteGlu, [3′,5′,9-^{3}H]pteroylglutamic acid; H_4PteGlu, tetrahydropteroylglutamic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

proteins in milk, plasma, leukocyte lysates, and hog kidney extracts [3]. Folate binding is pH dependent with a maximum at pH 7.4 where the affinity for unreduced folic acid is much greater than for reduced folates or methotrexate.

The location of folate-binding proteins in epithelial membranes is consistent with a possible role in folate transport. Folate transport in some cell systems has been reported to be affected by anions [4,5]. The effect of anions on folate binding in brush border membranes was examined in these studies.

Materials and Methods

[^{3}H]PteGlu (34—63 Ci/mmol), was purchased from Amersham/Searle Corporation (Arlington Heights, IL). It was stored at —30°C and purified by column chromatography [6], or adsorption and desorption from activated charcoal [7]. This compound was used as the source for preparation of [^{3}H]-labelled 5-methylH_4PteGlu according to the procedure described by Buehring et al. [8] which employed biosynthesis in *Lactobacillus casei* cells (ATCC 7469) followed by treatment with hog kidney conjugase and purification of the labelled methyl derivative on DEAE columns [7]. [^{14}C]-labelled 5-methyl-H_4PteGlu (53 Ci/mol) was purchased from Amersham/Searle Corporation. 10-FormylH_4PteGlu was prepared from 5-formylH_4PteGlu (GIBCO Grand Island, NY) as described by Brown et al. [9] and H_4PteGlu was prepared by reduction of PteGlu with sodium dithionite [10]. The latter four derivatives were used as standards to determine elution positions on analytical DEAE columns (see below).

Preparation of brush border membranes from rat kidney. Brush border membranes were prepared from rat kidney as previously described [1]. In this procedure the kidney was homogenized in 50 mM mannitol, treated with 0.01 M $CaCl_2$ and centrifuged at 2000 × *g* to remove cell debris, then at 20 000 × *g* to obtain a pellet enriched with brush border membranes. This pellet was resuspended in 50 mM mannitol (2 ml/original 1 g tissue) and used either as such (untreated membranes) or after removal of endogenous folates by acidification. Membrane folates were removed by adding acetic acid to the untreated membrane suspension (final concentration 5 mM, pH 4.0) and centrifuging immediately at 4°C for 15 min at 30 000 × *g*. The resulting pellet was resuspended in 50 mM mannitol to the original volume of the untreated membranes. The acid supernatant was made 1% in sodium ascorbate, adjusted to pH 6.0, and stored in evacuated tubes at 4°C (see below).

Determination of folate-binding activity. The assay method [1,2] is based on selective trapping of bound [^{3}H]PteGlu to cellulose nitrate filters (Schleicher and Schull, Dassel, F.R.G.). The membrane preparations (0.02—0.04 mg protein) were incubated in 20 μM Tris/Hepes (Sigma Chemical Co., St. Louis, MO), pH 7.4 for 30 min at room temperature with 4 nM [^{3}H]PteGlu. The volume of the incubation was 1 ml and a final osmolarity of 0.3 was maintained using mannitol alone or a combination of mannitol and inorganic salts as indicated. After incubation, the mixture was passed under vacuo, through a single 0.45 μm cellulose nitrate filter, and washed with 10 ml 0.05 M potassium phosphate buffer, pH 7.0. The filter was then dried for 20 min in a 70°C oven and counted as previously described.

Under these incubation conditions, the radioactivity adsorbed to the brush border membranes is exclusively due to the binding protein and not to vesicular transport. No radioactivity was released from the brush border membranes when the osmolarity of the bathing medium was increased to 1 using raffinose, conditions which were reported to cause the exit of small molecular weight substances from the intravesicular space [11,12]. Furthermore, solubilization of the membranes containing [^{3}H]PteGlu with Triton X-100 and subsequent chromatography on Biogel P-10, resulted in the exclusive elution of [^{3}H]PteGlu with the protein fraction.

Folate bioassay. Folates in the neutralized acid supernatant were analyzed after fractionation on analytical DEAE-celite columns [6]. A 5 ml sample was added to the column and eluted with a potassium phosphate gradient (0—0.5 M) containing 0.2% potassium ascorbate (pH 6.0). The type of folate in each fraction was determined by differential microbial assay using *L. casei* and *Streptococcus faecalis* (ATCC 8043) [13], and comparisons with the elution positions of synthetic folate derivatives fractionated under identical conditions.

Results

PteGlu binding by untreated membranes

Study of the time dependence of [^{3}H]PteGlu binding by untreated membranes shows the presence of three binding components (Fig. 1, upper curve). One component which makes up approximately 5% of the total binding essentially represents instantaneous association of the label with membranes. A second component which comprises 45% of the total binding is primarily active in the first 40 min of incubation, and shows a slower rate of binding ($k_{exp} = 5.8 \cdot 10^{-2}$ min^{-1}). A third component comprising 50% of the total binding shows a still slower binding rate ($k_{exp} = 4.4 \cdot 10^{-3}$ min^{-1}) and accounts for the prolonged incubation required to completely saturate the membrane binding sites.

The study in Fig. 1 (lower curve) further shows that this binding of [^{3}H]-PteGlu by untreated membranes is inhibited by NaCl at concentrations saturating at around 0.1 M salt. Inhibition by NaCl is greatest in the first 30—40 min of incubation amounting to 70—75% at 0.1 M NaCl. Inhibition decreases thereafter being negligible after 24 h of incubation. Most affected is the second binding component which diminishes in magnitude and disappears at high salt concentration (Fig. 1, insert). Consequently, the percent of total binding represented by the third component increases. Even this component, however, is affected by NaCl since the rate constant of its binding decreases by half at 0.1 M NaCl.

Inhibition by NaCl was found to be reversible when the salt was removed from the membranes by repeated washing with mannitol. Potassium chloride was found to be as effective an inhibitor as NaCl. The data in Fig. 2 indicate that it is the anion moiety which promotes this inhibition of PteGlu binding. Under standard conditions (i.e. after 30 min incubation), nearly 70% of the [^{3}H]PteGlu binding by untreated membranes is susceptible to inhibition depending on the concentration of salt and type of anion. Most active are Cl^-

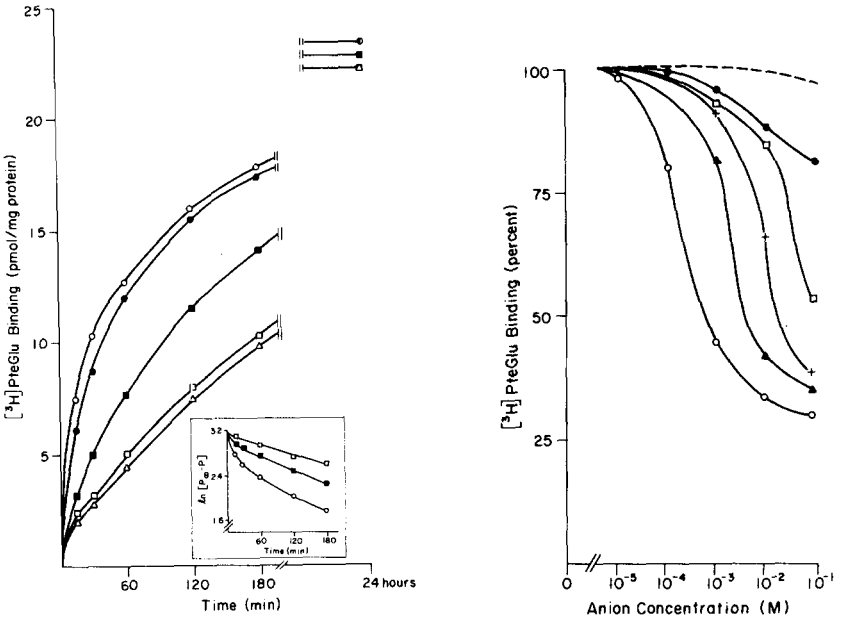


Fig. 1. Time and chloride dependence of [^{3}H]PteGlu binding by untreated brush membranes from rat kidney homogenates. Untreated membranes (0.04 mg protein) were incubated at room temperature with increasing concentrations of NaCl and 4 nM [^{3}H]PteGlu added 5 min after the salt. Mannitol was included to maintain a final osmolarity of 0.3. At the times indicated aliquots from these incubations were removed and analyzed for bound [^{3}H]PteGlu as described in Materials and Methods. ○, no NaCl; ●, 10^{-4} M NaCl; ■, 10^{-3} M NaCl; □, 10^{-2} M NaCl; △, 10^{-1} M NaCl. Insert depicts the plots of the natural logarithim of $(P_\infty - P)$ versus time where P_∞ represents maximum binding and P, binding at the time indicated both after subtraction of the binding value (i.e. 1 pmol/mg protein) due to the first binding component. Rate constant for the third binding component was estimated from the slope of the line drawn between the two data points at 120 and 180 min. The rate constant for the second binding component was estimated from the early points after subtraction of the binding value due to the third component.

Fig. 2. Effect of sodium salts on the binding of [^{3}H]PteGlu by untreated brush border membranes. Untreated membranes (0.04 mg protein) were mixed at room temperature with various sodium salt concentrations. Osmolarity was adjusted to 0.3 with mannitol. After 5 min [^{3}H]PteGlu was added to a final concentration of 4 nM and the mixture was incubated for 30 min before binding activity was determined as described in Materials and Methods. The sodium salts used were as follows: ●, sodium sulfate and sodium phosphate (pH 7.4); □, sodium iodide; +, sodium thiocyanate; ▲, sodium bromide; ○, sodium chloride. Values are expressed as percent of controls without salt. The broken line at the top of the figure represents [^{3}H]PteGlu binding by membranes after acid treatment in presence of increasing concentrations of NaCl.

which produce 50% of maximum inhibition at concentrations below 1 mM. Inhibitory activity of anions appears to bear some orderly relation to size and charge. Anions with large size or higher charge are less active. In the following study this chloride sensitivity of PteGlu binding was further analyzed.

Binding of PteGlu by acid-treated membranes

Treatment of the membranes with acid and subsequent removal of the acid-soluble fraction by centrifugation results in an enhancement of folate-binding activity under standard incubation conditions consistent with removal of endogenous folate by such treatment [1]. Adding back the neutralized supernatant results in a dose-dependent inhibition of [^{3}H]PteGlu binding as

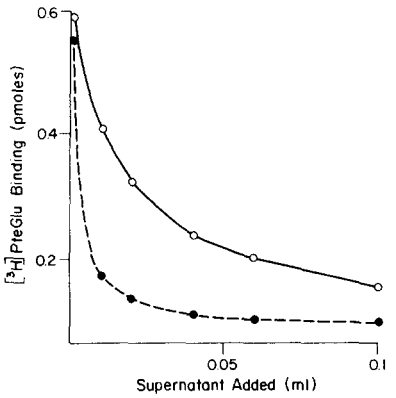


Fig. 3. Restoration of the chloride-dependent inhibition of [^{3}H]PteGlu binding to acid-treated membranes. The membranes (0.04 mg protein) were preincubated at room temperature with increasing amounts of the neutralized acid supernatant fraction (see Materials and Methods) in the presence (•) and absence (○) of 0.05 M NaCl. The final pH was 7.4. After 15 min 4 pmol of [^{3}H]PteGlu were added and the mixture was incubated for an additional 30 min. Bound [^{3}H]PteGlu was determined as described in Materials and Methods.

expected due to resaturation of the binding sites with endogenous folate in the supernatant (Fig. 3, upper curve).

Acid treatment also results in a loss of sensitivity to Cl^- (Fig. 2, upper curve and Fig. 3). This is reversed by adding back the neutralized supernatant. As shown in Fig. 3 (lower curve), the inhibitory effect of the neutralized supernatant on the binding of [^{3}H]PteGlu by acid-treated membranes is increased several fold when NaCl is present. Under these conditions relatively low quantities of supernatant are required to produce maximal inhibition.

The following studies explore the possibility that both effects of supernatant and NaCl on PteGlu binding by acid-treated membranes are explicable on the basis of behavior of endogenous membrane-bound folate. Folate extracted from membranes by acid is resolved into three peaks of monoglutamate derivatives when chromatographed on DEAE-celite columns (Fig. 4a). Peaks I and III each exhibit equal activity for *L. casei* and *S. faecalis* and elute in positions corresponding to 10-formylH_4PteGlu and unsubstituted H_4PteGlu. Peak II consists of 5-methylH_4PteGlu as judged from the growth of *L. casei* compared with *S. faecalis* and from identical elution pattern of synthetic 5-methylH_4PteGlu. Polyglutamyl derivatives of folate were not present in these preparations.

The effect of adding back various fractions on the binding of [^{3}H]PteGlu by acid-treated membranes in the presence and absence of Cl^- is presented in the study shown in Fig. 4b. Inhibition of the binding occurs only when fractions that contain folate activity are added back. Inhibition of binding by fractions containing 10-formylH_4PteGlu (peak I) is proportional to the concentration of the folate derivative and is independent of Cl^-. Fractions containing unsubstituted H_4PteGlu (peak III) are similarly inhibitory, however, Cl^- approximately double this inhibition. In the absence of Cl^- there is virtually no inhibition by fractions containing 5-methylH_4PteGlu. However, in the presence of

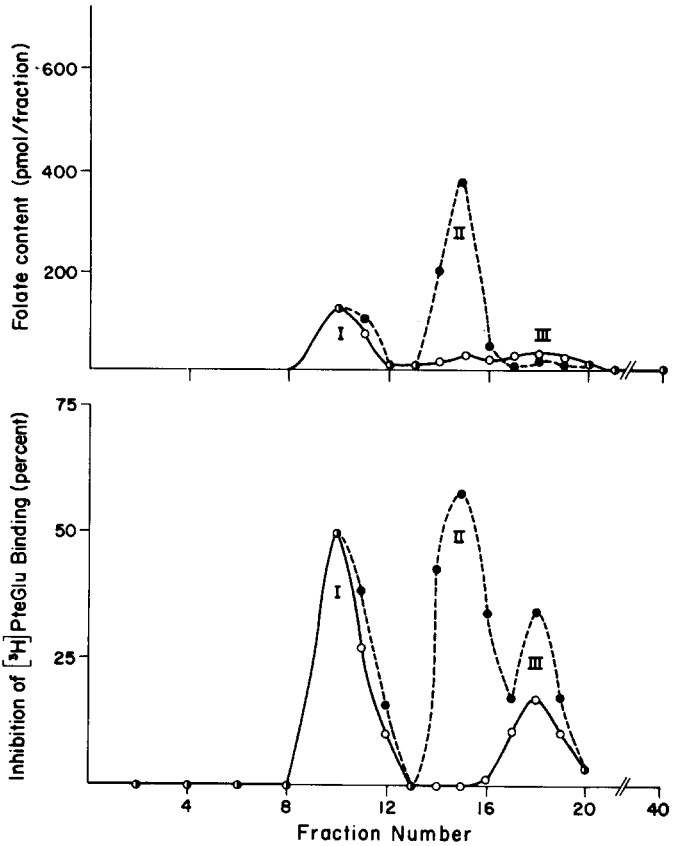


Fig. 4. Fractionation of folate in the acid supernatant on DEAE-celite column. An aliquot (5 ml) of the acid supernatant, neutralized to pH 6.0, was fractionated on a DEAE-celite column with a potassium phosphate gradient as described. Fractions were analyzed (top) for *L. casei* (- - - - - -) and *S. faecalis* (———) activities, and (bottom) for the ability to inhibit [^{3}H]PteGlu binding. To determine binding inhibition, 0.05 ml of each fraction was preincubated for 15 min with acid-treated membranes (0.04 mg) at pH 7.4 with (- - - - - -) and without (———) 0.05 M NaCl. This was followed by a 30 min incubation with 4 pmol [^{3}H]PteGlu and analysis as described in Materials and Methods.

Cl^-, adding back the fractions with 5-methylH_4PteGlu causes a marked inhibition in the [^{3}H]PteGlu binding.

Adding synthetic (±)-5-methylH_4PteGlu to acid-treated membranes (Fig. 5) results in the same type of chloride-dependent inhibition of [^{3}H]PteGlu binding, although there appears to be a greater inhibition of the binding in the absence of the anion than with the natural folate. This may reflect differences between (—)- versus (±)-stereoisomers. Concentration dependence studies with natural [^{3}H]-labelled methylH_4PteGlu (Fig. 6) prepared biosynthetically from *L-casei* demonstrates that 1 mM chloride enhances considerably the binding of this folate to acid-treated membranes. The Klotz plots derived from these data (Fig. 6, insert) show an anion-induced increase in the association constant from $0.5 \cdot 10^9$ mol^{-1} to $2.5 \cdot 10^9$ mol^{-1}.

Time course of PteGlu binding by acid-treated membranes

Acid-treated membranes, unlike the untreated preparation, bind [^{3}H]PteGlu

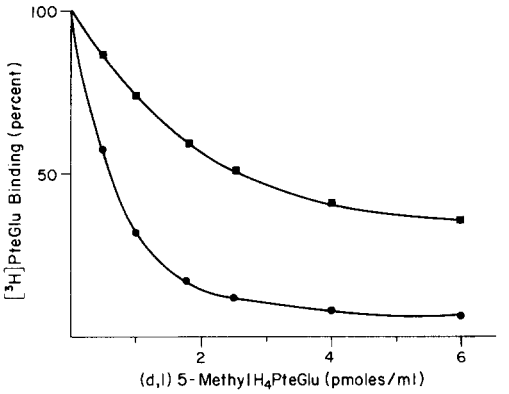


Fig. 5. Effect of chloride on the [^{3}H]PteGlu binding by acid-treated membranes preincubated with synthetic (±)-5-methylH$_4$PteGlu. Membranes (0.02 mg protein) were preincubated 15 min at room temperature with and without increasing amounts of synthetic (±)-5-methylH$_4$PteGlu in presence (•) and absence (■) of 0.5 M NaCl. The mixtures were then reincubated for 30 min at room temperature with 4 pmol [^{3}H]PteGlu before analysis for [^{3}H]folate binding.

rapidly (k_{exp} = 0.88 min^{-1}) and NaCl causes a slight increase in the rate of binding (Fig. 7a and Table I). When preincubated with 5-methylH$_4$PteGlu without Cl$^-$, the binding of [^{3}H]PteGlu by these acid-treated membranes proceeds by a slower, first-order process which requires 30 min for completion (Fig. 7b). At this time point the [^{3}H]PteGlu binding value is the same as that

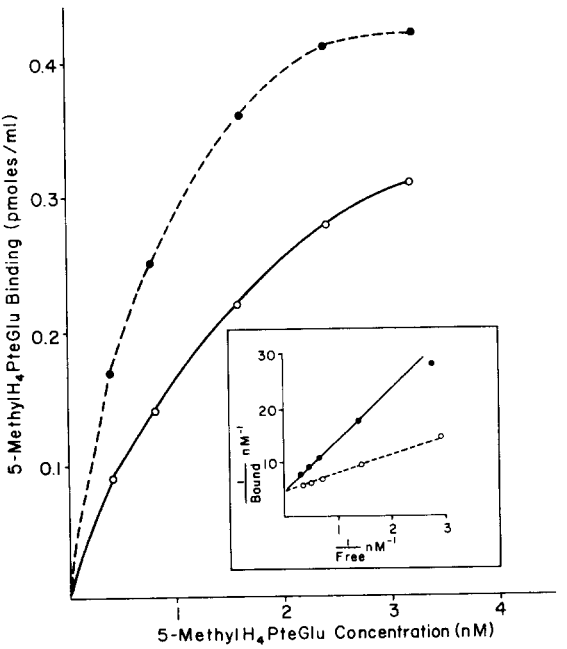


Fig. 6. Effect of chloride on the binding of 5-methylH$_4$PteGlu by acid-treated membranes. Membranes (0.02 mg protein) were preincubated with (- - - - - -) and without (———) 1 mM NaCl. After 15 min various concentrations of [^{3}H]-labelled 5-methylH$_4$PteGlu were added and binding was determined after another 30 min. Insert shows the Klotz plot [14] of the data obtained.

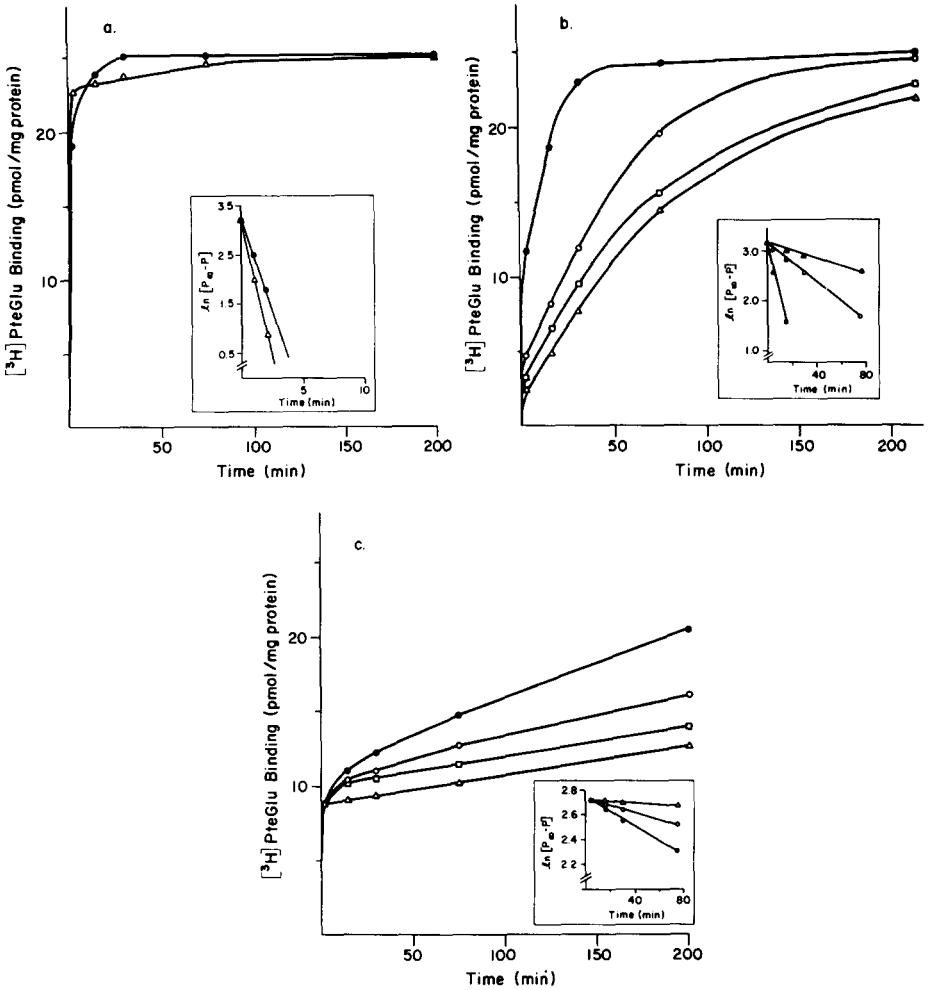


Fig. 7. Time and chloride dependence of [^{3}H]PteGlu binding by acid-treated membranes before and after preincubation with endogenous folates from DEAE fractionation. [^{3}H]PteGlu binding by acid-treated membranes (0.02 mg protein) was determined at various times, in presence of increasing concentrations of chloride; (a) before incubation with endogenous folates; (b) after incubation with aliquots of fraction 15 containing 1.25 pmol of 5-methylH$_4$PteGlu, and (c) after incubation with aliquots of fraction 9 containing 0.75 pmol of 10-formylH$_4$PteGlu. •, No NaCl; ○, 10^{-3} M NaCl; □, 10^{-2} M NaCl; △, 10^{-1} M NaCl. Inserts depict the plots of $\ln(P_\infty - P)$ versus time where P_∞ represents maximum binding as obtained in (a) and P binding of [^{3}H]PteGlu at the period indicated.

obtained by these treated membranes alone, thus indicating that the label has displaced bound 5-methylH$_4$PteGlu. The rate constant determined for this [^{3}H]PteGlu binding (Table I) is of the same magnitude as that estimated for the second binding component in the untreated preparation (Fig. 1).

The presence of NaCl during preincubation with 5-methylH$_4$PteGlu causes a yet slower rate of [^{3}H]PteGlu binding and delay in reaching complete displacement of the methyl derivative by the label (Fig. 7b). The decrease in the rate of binding caused by 0.1 M NaCl is of such a magnitude that the rate constant estimated (Table I) is now in the same order of magnitude as that estimated for

TABLE I

RATE CONSTANTS (k_{exp}) OF [^{3}H]PteGlu BINDING BY ACID-TREATED MEMBRANES UNDER VARIOUS CONDITIONS

k_{exp} was calculated from the inserts in Fig. 7.

Endogenous folate added	Chloride ion concentrations (M)	k_{exp} (min^{-1})
None	0	$0.88 \cdot 10^{-1}$
	10^{-1}	$1.1 \cdot 10^{-1}$
5-MethylH$_4$PteGlu	0	$9.4 \cdot 10^{-2}$
	10^{-3}	$1.24 \cdot 10^{-2}$
	10^{-2}	$1.00 \cdot 10^{-2}$
	10^{-1}	$0.85 \cdot 10^{-2}$
10-FormylH$_4$PteGlu	0	$4.2 \cdot 10^{-3}$
	10^{-3}	$2.2 \cdot 10^{-3}$
	10^{-2}	$1.6 \cdot 10^{-3}$
	10^{-1}	$1.1 \cdot 10^{-3}$

the third binding component in the untreated membranes.

When preincubated with 10-formylH$_4$PteGlu binding of [^{3}H]PteGlu by the acid-treated membrane is much slower than the membranes with 5-methyl-H$_4$PteGlu and complete saturation of binding sites with the label is achieved after several hours of incubation (Fig. 7c). The rate constant determined for this binding (Table I) is similar to that estimated for the third binding component in the untreated membranes. As with the latter, NaCl causes a small yet significant decrease in the rate of [^{3}H]PteGlu binding by membranes containing 10-formylH$_4$PteGlu (Fig. 7c).

Discussion

The observation that untreated brush border membranes bind [^{3}H]PteGlu by a process which includes three components (Fig. 1) is best attributed to the need to occupy binding sites which are in part unsaturated (rapid binding component) and in part occupied by 5-methylH$_4$PteGlu (second binding component) and non-methyl-reduced folates, mostly 10-formylH$_4$PteGlu (slowest binding component). In the absence of NaCl, endogenous 5-methylH$_4$PteGlu exchanges rapidly with [^{3}H]PteGlu resulting in complete displacement within 30 min of incubation. This explains the earlier data from this laboratory which assumed that about 60% of the binding sites in the untreated kidney homogenates and brush border membrane preparation were unoccupied [1]. Non-methylated folates are more tightly bound and their exchange with the labelled folate is much slower.

These results are not unexpected. At neutral pH folic acid exhibits a higher affinity for the membrane folate-binding protein than reduced folates with a methyl or formyl substitution in the 5 position [1]. The rate of exchange between prebound 5-methylH$_4$PteGlu and PteGlu has been employed by several investigators to characterize soluble folate-binding proteins from different [15] or from the same tissues [16]. The novel aspect of the present study lies in the observed effect of NaCl and other salts on the binding of folates by the mem-

brane folate-binding protein. This effect is largely due to the anion moiety. The capacity of sodium or potassium salts to inhibit the binding of [^{3}H]PteGlu by untreated membranes appears to depend on the type of anion and its concentration. The observation that this anion effect is saturable and effective at concentrations of Cl^- below 1 mM, suggests the existence of anion sites, more specific for chloride than other anions, which influence the folate binding to the membranes.

This influence is seen in the chloride-induced enhancement of the association of folates, particularly 5-methylH_4PteGlu, with the membrane folate-binding protein, in studies which restore chloride-dependent inhibition to acid-treated membranes by adding back the acid supernatant (Fig. 3), DEAE-celite fractions containing endogenous 5-methylH_4PteGlu (Figs. 4 and 7b) or, synthetic (±)-5-methylH_4PteGlu (Fig. 5). Additional evidence derives from direct studies with [^{3}H]-labelled 5-methylH_4PteGlu were 1 mM NaCl increased the association constant by 2.5 fold (Fig. 6). Stabilized association of endogenous 5-methyl-H_4PteGlu with the membrane binding protein would account for the observed anion-dependent inhibition of [^{3}H]PteGlu binding by untreated membranes as the rate of exchange between the label and endogenous folate would be decreased.

Evidence that Cl^- exert a similar, though smaller, effect on the binding of other folate derivatives, comes from the observations that this anion slightly enhances the initial rate of [^{3}H]PteGlu binding by acid-treated membranes (Fig. 7a); that chloride causes a greater inhibition of binding by acid-treated membranes preincubated with fractions containing endogenous H_4PteGlu (Fig. 4); and that chloride decreases further the rate of [^{3}H]PteGlu binding by membranes preincubated with 10-formylH_4PteGlu (Fig. 7c).

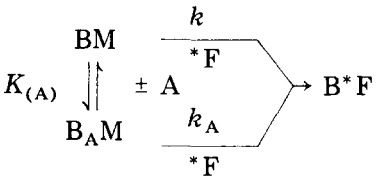
The data presented here conforms to a model based on the existence of two types of complexes: (1) a binary complex of binding protein and folate, particularly 5-methylH_4PteGlu, which is relatively unstable, and (2) a ternary complex of binding protein, anion, and folate which is more stable. (See Appendix for a more complete treatment of the relationship between the complexes).

These properties of the membrane folate-binding protein may provide a link between binding and a possible function in folate transport. Some participation of anions in folate transport has been recently suggested from studies with Ehrlich ascites tumor cells [4] and L1210 murine leukemia cells [5]. These studies have shown that the partial or total replacement of Cl^- with inorganic (NO_3^-, SO_4^{2-}, PO_4^{2-}) or organic (glucose 6-phosphate, ATP, ADP, and AMP) anions is accompanied by a decrease in the flux of 5-methylH_4PteGlu and methotrexate into cells. The model that was proposed to interpret these observations [4] takes into account the interaction of a folate 'carrier' with anions either through the same site that recognizes folates or through an alternate site which implies a ternary complex.

Appendix

On the basis of the data presented, the observed difference between various anions in promoting inhibition of [^{3}H]PteGlu binding (Fig. 2) can be quantitatively expressed by the model described below which makes the following

assumptions: (a) The effect of the anion is exerted on



the rate of dissociation of bound 5-methylH_4PteGlu alone (the effect of anions on dissociation of 10-formylH_4PteGlu is relatively small and is therefore neglected here). (b) The association of 5-methylH_4PteGlu with the binding protein is either an unstable binary complex (BM) (rate consant for dissociation = k) or a relatively stable ternary complex (B_AM) (rate constant of dissociation = k_A) which is brought about by the association of anions to secondary binding sites. (c) The equilibrium constant (K_A) between binary and ternary complexes is determined by the type of anion present. (d) Regardless of whether or not anions are present the formation of the complex between [^{3}H]PteGlu and binder (B*F) is rapid, preferred and irreversible, hence the rate of formation of this complex is a direct measure of the dissociation rate of 5-methylH_4PteGlu. (This is justifiable because [^{3}H]PteGlu is added in excess to its K_m and to the concentration of endogenous folates. It also competes more efficiently than 5-methylH_4PteGlu, and its binding is not inhibited by anions.)

The relationships between BM, B_AM, and B*F are expressed below.

$$\frac{dB^*F}{dt} = k(BM) + k_A(B_AM) \tag{1}$$

$$K_A = \frac{[BM][A]}{[B_AM]} \tag{2}$$

$$[BM] + [B_AM] = [BM_0] \tag{3}$$

dB^*F/dt is the rate of formation of B*F, and BM_0 is the initial concentration of 5-methylH_4PteGlu binder complex. From Eqns. 2 and 3 BM and B_AM are expressed as follows:

$$BM = B_AM\,\frac{K_A}{[A]} = \frac{BM_0}{1 + [A]/K_A} \tag{4}$$

$$B_AM = BM\,\frac{[A]}{K_A} = \frac{B_0}{1 + K_A/[A]} \tag{5}$$

Replacing these expressions in Eqn. 1

$$\frac{dB^*F}{dt} = B_0\left(\frac{k}{1 + [A]/K_A} + \frac{k_A}{1 + K_A/[A]}\right) \tag{6}$$

or

$$\frac{dB^*F}{dt} = B_0\left(\frac{kk_A + k_A[A]}{K_A + [A]}\right) \tag{7}$$

TABLE II

EQUILIBRIUM CONSTANTS (K_A) FOR VARIOUS ANIONS

K_A was calculated from Eqn. 8 in the appendix and the data in Fig. 2.

Anion	K_A (M)
Cl^-	$5 \cdot 10^{-5}$
Br^-	$8.5 \cdot 10^{-4}$
SCN^-	$3.8 \cdot 10^{-3}$
I^-	$4 \cdot 10^{-2}$
PO_4^{2-}	$3 \cdot 10^{-1}$
SO_4^{2-}	$3 \cdot 10^{-1}$

and upon integration

$$B^*F = B_u + BM_0(1 - e^{-(k_A + k_A[A])/(K_A + [A]) \cdot t}) \quad (8)$$

where B_u represents sites which bind independently of anions and include unsaturated sites as well as those that are saturated with 10-formylH_4PteGlu that dissociate during the period of incubation.

In Eqn. 7 the expression in parenthesis represents the experimental rate constant and is therefore equal to k in the absence of added anions and k_A in the presence of the highest concentration of the anion with greatest effect, namely Cl^- at 0.1 M. Between these two extremes this expression take into account both [A] and k_A. The terms B_u and BM_0 are determined from experimental binding values after a time, t, in the absence (B^*F_{max}) and, presence (B^*F_{min}) of a high concentration of Cl^-

$$B^*F_{max} = B_u + BM_0 \quad (9)$$

$$B^*F_{min} = B_u + BM_0(1 - e^{-k_A t}) \quad (10)$$

$$BM_0 = \frac{B^*F_{max} - B^*F_{min}}{e^{-k_A t}} \quad (11)$$

$$B_u = B^*F_{max} - BM_0 \quad (12)$$

K_A values of the various anions tested in the experiment described in Fig. 2 were calculated using Eqn. 8 (Table II). Values for k and k_A were determined from the experiment in Fig. 7b as $7.4 \cdot 10^{-2}$ min^{-1} and $0.85 \cdot 10^{-2}$ min^{-1}, respectively. Replacement of chloride with other anions increases the K_A. The biggest difference being seen with the polyanions which have a K_A four orders of magnitude larger.

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References

1 Selhub, J. and Rosenberg, I.H. (1978) Proc. Natl. Acad. Sci. U.S. 75, 3090—3093
2 Selhub, J., Gay, A.C. and Rosenberg, I.H. (1978) in The Chemistry and Biology of Pteridines (Kisliuk, R.Y. and Brown, G.M., eds.), pp. 593—596, Elsevier/North-Holland, Inc., New York
3 Rothenberg, S.P. and da Costa, M. (1976) Clin. Haematol. 5, 569—587
4 Goldman, I.D. (1977) in Membrane Toxicity (Miller, M.E. and Shamos, A.E., eds.), pp. 85—113, Plenum Press, New York
5 Henderson, G.B. and Zevely, E.M. (1978) in The Chemistry and Biology of Pteridines (Kisliuk, R.Y. and Brown, G.M., eds.), pp. 549—554, Elsevier/North-Holland, Inc., New York
6 Selhub, J., Brin, H. and Grossowicz, N. (1973) Eur. J. Biochem. 33, 433—438
7 Ritari, S.J., Sakami, W., Black, C.W. and Rezepka, J.A. (1975) Anal. Biochem. 63, 118—129
8 Buehring, K.U., Tamura, T. and Stokstad, E.L.R. (1974) J. Biol. Chem. 249, 1081—1089
9 Brown, J.P., Davidson, G.E. and Scott, J.M. (1974) Biochim. Biophys. Acta 343, 78—88
10 Davis, L. (1968) Anal. Biochem. 26, 459—460
11 Hopfer, U. (1977) Am. J. Physiol. 233, E445—E449
12 Hopfer, U. (1978) Am. J. Physiol. 234, F89—F96
13 Bird, O.D., McGlohon, M.V. and Vaitkus, J.W. (1968) Anal. Biochem. 12, 18—55
14 Klotz, I.M. (1974) Acc. Chem. Res. 7, 162—168
15 Kamen, B.A. and Caston, J.D. (1975) J. Biol. Chem. 250, 2203—2205
16 Fischer, C.D., da Costa, M. and Rothenberg, S.P. (1975) Blood 46, 855—867